G PROTEIN-COUPLED RECEPTOR KINASES (GRKS) AND BETA-ARRESTINS: NEW INSIGHTS INTO DISEASE REGULATORS

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G PROTEIN-COUPLED RECEPTOR KINASES (GRKS) AND BETA-ARRESTINS: NEW INSIGHTS INTO DISEASE REGULATORS

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EDITORIAL
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Editorial: G Protein-Coupled Receptor Kinases (GRKs) and β-Arrestins: New Insights Into Disease Regulators

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Editorial on the Research Topic

G Protein-Coupled Receptor Kinases (GRKs) and β-Arrestins: New Insights Into Disease Regulators

G protein-coupled receptors (GPCRs) are the largest family of plasma membrane proteins mediating cellular responses to a wide variety of external stimuli and can be crucially involved in a multitude of physiological process and their dysregulation contributes to many diseases. G protein-coupled receptor kinases (GRKs) and β-arrestins were initially identified as a pivotal player in the process of desensitization of agonist-activated GPCRs (Premont and Gainetdinov, 2007; Black et al., 2016): GRKs specifically phosphorylate agonist-activated GPCRs, and receptor phosphorylation triggers the binding of cytoplasmic β-arrestin molecules, which sterically block the activation of heterotrimeric G proteins, leading to rapid desensitization of G protein-mediated signaling cascades. However, growing evidence suggests GRKs and β-arrestins fulfill a vital role in regulating a variety of cellular proteins involved in signal transduction independently of GPCRs (Penela et al., 2010). Thus, GRKs and β-arrestins can interact with non-GPCRs. GRKs and β-arrestins may directly affect functioning of non-GPCRs or indirectly regulate non-GPCR signaling. In addition, incoming evidence supports that changes in function and/or expression of GRKs and β-arrestins may be important in cardiovascular, inflammatory, metabolic, or cancer pathologies (Vroon et al., 2006; Schumacher and Koch, 2017; Steury et al., 2018; Yu et al., 2018). A better understanding of the pathological roles of GRKs and β-arrestins would provide a basis for new therapeutic targets in different human diseases.

We organized the Research Topic entitled "G protein-coupled receptor kinases (GRKs) and β-arrestins: new insights into disease regulators" in *Frontiers in Pharmacology*, which started at February, 2018. A total of 12 articles, consisting of 3 original papers and 9 review papers, has been published in *Frontiers in Pharmacology*. Our Research Topic has been well received by the readership of the journal with about 20,000 views.

In this Research Topic, Sun et al. advocated the unconventional role of β-arrestin 2 in promoting hepatocyte apoptosis in alcoholic liver disease. It is documented that apoptosis of massive hepatocytes is a prominent feature of the initiation and progression stages of alcoholic liver disease (Ceni et al., 2014). Sun et al. demonstrated that β-arrestin 2 levels in liver tissues from ethanol-fed mice were markedly higher than those from control diet-fed mice and knockdown of β-

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arrestin 2 inhibited hepatocyte apoptosis induced by ethanol *in vivo*. As deficiency of β-arrestin 2 increased phosphorylation of Akt and overexpression of β-arrestin 2 suppressed Akt activation in AML-12 mouse hepatic cell line which exhibited a reduction in Akt phosphorylated levels in response to ethanol exposure, β-arrestin 2 appears to promote hepatocyte apoptosis through suppression of the cell survival regulator Akt.

Palikhe et al. have demonstrated that GRK2 can function as Toll-like receptor (TLR) signaling to elicit inducible nitric oxide synthase (iNOS) expression in mouse MG6 microglial cells. They revealed that GRK2 strongly regulated the expression/activation of IRF1as well as the activation of the STAT pathway, leading to increased transcription of iNOS, when TLR-3, TLR-4, or TLR-9 was stimulated in microglial cells. This extends their previous report showing that GRK2 plays a critical role in iNOS gene transcription in microglial cells stimulated with lipopolysaccharide (Kawakami et al., 2018). Their findings highlight a novel pathological role of GRK2 in regulating inflammatory signaling in microglia with a potential therapeutic window for some neuroinflammatory disorders.

Cannavo et al. have identified GRK2 as a relevant player in the aldosterone signaling pathway. Aldosterone is produced not only in adrenals but also in cardiovascular tissues, and has been implicated in the development of cardiac hypertrophy and fibrosis (Jewell et al., 2006). Cannavo et al. (2016) have previously reported that GRK2 and GRK5 lie downstream of the aldosterone/mineralocorticoid receptor system. In this study, using *in vivo* two mouse models of hyperaldosteronism, chronic aldosterone infusion and surgical myocardial infarction, and *in vitro* 3T3-L1 fibroblast cultures, they demonstrated that canonical and noncanonical actions of GRK2 are involved in aldosterone-triggered attenuation of insulin- and β-adrenoceptor-mediated effects at the heart levels. Their finding that spironolactone, a mineralocorticoid receptor antagonist, offset cardiac insulin-signaling dysfunction and β_1-adrenoceptor downregulation in mice with myocardial infarction by blunting canonical and noncanonical effects mediated by GRK2 levels may provide a novel mechanism for the cardioprotective effect of aldosterone antagonists.

Several review articles highlighted the novel roles of GRKs and β-arrestins as crucial modulators in the pathogenesis of a variety of diseases. Murga et al. summarized the pathophysiological roles of GRK2 in cardiovascular and metabolic diseases, such as heart failure, hypertension, obesity and insulin resistance conditions, and non-alcoholic fatty liver disease (NAFLD). Furthermore, they discussed different strategies to target GRK2 functionality as a potentially relevant approach to treat cardiovascular disease, obesity, type 2 diabetes, or NAFLD. Mangmool et al. summarized evidence that GRKs and β-arrestins could be potential candidates for novel therapeutic strategies for heart failure, including the view that carvedilol, alprenolol, and nebivolol are identified as β-arrestin-biased β-blockers, based on the roles of GRKs and β-arrestins on how they affect cardiac β-adrenoceptor signaling regarding the molecular and cellular pathophysiology. Bagnato and Rosanò highlighted the role of GPCR/β-arrestins-dependent signal pathways in cancer growth, invasion, and metastasis, although it remains to be learned both about how β-arrestins mediate gene expression changes to execute the GPCR-induced pro-metastatic effects in tumor cells and about how β-arrestins and G protein-mediated effects may differ in this regard. Bond et al. summarized that β-arrestins are involved in the pathophysiology of numerous and wide-ranging diseases, including asthma and cancer, and described the mechanisms by which β-arrestins regulate GPCR signaling, including the functional cellular mechanisms modulated by β-arrestins and related this to observed pathophysiological responses associated with β-arrestins.

Recent technological advancements in molecular and structural biology have provided new insights into the roles of GRKs and β-arrestins in not only GPCR-dependent but also non-GPCR-mediated signaling mechanisms. Given that GRKs and β-arrestins are important in signaling pathways and processes related to a variety of disease conditions, they could be expected as a promising therapeutic target in some diseases. A number of small molecules, peptides, and inhibitory constructs are being developed to target GRKs and β-arrestins, but we face the issues that need to be addressed in this drug discovery in light of the complexity of GPCR signaling pathways and the pleitropy of GRK and β-arrestin functions. However, the unwavering search for modulators targeting GRKs and β-arrestins will bestow a great impact on future therapies for a variety of nasty diseases.

AUTHOR CONTRIBUTIONS

YH and MM initiated the Research Topic. YH drafted the editorial and MM revised it for critical content. YH and MM have read and approved the final manuscript and take full responsibility for it.

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ORIGINAL RESEARCH
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β-Arrestin 2 Promotes Hepatocyte Apoptosis by Inhibiting Akt Pathway in Alcoholic Liver Disease

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Alcoholic liver disease (ALD) is a complex process that includes a wide range of hepatic lesions, from steatosis to cirrhosis, and even hepatocellular carcinoma (HCC). Accumulating evidence shows that the cytotoxic effects of ethanol metabolism lead to cell apoptosis and necrosis in ALD. Recently, several studies revealed that multifunctional protein β-arrestin 2 (Arrb2) modulated cell apoptosis in liver fibrosis and HCC, but its role in ALD has not been fully understood. The aim of this study is to explore the function and underlying mechanism of Arrb2 in hepatocyte survival and apoptosis in ALD. In our study, the primary hepatocytes were isolated from the livers of C57BL/6 mice fed EtOH-containing diet, it showed an increased level of Arrb2. EtOH also significantly up-regulated Arrb2 production in AML-12 cells *in vitro*. Furthermore, TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) and FCM results demonstrated that knockdown of Arrb2 could inhibit hepatocyte apoptosis induced by EtOH *in vivo* and *vitro* while over-expression of Arrb2 induced apoptosis in ALD. In addition, western blot results revealed that Arrb2 remarkably suppressed the Akt signaling. Taken together, our data suggested that Arrb2 may serve as a potential therapeutic target for ALD by promoting hepatocyte apoptosis via Akt suppression.

Keywords: alcoholic liver disease (ALD), β-arrestin 2 (Arrb2), hepatocyte, apoptosis, Akt

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INTRODUCTION

Alcoholic liver disease (ALD) is a complex disease that becomes one of the leading cause of severe liver-related morbidity and significant mortality worldwide.

Every year, about 3.3 million deaths occur in worldwide because of the prolonged alcohol abuse according to the World Health Organization (Arsene et al., 2016). ALD includes a wide spectrum of hepatic lesions, from steatosis, alcoholic steatohepatitis, progressive fibrosis, cirrhosis to even hepatocellular carcinoma (HCC) due to a consequence of susceptibility factors and degree of alcohol consumption (Ju and Mandrekar, 2015).

So far, the pathogenic mechanisms of ALD include the direct cytotoxic effect of alcohol and its metabolites like acetaldehyde, they may induce oxidative stress in hepatocytes and finally lead to cell inflammation, injury, and death (Louvet and Mathurin, 2015). Accumulating evidence demonstrated that apoptosis of massive hepatocytes is a prominent feature of the initiation and progression stages of ALD (Verma et al., 2016). In this regard, inhibition of apoptotic hepatocytes is critical in relieving the degree of ALD, and it is essential to find a potential therapeutic target for treating disease (Wu et al., 2016).

β-Arrestin, also termed 48-kDa protein, was found in 1986 as a cytosolic protein initially (Wilden et al., 1986). It not only serves as a co-factor in restraining the process of the light recept or rhodopsin to photoactivation by rhodopsin kinase but also suppresses the activation of cGMP phosphor-diesterase in retinal rod disk membranes (Kingsmore et al., 1995). There are four members in the arrestin family. Arrestin 1 and 4, which called the visual arrestins, can be found in the visual system exclusively, whereas the arrestins 2 and 3 (also called β-arrestin 1 and 2) are confirmed to exist in mammalian tissue ubiquitously (Barki-Harrington and Rockman, 2008).

It is well documented that multifunctional adaptor β-arrestin 2 (Arrb2) modulates cell apoptosis and it may have either a pro- or anti-apoptotic effect in different diseases. For instance, Arrb2 promotes hepatocyte apoptosis in bile duct ligation (BDL) while blocking hepatic stellate cells (HSCs) apoptosis in liver fibrosis (Sun et al., 2013; Yin et al., 2016; Zhang et al., 2017). However, the role of Arrb2 in modulating apoptosis during ALD remains unclear.

In this study, our data showed that Arrb2, but not Arrb1, significantly induced hepatocyte apoptosis in ALD by inhibiting Akt signaling pathway. Arrb2 depletion could inhibit hepatocyte apoptosis induced by EtOH both *in vivo* and *vitro*. Taken together, our data suggested Arrb2 plays a critical role in ALD via Akt signal, it may be a potential therapeutic target for ALD.

MATERIALS AND METHODS

Animal, Mouse Model of ALD

Eight-week-old male C57BL/6J mice were provided by the Experimental Animal Center of Anhui Medical University. All the animal experiments were approved by the Ethics Committees of Anhui Medical University and all procedures were performed under the permission of the Guideline of Animal Care and Use Committee of Anhui Medical University. For all experiments, mice were divided randomly into four groups, CD-fed + pGLV2-NC, EtOH-fed + pGLV2-NC, CD-fed + pGLV2-Arrb2, EtOH-fed + pGLV2-Arrb2, and all mice were fed at comfortable environment for at least 1 week. Modeling process of Gao-Binge protocol has a total of 16 days including adaptation period for 5 days and modeling for 10 days with 5% v/v ethanol liquid diets, EtOH-fed mice were gavaged with one time binge ethanol administration (5 g/kg, body weight, 20% ethanol) at the last day (Bertola et al., 2013). Mice were injected with lentivirus in caudal vein at the beginning and the middle of the modeling period. All mice were anesthetized after 9 h since the last time of gavage alcohol. The mice blood and liver tissue were separated for preparing further experiments.

Isolation of Liver Hepatocytes

Isolated liver hepatocytes were perfused from liver tissue of mice by using collagenase (type I; Sigma-Aldrich, St. Louis, MO, United States) perfusion. We referred to the previous papers to find the perfusion methods (Smedsrod and Pertoft, 1985; Hansen et al., 2002; Cassim et al., 2017). First, cannulating the cannula and then cutting out inferior vena cava. Next, perfusing the liver with 1% EGTA solution [1 × EGTA, NaCl (80 g), KCl (4 g), KH_2PO_4 (0.6 g), NA_2HPO_4 (0.48 g) and EGTA (1.9 g) in H_2O (1000 ml)], and then via recirculation with collagenase until the hepatic parenchyma appeared liquefied. Afterward, removing and placing liver in a sterile dish and add digestion buffer [0.075% collagenase 3 ml and 24 ml GBSS (Gey's Balanced Salt Solution)]. Cutting liver into very small pieces and shaking for 30 min at 200 rpm in 37°C incubator. Finally, adding GBSS and then centrifuging 400 rpm for 5 min to collect hepatocytes for further RNA and protein analysis.

Cell Culture and Treatment

AML-12 cells were obtained from American Type Culture Collection (ATCC) (Shanghai, China), cells were cultured in F-12 medium (Gibco, United States) supplemented with 8% fetal bovine serum (Clark, United States) and incubated at 37°C at an atmosphere of 5% CO_2. AML-12 cells were cultured in F-12 medium with EtOH for 24 h while the non-treated AML-12 cells were used as control (Aller de la Fuente et al., 2018).

Histopathological Examination and Immunofluorescent Staining

For histologic analysis, liver tissues were fixed with 10% neutral formalin solution and dehydrated with different concentration of ethanol. After then treated with xylene and embedded in paraffin. The paraffin liver tissue were cut into 5 μm thick sections and stained with hematoxylin and eosin (H & E). For immunofluorescent staining, liver tissues were fixed with 4% paraformaldehyde and then blocked with 10% BSA for 10 min. To investigate the expression of Arrb2 in mice liver, the tissue were incubated with mouse anti-Arrb2 antibody (1:100) overnight at 4°C and anti-mouse FITC (1:200) at temperature for 2 h. At last, the section was mounted with DAPI and images were taken using fluorescence microscopy.

The ALT/AST Activity Assay and the Serum Levels of TG/TCH Analysis

The serum levels of ALT, AST, TG, and TCH in mice with alcohol-induced ALD were assayed by using alanine aminotransferases (ALT) assay kit, aspartate aminotransferases (AST) assay kit, triglyceride (TG) and total cholesterol (TCH) assay kits. All kits were from Nanjing Jiancheng Bioengineering Institute. The absorbance was measured with a micro-plate reader model 680 (Bio-Rad Laboratories, Hercules, CA, United States).

Flow Cytometry Analysis

The level of Apoptosis was quantified by FITC-Annexin V apoptosis detection kit (BestBio, China). Firstly, AML-12 cells were washed by cold PBS three times. Then cells sedimentation were resuspended in binding buffer at a density of 1×10^6/ml. Next, adding 10 μl PI and 5 μl Annexin V-FITC to stain apoptosis cells. Staining cells were calculated with BD LSR flow cytometer (BD Biosciences, San Jose, CA, United States) and the data was

analyzed by a software named FlowJo (Gondhalekar et al., 2018; Libregts et al., 2018).

TUNEL Staining

To visualize apoptotic bodies, cells slides were firstly fixed in 10% buffered formalin at room temperature for 25 min and then supplemented with 0.2% Triton X-100 solution in PBS after washing twice with PBS at room temperature for 5 min. Subsequently, cells slides were covered with 100 μl equilibration buffer and then equilibrated for 10 min. Add rTdT reaction mix to the slides and the slide was covered with coverslip. Next, remove the coverslip and terminate the reaction using saline-sodium citrate. After that, cell slides were immersed in 0.3% H_2O_2 for 3–5 min. After being washed three times with PBS, slides were immersed in 50 μl of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) detection solution at 15–25°C for 60 min. At last, slides were incubated with DAPI (Bi Yuntian Biological Technology, China) for 10 min. TUNEL positive cells were visualized with a fluorescence microscope.

Small Interfering RNA Analysis

According to the manufacturer's protocol, transfection of AML-12 cells was carried out with 100 nM of small interfering RNA (siRNA) by using Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA, United States). The Arrb2-siRNA and a negative scrambled siRNA were both synthesized by GenePharma (Shanghai, China). The siRNA sequences were as follows: siRNA-Arrb2 (mouse), 5′-GGACCAGGGUCUUCAAGAATT-3′ (sense) and 5′-UUCUUGAAGACCCUGGUCCTT-3′ (antisense). The AML-12 cells were cultured in F-12 for 12 h, and then subjected to reverse transfection by using Opti-MEM (Gibco, United States). Next, the culture medium was changed after 6 h transfection. Quantitative real-time PCR and western blot analysis were used after siRNA transfection. It was worth noting that all experiments were repeated three times.

Plasmid Construction

The mouse pEX3-Arrb2 was purchased from GenePharma (Shanghai, China). Using pEX3-Arrb2 transfection made ectopic high expression of Arrb2 and empty vector pEX3 was used as control. Firstly, the constructed plasmid was transfected into AML-12 cells and then using quantitative real-time PCR and western blot for further analysis. All experiments were repeated three times in the same way.

Total RNA Extraction and Quantitative Real-Time PCR

Total RNA was extracted from liver hepatocyte and AML-12 cells by using TRIzol (Invitrogen, United States), and then reverse transcribed to the first-strand cDNA by using TaKaRa kit (QIAGEN, Japan). The mRNA expression of Arrb2 was detected by quantitative real-time PCR analyses kits (Applied Biosystems, United States). The mRNA level of β-actin was used as an internal control. Quantitative real-time PCR was performed at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The primers sequences were listed as follows: Arrb2 (forward: GGCAAGCGCGACTTTGTAG and reverse: GTGAGGGTCACGAACACTTTC).

Western Blot Analysis

Isolated mouse hepatocyte and AML-12 cells were lysed with RIPA lysis buffer containing PMSF (100:1). The concentration of extract protein was determined using BCA protein assay kit (Beyotime, Jiangsu, China). Equal amounts of extracted protein were separated by SDS-PAGE and blotted onto PVDF membranes. Firstly, block non-specific binding with TBST containing 5% skim milk for 1 h at room temperature. Then nitrocellulose blots were incubated with the primary antibody against Arrb2 for 12 h at 4°C. The next day, the membranes were incubated with HRP-conjugated secondary antibodies at 37°C for 1 h after washed three times with TBS/Tween 20 (0.075%). Signals of bands were visualized with ECL-chemiluminescent kit. The characteristics of antibodies were listed in **Supplementary Table 1**.

Statistical Analysis

All data were presented as means ± SD analyzed using Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, United States, version 13.0). Two samples were analyzed by using *t*-test, multiple samples were analyzed by using Kruskal–Wallis one-way analysis of variance (ANOVA). In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Pathological Characteristics and Characterization of a Mouse Model of ALD After Binge Ethanol Feeding

As described in Section "Materials and Methods," all the male C57BL/6J mice fed with alcohol for 16 days displayed significant immune cell inflammation, injury and plenty of lipid accumulation in the liver, while the CD-fed mice showed normal cell state. The degree of liver injury and histological features were assessed by using hematoxylin eosin (H & E) staining and Oil Red O staining. As shown in **Figure 1A**, liver tissues in EtOH-fed mice displayed liver cell cord derangement, fat lipid vacuoles, cell spaces dilatation, and inflammatory cell infiltration compared to the CD-fed mice that showed normal radiating hepatic cord. Moreover, the liver tissue of EtOH-fed mice exhibited abundant lipid droplet by using Oil Red O staining (shown in **Figure 1B**). In the initial stage, body weights of both EtOH-fed and CD-fed mice were decreased slightly. After a short-time adaptive phase, body weight in both group was gradually increased, but the body weight of EtOH-fed mice was significantly decreased at the end of the stage and still lower than the beginning (**Figure 1C**). Interestingly, the liver to body weight ratio of EtOH-fed group was notably higher than CD-fed group (**Figure 1D**). The serum levels of ALT and AST in EtOH-fed mice was remarkably increased compared to the CD-fed mice (**Figures 1E,F**). The metabolic changes in the above

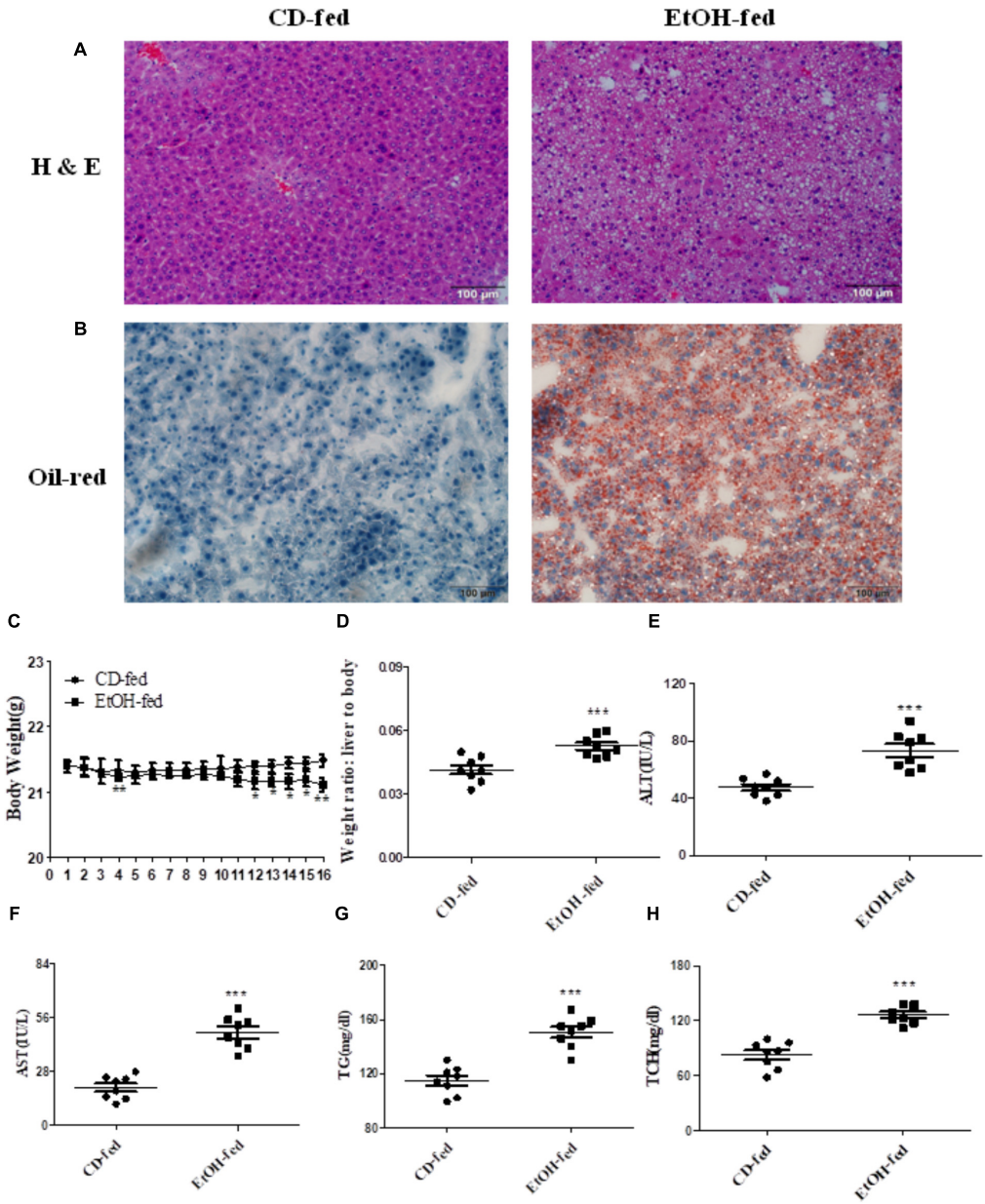

FIGURE 1 | Pathological characteristics in ALD mouse model after binge ethanol feeding. **(A)** Representative views of hematoxylin and eosin (H & E) staining of liver tissues (original magnification, ×20). **(B)** Representative views of Oil Red O staining of liver tissues (original magnification, ×20). **(C)** Body weights after ethanol feeding. **(D)** The liver to body weight radio after ethanol feeding. **(E)** The serum levels of ALT. **(F)**The serum levels of AST. **(G)** Hepatic triglyceride (TG) levels. **(H)** Hepatic total cholesterol (TCH) levels. The values represent means ± SD. (n = 6 in CD-fed group, n = 6 in EtOH-fed group) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CD-fed group.

result were further confirmed by measuring the degree of TG and TCH (**Figures 1G,H**).

Expression Level of Arrb2 in Mouse Liver Is Significantly Increased After Binge Ethanol Feeding

To investigate the expression profile of Arrb1 and Arrb2 between the EtOH-fed group and CD-fed group, real-time PCR and western blot were used to detect the mRNA level and protein level of liver tissues, respectively. As shown in **Figures 2A,B**, both mRNA levels of Arrb1 and Arrb2 were increased, and the upregulation of Arrb2 is more significant than Arrb1. So we presumed that Arrb2 may play more important role in ALD. Next, we detected the expression of Arrb2 in the hepatocytes isolated from the mice liver. mRNA and protein level of Arrb2 were increased by more than onefold compared with CD-fed group (**Figures 2C,D**). Immunofluorescent (IF) analysis further

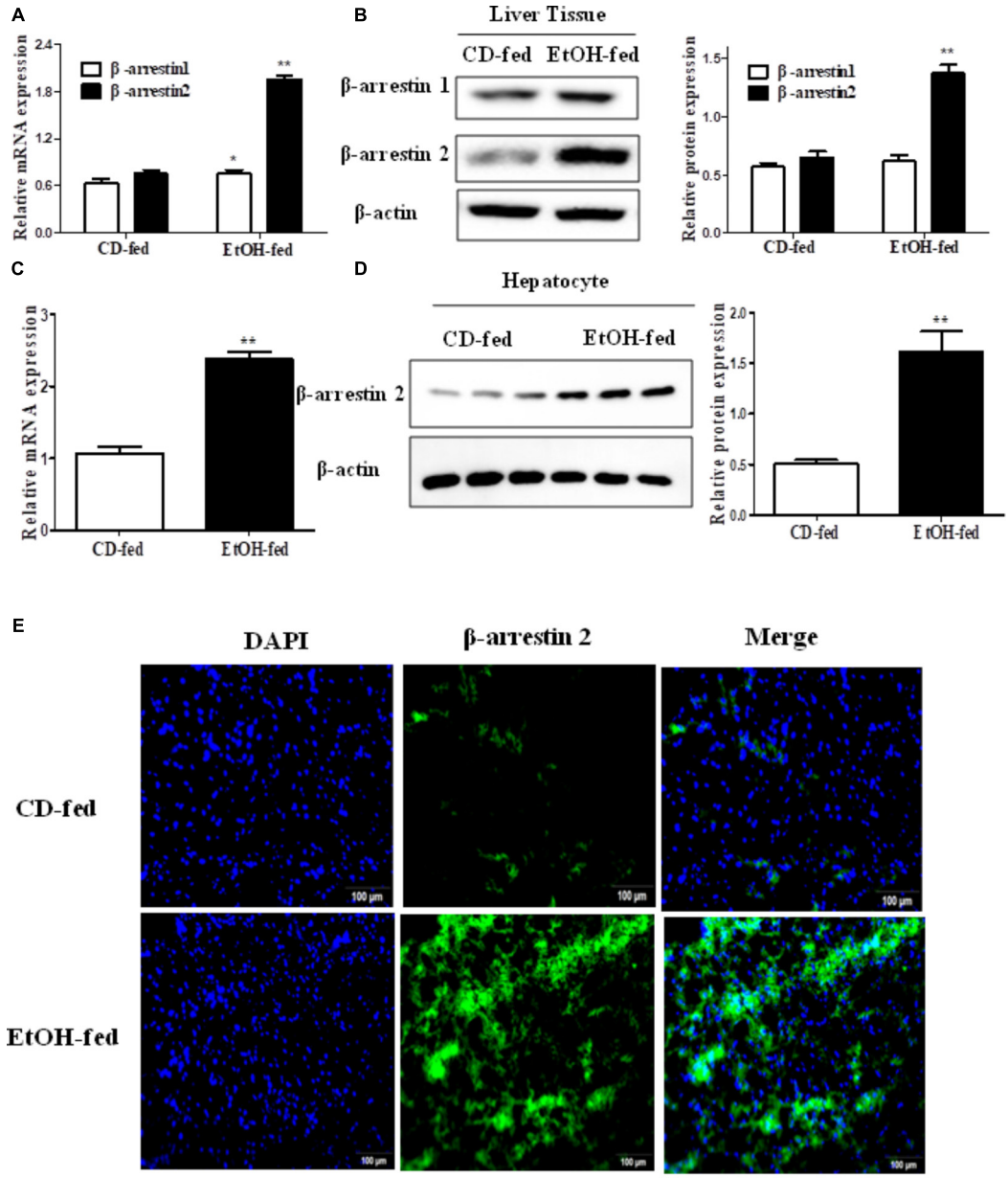

FIGURE 2 | Effect of alcohol on Arrb2 expression in liver tissues and hepatocytes in ALD mouse model. **(A)** Total Arrb1 and Arrb2 mRNA levels in liver tissues were analyzed by real-time PCR. **(B)** The protein levels of Arrb1 and Arrb2 in liver tissues were analyzed by western blot in ALD mouse model. **(C)** Total Arrb2 mRNA levels in hepatocytes isolated from liver tissues were analyzed by real-time PCR. **(D)** The protein levels of Arrb2 in hepatocytes isolated from liver tissues were analyzed by western blot. **(E)** The expression of Arrb2 in liver tissues was analyzed by immunofluorescence (IF) assay (original magnification, ×20). The values represent means ± SD. (n = 6 in CD-fed group, n = 6 in EtOH-fed group) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CD-fed group.

showed the changes of Arrb2 and the results in **Figure 2E** showed a prominent increase in EtOH-fed group mice.

Arrb2 Induces Hepatocytes Apoptosis *in vivo*

The observation above indicated that up-regulation of Arrb2 may play key roles in the progression of ALD. Hence, to explore whether Arrb2 affects the development of ALD by regulating hepatocytes apoptosis, the lentivirus were used to knockdown the expression of Arrb2 in ALD mouse model. The lentivirus vector have fluorescence and glow green by using microscope. Firstly, the changes of ALT, AST, TG, and TCH in serum were detected to evaluate the effect of Arrb2 on alcoholic liver injury. The results in **Figure 3A** showed that the degree of liver injury was decreased in pGLV2-Arrb2 mice group. Secondly, the TUNEL staining and apoptosis assay by flow cytometric analysis were carried out. As shown in **Figure 3C**, the sum of B2 and B4 quadrant fraction

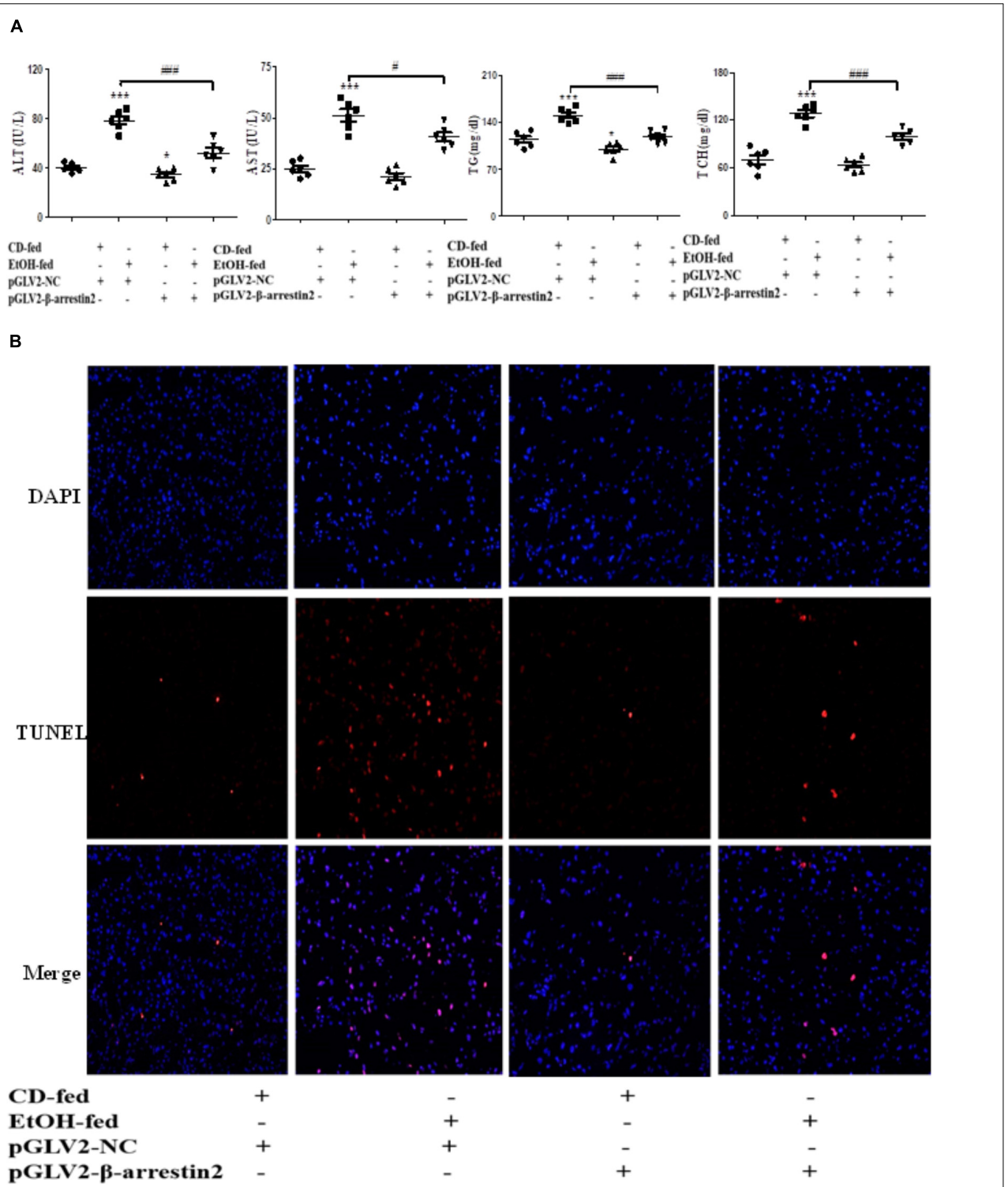

FIGURE 3 | Continued

displayed apoptotic cells. Therefore, the results in **Figures 3B,C** demonstrated that down-regulated expression of Arrb2 could suppress hepatocyte apoptosis. To further investigate the effect of Arrb2 in regulating hepatocyte apoptosis, real-time PCR, and western blot were used to detect the mRNA levels of Arrb2 after lentivirus injection and apoptosis related proteins levels in pGLV2-Arrb2 mice group. The results in **Figures 3D,E** further indicated that Arrb2 could induce hepatocytes apoptosis *in vivo*.

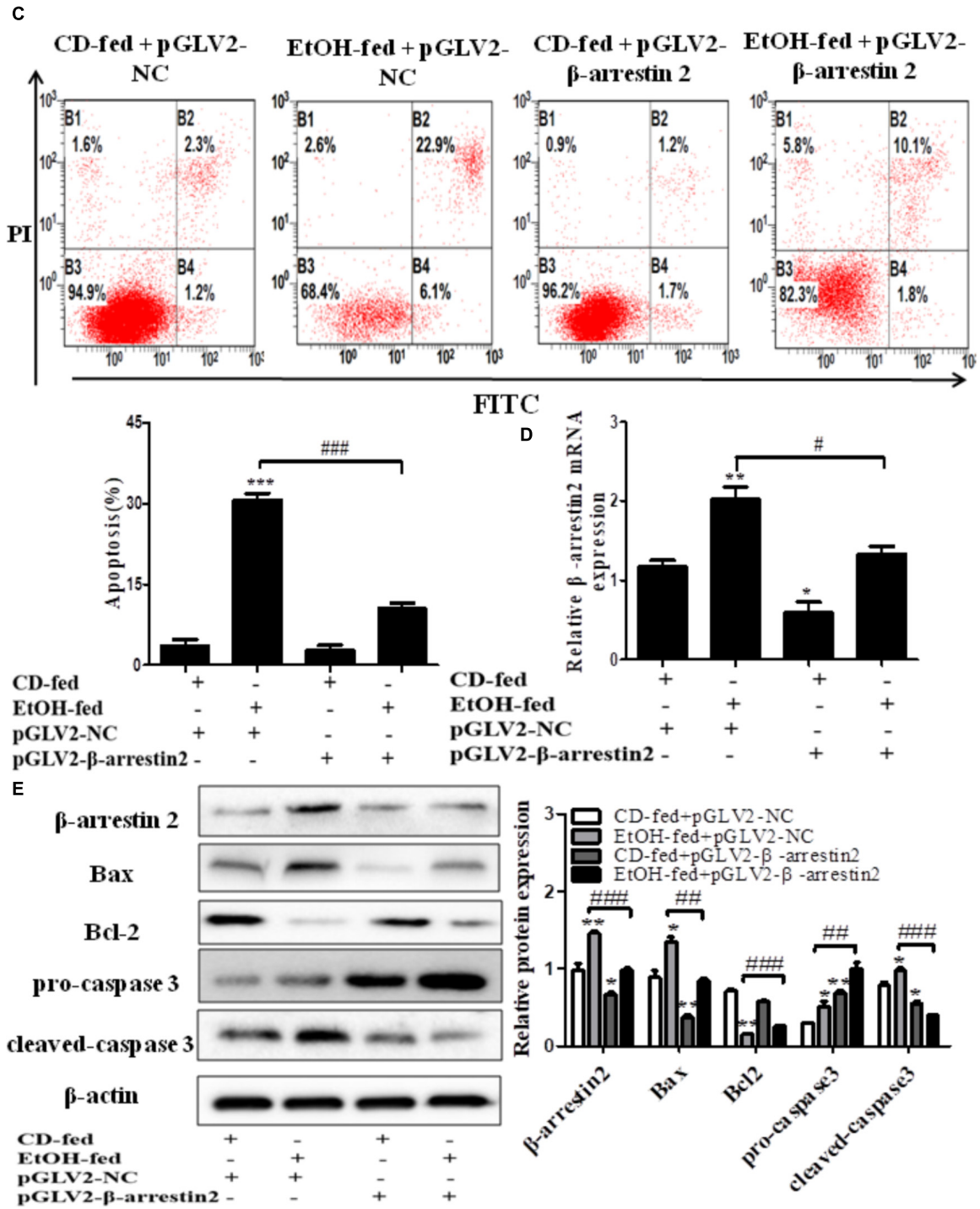

FIGURE 3 | Effect of Arrb2 silencing on ALD mouse model in hepatocytes. **(A)** The serum levels of ALT, AST, TG, TCH were detected after injecting lentivirus to knockdown Arrb2 expression. **(B)** Representative images of TUNEL staining in different groups by using fluorescence microscope (original magnification, ×20). **(C)** Apoptosis of hepatocytes isolated from ALD model mice liver tissues were analyzed by flow cytometry with Annexin V-FITC and PI staining. **(D)** The mRNA levels of Arrb2 were detected by real-time PCR. **(E)** The apoptosis relative protein expression were observed by western blot. The results are shown as relative expression against control expression without treatment. The values represent means ± SD. (n = 3 in CD-fed group, n = 3 in EtOH-fed group). Data shown are the mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. CD-fed + pGLV2-NC. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. EtOH-fed + pGLV2-NC.

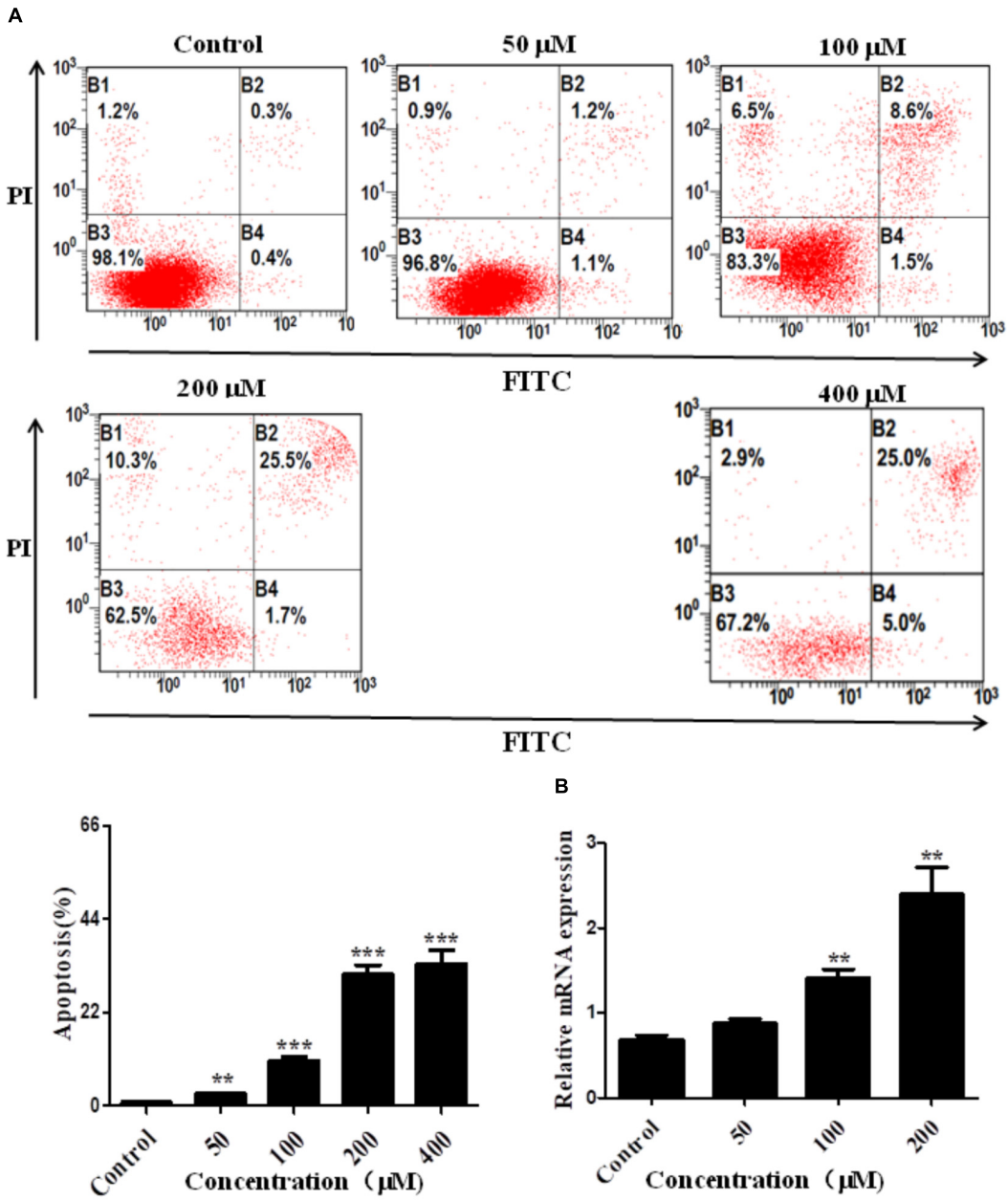

FIGURE 4 | Effect of alcohol on Arrb2 expression *in vitro*. **(A)** Effect of different concentrations of ethanol on AML-12 cells apoptosis were detected by flow cytometry with Annexin V-FITC and PI staining. **(B)** The mRNA levels of Arrb2 at different concentrations were detected by real-time PCR. The results are shown as relative expression against control expression without treatment. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control.

Arrb2 Induces Hepatocyte Apoptosis *in vitro*

To confirm the pro-apoptosis roles of Arrb2 in ALD, the experiment *in vitro* were carried out to further verify the results *in vivo* above. First of all, flow cytometric analysis combined with real-time PCR were used to find the optimal concentration of ethanol which can stimulate AML-12 cells to imitate acute alcohol treatment of ALD. The results in **Figures 4A,B** showed that ethanol enhanced mRNA levels of Arrb2 in a concentration-dependent manner and 200 μM of ethanol was selected as the optimal concentration. IF analysis further showed the changes of Arrb2 and the results in **Supplementary Figure 1A** showed a significantly increase in EtOH (200 μM)-stimulated group. Secondly, As shown in **Figure 5A**, Arrb2 siRNA was transfected into AML-12 cells with or without the treatment of ethanol transiently and the results of real-time PCR demonstrated that

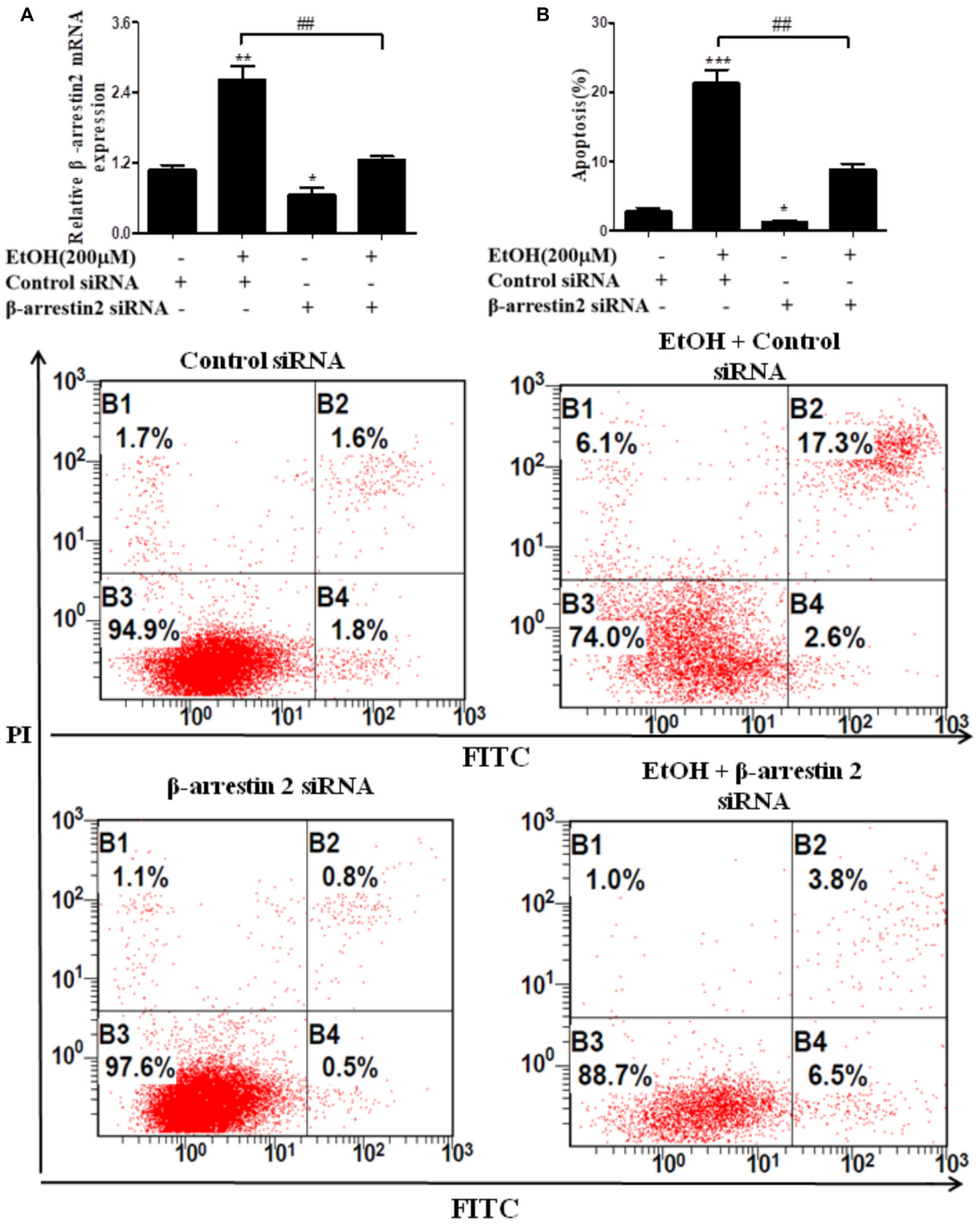

FIGURE 5 | Continued

Arrb2 was successfully transfected into AML-12 cells because the mRNA levels was decreased obviously. In line with the above date *in vivo*, the hepatocyte apoptosis was substantially decreased by using TUNEL staining, flow cytometric analysis, and western blot when Arrb2 was blocked *in vitro* (**Figures 5B–D**). On the contrary, pEX3-Arrb2 was utilized to over-express Arrb2 and it was successfully up-regulated the mRNA levels of Arrb2 by real-time PCR (**Figure 6A**). Result showed that over-expression of Arrb2 led to significant hepatocyte apoptosis (**Figures 6B–D**).

Arrb2 Promotes Hepatocyte Apoptosis by Inhibiting Akt Signaling Pathway

In recent years, evidence has shown that Arrb2 modulated Akt signaling pathway, but whether Akt signaling is associated

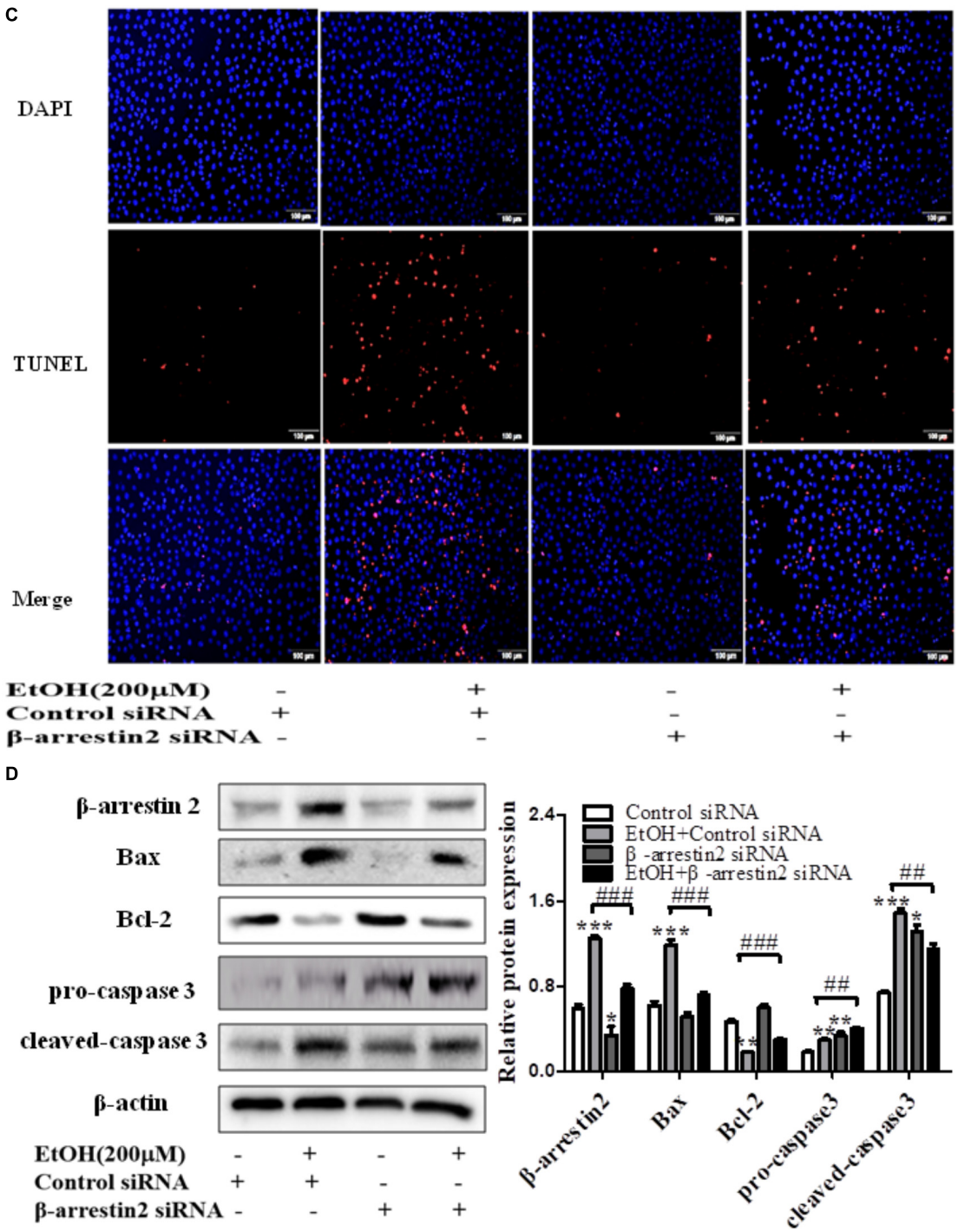

FIGURE 5 | Effect of Arrb2 siRNA on EtOH-stimulated AML-12 cells. **(A)** The Arrb2 levels were detected by real-time PCR after transfection. The results are shown as relative expression against control expression without treatment. **(B)** Apoptosis of AML-12 cells were analyzed by flow cytometry with Annexin V-FITC and PI staining. **(C)** Representative images of TUNEL staining in different groups (original magnification, ×20). **(D)** The apoptosis relative protein expression were detected by western blot. A representative image of the three independent experiments were demonstrated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control siRNA. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. EtOH + Control siRNA.

with hepatocyte apoptosis in ALD remains unknown (Povsic et al., 2003; Beaulieu et al., 2005). To better understand the mechanism by which EtOH up-regulated Arrb2 expression in ALD and Arrb2 promoted hepatocyte apoptosis *in vivo* and *in vitro*, we next determined the protein levels of phospho-Akt in Arrb2-siRNA hepatocyte and pEX3-Arrb2 hepatocyte with or

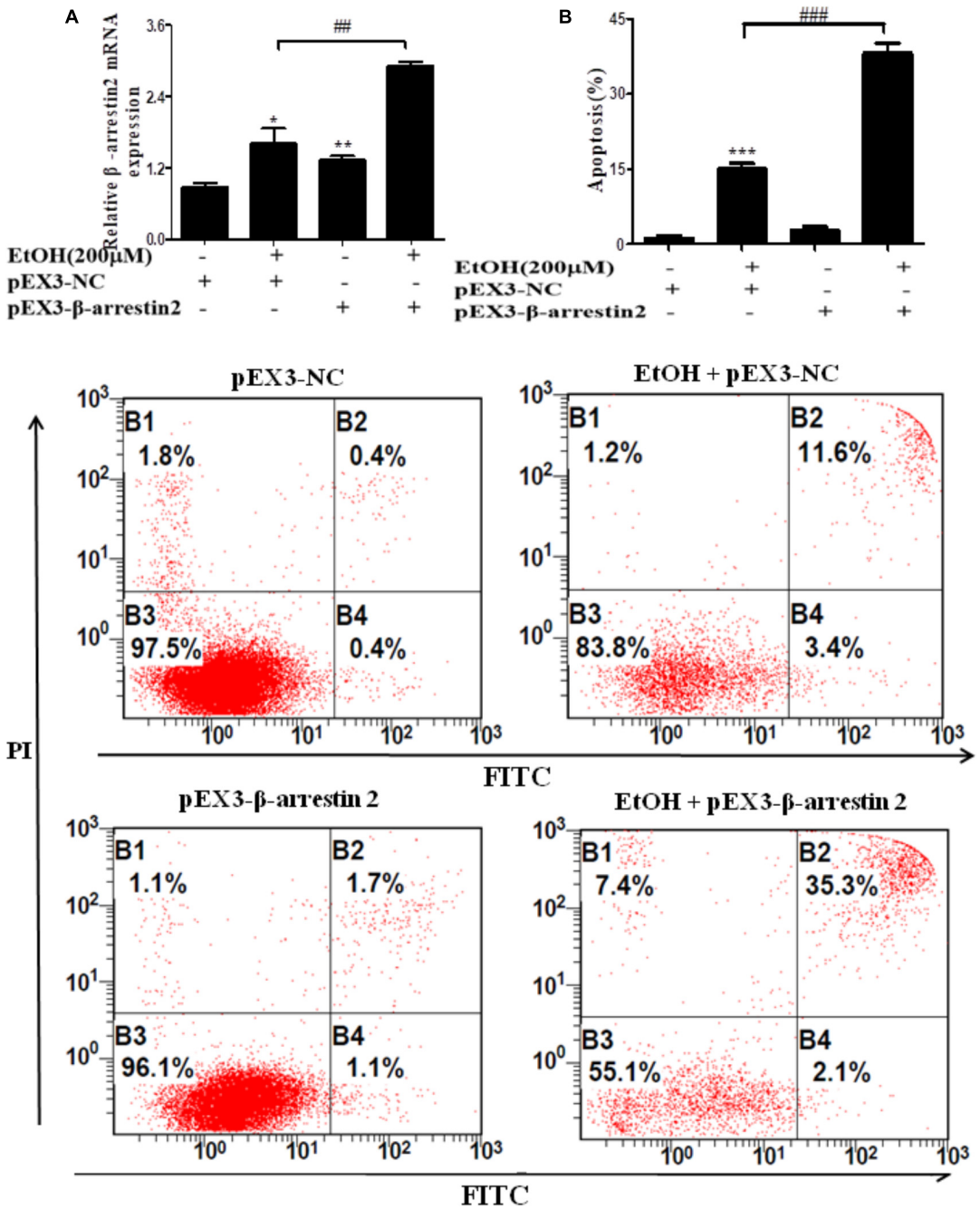

FIGURE 6 | Continued

without the treatment of ethanol. Western blot results showed that Arrb2 increased hepatocyte apoptosis by inhibiting Akt signaling pathway (**Figures 7A,B**). To further confirm the effect of Akt signaling pathway on AML-12 cells apoptosis in ALD, a chemical inhibitor for Akt named LY249002 was used to suppress Akt pathway in EtOH-stimulated AML-12 cells. As shown in **Supplementary Figure 2**, results of western blot showed that treatment of 25 μM LY249002 significantly decreased p-Akt protein, leading to enhanced hepatocyte apoptosis.

DISCUSSION

Liver is the most important metabolic organ. Hepatocytes are damaged in response to the toxic metabolites, cytokines, chemokines, and reactive oxygen species (ROS) which cause cell apoptosis and death (Nassir and Ibdah, 2014). Alcohol abuse is a major cause for liver-related morbidity and mortality which can lead to steatosis, progressive fibrosis, cirrhosis, and ultimately HCC. Although the pathogenesis of ALD is poorly

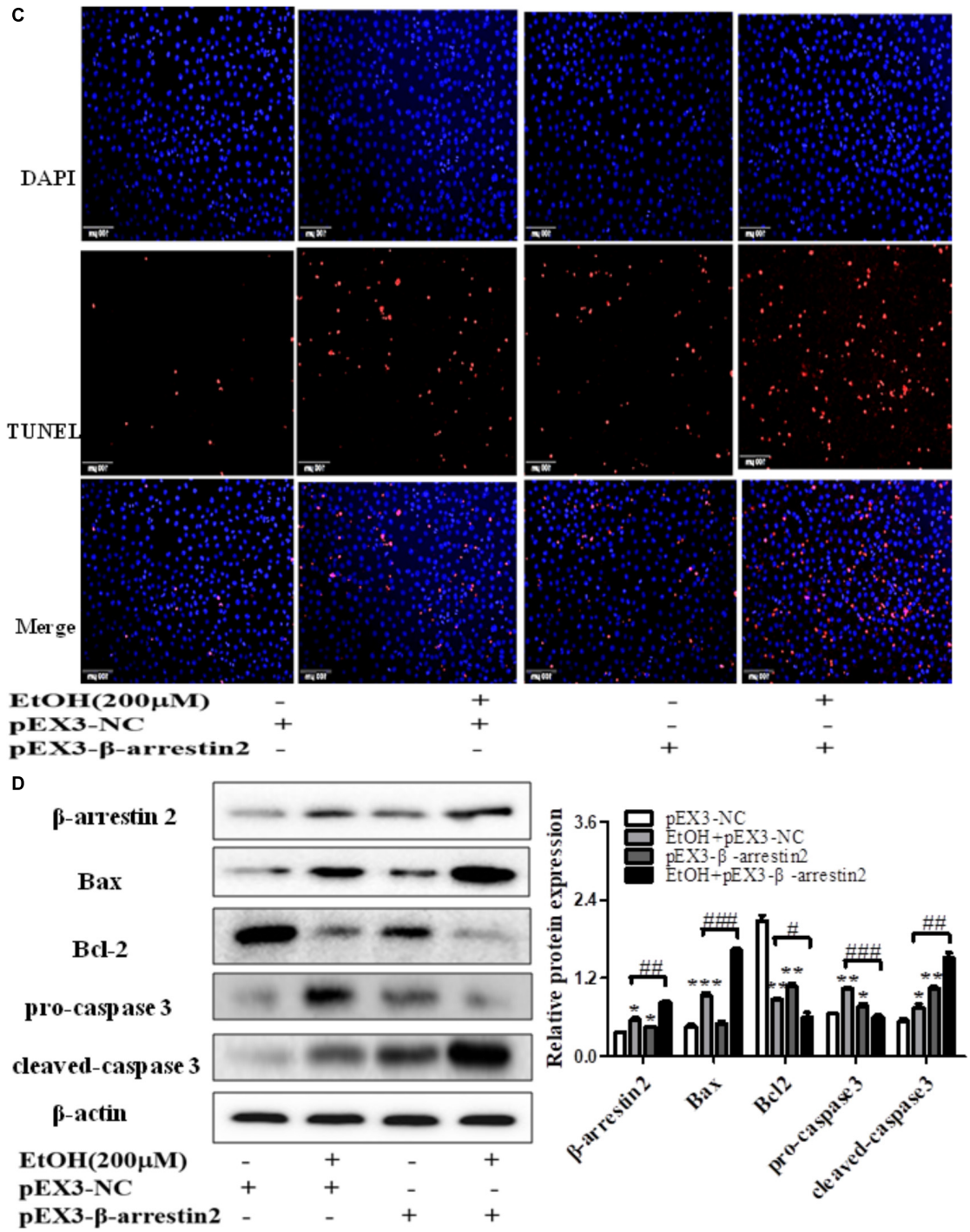

FIGURE 6 | Effect of pEX3-Arrb2 on EtOH-stimulated AML-12 cells. **(A)** The Arrb2 levels were detected by real-time PCR after over-expression plasmid construction. The results are shown as relative expression against control expression without treatment. **(B)** Apoptosis of AML-12 cells were analyzed by flow cytometry with Annexin V-FITC and PI staining. **(C)** Representative images of TUNEL staining in different groups (original magnification, ×20). **(D)** The apoptosis relative protein expression were detected by western blot. A representative image of the three independent experiments were demonstrated. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. pEX3-NC. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. EtOH + pEX3-NC.

understand, several studies demonstrated that apoptosis of massive hepatocytes is a prominent feature of the initiation and progression stages of ALD (Ceni et al., 2014; Verma et al., 2016).

Available evidence suggested that apoptosis can either be triggered by intrinsic (mitochondria) or extrinsic (death receptor) pathway. The alcohol metabolism plays a key role

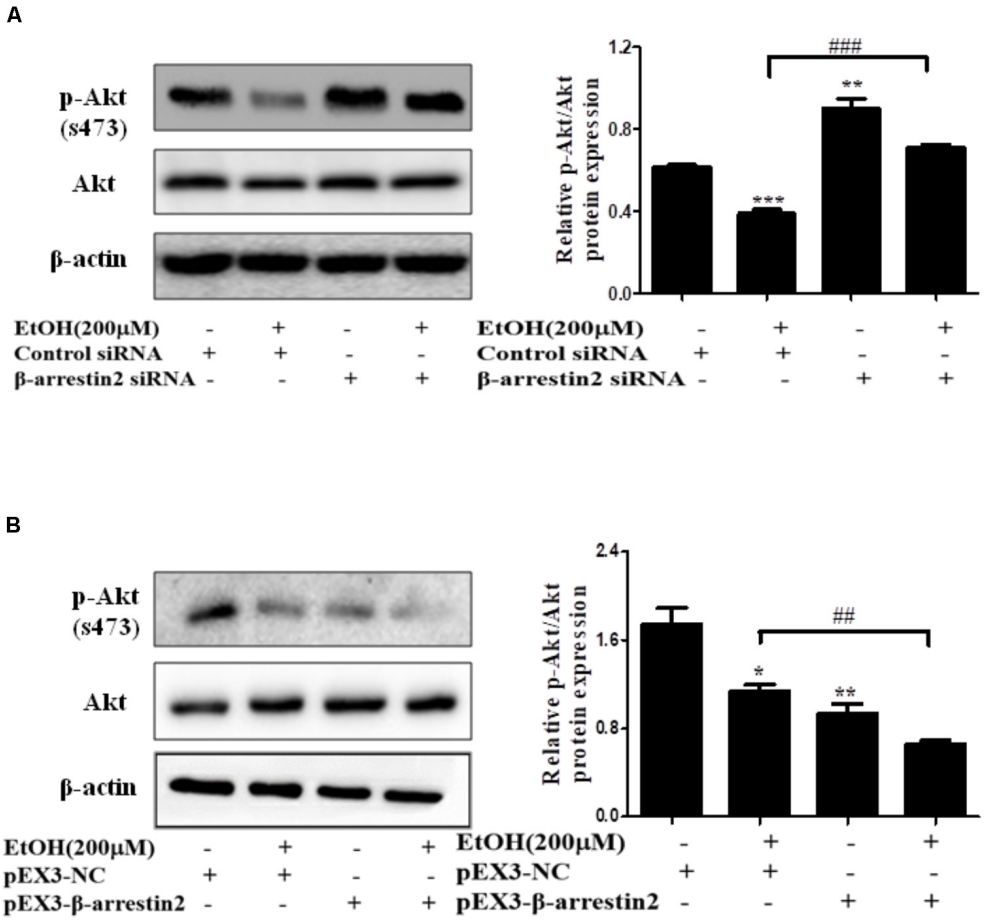

FIGURE 7 | The interaction of Arrb2 with Akt in EtOH-stimulated AML-12 cells. **(A)** The protein levels of phospho-Akt in EtOH + Arrb2 siRNA transfected to AML-12 cells were assessed by Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control siRNA. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. EtOH + Control siRNA. **(B)** The protein levels of phospho-Akt and in EtOH + pEX3-Arrb2 transfected to AML-12 cells were assessed by Western blot. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. pEX3-NC. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. EtOH + pEX3-NC. Data shown are the mean ± SD from three independent experiments.

in activation of mitochondria-dependent apoptotic pathway. Many apoptotic stimuli, such as DNA damage or growth factor, were involved in regulating mitochondria-dependent apoptotic pathway (Wang S. et al., 2016). Bailey and Cunningham (2002) demonstrated that the process of alcohol metabolism accelerated the flow of electrons and accumulated leakage of electrons in mitochondrial respiratory chain, thereby produced ROS. In addition, several studies revealed that alcohol exposure destroyed mitochondrial DNA resulting in the reduction of mitochondrial protein and ATP synthesis (Coleman and Cunningham, 1991). Furthermore, chronic alcohol exposure suppressed the rate of mitochondrial oxygen consumption, leading to increased sensitivity of hepatocytes in response to alcohol, and eventually caused the hypoxia and hepatocytes apoptosis or even death (Zelickson et al., 2011). It has been demonstrated that the mitochondria-dependent apoptotic pathway was mainly regulated by the Bcl-2 family proteins. Bcl-2-family members included three clusters: pro-survival subfamily (Bcl-2, Bcl-w, Bcl-XL, cl-1, and A1), pro-apoptotic subfamily (Bax, Bok, and Bak), and BH3-only group. The anti-apoptotic gene, Bcl-2, and the pro-apoptotic gene, Bax and caspase-3 played critical roles in ALD (Renault and Manon, 2011; Vervliet et al., 2016). It is now clear that caspases family became the main regulator in apoptosis and survival signals. In response to death signals, caspases are synthesized and processed to form active heterotetrameric enzymes (Wang S. et al., 2016). It was reported that activated caspase also cleaved pro-apoptotic Bcl-2 family protein (Yin and Ding, 2003).

In our study, the results showed that Arrb2 levels in liver tissue from EtOH-fed mice were notably higher than those in CD-fed mice. Furthermore, hepatocyte were isolated from the liver, and the results illustrated that the expression of Arrb2 was significantly increased in EtOH-fed mice compared to CD-fed mice. Arrb2 was also remarkably up-regulated in AML-12 cells in response to alcohol exposure. All above findings suggest that up-regulation of Arrb2 may play an important role in the development of ALD. However, the possible mechanisms for the EtOH-mediated upregulation of Arrb2 in ALD remains unknown. Arrb2 played an anti-inflammatory role of fenoterol in AICAR-treated THP-1 cells by activating nuclear transcription

factor NF-κB (Wang W. et al., 2016). In systemic diseases, Arrb2 decreased the secretion of NF-κB-dependent gene products from fibroblast-like synoviocytes (Yu et al., 2015). Additionally, the bone marrow-derived macrophages from Arrb2 knockout mice showed a greater IκB kinase activity than WT mice macrophages (Liu et al., 2016). Therefore, we supposed that the nuclear transcription factor NF-κB may be involved in EtOH-mediated upregulation of Arrb2 and this possibility remained to be further explored. Then we further evaluated the function of Arrb2 in hepatocyte apoptosis. Our data demonstrated that knockdown of Arrb2 diminished hepatocyte apoptosis *in vivo*. In line with the *in vivo* data, the results in our study demonstrated that Arrb2 largely promoted apoptosis of hepatocyte *in vitro*.

It has been shown that the phosphatidylinositol 3-kinase (PI3K)/Akt pathway modulates cell survival, apoptosis, autophagy, and differentiation (Ouyang et al., 2017; Zhou et al., 2018). The mechanisms of PI3K/Akt/mTOR signaling pathway activation include the extension or mutation of PI3K and Akt, the loss of phosphatase and tensin homolog (PTEN) function and activation of growth factor receptor (LoPiccolo et al., 2008). The PI3K/Akt/mTOR signaling pathway is a prototypic survival pathway and is activated in many types of cancer disease (Mayer and Arteaga, 2016; Li et al., 2018). For example, the PI3K regulatory subunit p85 alpha can suppress tumor extension through negative regulation of growth factor signaling in breast cancers and HCCs. *N*-glycosylation of β4-integrin can promote tumor development and progression through activating PI3K in human cutaneous squamous cell carcinoma (Engelman, 2009; Taniguchi et al., 2010; Kariya et al., 2018). Consistently, activation or inhibition of PI3K/Akt/mTOR signaling pathway also played a critical role in many types of liver diseases. Zhang et al. (2018) demonstrated that long non-coding RNA lncARSR promoted hepatic lipogenesis by activating PI3K/Akt/mTOR pathways in non-alcoholic fatty liver disease (NAFLD). Conversely, Wei et al. (2018) revealed that asiatic acid attenuated HSC activation and extra cellular matrix (ECM) synthesis by inhibiting PI3K/Akt/mTOR signaling pathways in liver fibrosis (Zhang et al., 2018). In recent papers, it has been shown that Arrb1 and Arrb2 modulated Akt signaling pathway in HCC but it remains unknown that whether Akt signaling is associated with hepatocyte apoptosis in ALD (Yang et al., 2015; Yin et al., 2016). Given the above, the protein levels of phospho-Akt was detected in Arrb2 knockdown and overexpression hepatocytes with or without the treatment of ethanol. Our data showed that deficiency of Arrb2 increased phosphorylation of Akt while over-expressing Arrb2 suppressed Akt activation. In conclusion, our study is the first report in exploring the potential role of Arrb2 and the molecular mechanisms for the regulation of Arrb2 on hepatocyte apoptosis in ALD. This finding gained the support from the study that Arrbs can scaffold different molecules thereby playing different, even opposite effects on the same signaling pathway (Ma and Pei, 2007; Qi et al., 2016). More importantly, our study illustrates the cross-talk of Arrb2 with Akt signaling pathway and provides new insights of Arrb2 in hepatocyte apoptosis. Furthermore, Arrb2 may be used as a novel therapeutic target for ALD. However, the upstream modulators for Arrb2 in ALD remain to be further investigated.

AUTHOR CONTRIBUTIONS

JL contributed the experimental materials. Y-YS designed the experiments and research. Y-XZ performed the experiments. X-FL analyzed the data. CH and X-MM performed the experiments. All authors reviewed and approved this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01031/full#supplementary-material

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REVIEW
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Therapeutic Targets for Treatment of Heart Failure: Focus on GRKs and β-Arrestins Affecting βAR Signaling

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Heart failure (HF) is a heart disease that is classified into two main types: HF with reduced ejection fraction (HFrEF) and HF with preserved ejection fraction (HFpEF). Both types of HF lead to significant risk of mortality and morbidity. Pharmacological treatment with β-adrenergic receptor (βAR) antagonists (also called β-blockers) has been shown to reduce the overall hospitalization and mortality rates and improve the clinical outcomes in HF patients with HFrEF but not HFpEF. Although, the survival rate of patients suffering from HF continues to drop, the management of HF still faces several limitations and discrepancies highlighting the need to develop new treatment strategies. Overstimulation of the sympathetic nervous system is an adaptive neurohormonal response to acute myocardial injury and heart damage, whereas prolonged exposure to catecholamines causes defects in βAR regulation, including a reduction in the amount of βARs and an increase in βAR desensitization due to the upregulation of G protein-coupled receptor kinases (GRKs) in the heart, contributing in turn to the progression of HF. Several studies show that myocardial GRK2 activity and expression are raised in the failing heart. Furthermore, β-arrestins play a pivotal role in βAR desensitization and, interestingly, can mediate their own signal transduction without any G protein-dependent pathway involved. In this review, we provide new insight into the role of GRKs and β-arrestins on how they affect βAR signaling regarding the molecular and cellular pathophysiology of HF. Additionally, we discuss the therapeutic potential of targeting GRKs and β-arrestins for the treatment of HF.

Keywords: β-adrenergic receptor, β-arrestin, G protein-coupled receptor kinase, heart failure, β-blocker

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HEART FAILURE

Heart failure (HF) is a heart disease with high morbidity and mortality. Based on measurement of the left ventricular ejection fraction (LVEF), HF with an LVEF less than 40% corresponds to HF with reduced ejection fraction (HFrEF) whereas HF with normal LVEF (≥50%) is termed HF with preserved ejection fraction (HFpEF). The therapeutic goals in patients with HF are to improve the clinical outcome and quality of life of HF patients, and also to reduce hospitalization and mortality rates. Angiotensin-converting enzyme inhibitors (ACEIs) and β-adrenergic receptor antagonists (β-blockers) have been shown to improve clinical outcomes and survival in patients with HFrEF and, therefore, are recommended for HFrEF treatment according to the ESC 2016 guideline for treatment of acute and chronic HF (Ponikowski et al., 2016). In several clinical trials,

ACEIs, angiotensin receptor blockers (ARBs), mineralocorticoid receptor antagonists (MRAs), and β-blockers have been shown to reduce mortality and morbidity in patients with HFrEF (Ponikowski et al., 2016). However, none of these drugs have convincingly improved clinical outcomes and reduced morbidity/mortality in patients with HFpEF (Andersen and Borlaug, 2014; Ponikowski et al., 2016; Yamamoto, 2017; Bonsu et al., 2018).

Although β-blockers have dramatically reduced morbidity and mortality rates, β-blockers have limited effectiveness in some HF patients and have adverse effects. Thus, several barriers remain in the management of HF and new treatment strategies for HF need to be developed. In this review, we provide insight into the potential therapeutic targets for the treatment of HF, focusing in particular on G protein-coupled receptor kinases (GRKs) and β-arrestins.

G PROTEIN-COUPLED RECEPTOR KINASE

Upon agonist binding to β-adrenergic receptor (βAR), the heterotrimeric G proteins dissociate into Gα and Gβγ subunits which activate diverse downstream effectors and play fundamental roles in numerous cellular functions. Stimulation with an agonist simultaneously triggers the termination of the βAR signaling and the rapid reduction of the receptor responsiveness through a process called "receptor desensitization." The phosphorylation by GRKs of agonist-occupied βAR corresponds to the first step of desensitization occurring within seconds to minutes which induces the recruitment of cytosolic β-arrestins to the receptor complex located on the plasma membrane. After binding, β-arrestins sterically inhibit further the interaction of βAR with Gαs resulting in their uncoupling of βAR. GRKs and β-arrestins also play an important role in βAR internalization, trafficking, and resensitization (Moore et al., 2007). In addition, GRKs can directly interact as scaffolding proteins with many signaling proteins, resulting in modulation of various physiological responses, and GRKs also phosphorylate several proteins other than receptors (Kurose, 2011; Watari et al., 2014).

GRKs preferentially phosphorylate GPCRs in an activated (agonist-bound) state at serine and threonine residues localized within either the third intracellular loop (ICL3) or C-terminal tail (Ferguson, 2001). This GRK-mediated phosphorylation of GPCR at these residues may regulate the stability of β-arrestin/GPCR complexes (Oakley et al., 1999). Even though GRK phosphorylation sites have been identified for some receptors, no distinct GRK phosphorylation consensus sequence/motif has been identified. The β_2AR has a short ICL3 and a long C-terminal tail containing several serine and threonine residues. Mutation of all phosphorylation sites within the ICL and the C-terminal tail of β_2AR attenuates GRK-mediated phosphorylation of receptors (Bouvier et al., 1988). In addition, the ICL3 of β_2AR is associated with G protein activation and the specificity of the interaction between receptor and G protein (Reiter and Lefkowitz, 2006). On the contrary, human β_1AR is resistant to GRK-mediated desensitization and internalization. Human β_1AR does not internalize upon agonist stimulation and has lower affinity for β-arrestins than β_2AR (Suzuki et al., 1992; Shiina et al., 2000). However, mouse β_1AR is internalized by agonist stimulation (Volovyk et al., 2006). The physiological meaning of this species difference is unknown. Among GRKs, GRK2 has a fairly strict dependency of agonist binding for receptor phosphorylation, while GRK5 has the higher ability for phosphorylating agonist-unbound receptor as compared to GRK2 (Tran et al., 2007). GRK5-promoted phosphorylation of agonist-unbound receptor may help the receptor to activate β-arrestin-biased signaling that is primarily activated by antagonists. GRK-catalyzed phosphorylation of β_1AR enhances β-arrestin-mediated signaling. It has been reported that β_1AR-mediated biased signaling in the heart requires GRK5-promoted phosphorylation (Nakaya et al., 2012).

Interaction between GRKs and the activated βARs on the plasma membrane is necessary for GRK-catalyzed receptor phosphorylation. A recent study revealed a dynamic mechanism of complex formation between GRK5 and β_2AR (Komolov et al., 2017). Two major domains of GRK5 [the regulator of G protein signaling homology (RH) and the catalytic domain] are able to dissociate following binding to activated β_2AR causing disruption of a transient electrostatic contact between these two domains. These changes facilitate contacts between ICL2, ICL3, and the C-terminal tail of β_2AR with the GRK5 RH bundle domain, the membrane-binding surface, and the kinase catalytic pocket, respectively (Komolov et al., 2017).

GRK FAMILY

According to their amino acids sequence and ternary structural homology, GRK family members can be divided into three groups as follows: rhodopsin kinase subfamily (GRK1 and GRK7), βAR kinase (βARK) subfamily (GRK2 and GRK3), and GRK4 subfamily (GRK4, GRK5, and GRK6) (Penn et al., 2000; Penela et al., 2003). The structure of GRKs consists of three distinct domains: an amino terminal (N-terminal) domain, a central highly conserved catalytic domain, and a carboxyl terminal (C-terminal) domain. The N-terminal domain involved in receptor binding and recognition of the activated receptor contains a region of homology to the regulator of G protein signaling protein and Ca^{2+}/calmodulin-binding domain (Penn et al., 2000; Penela et al., 2003). The N-terminal domain of GRK4, GRK5, and GRK6 contains a phosphatidylinositol 4,5-bisphosphate (PIP_2)-binding site allowing the amplification of their kinase activities (Pitcher et al., 1998), whereas the N-terminus of GRK2 contains a Gβγ-binding site causing GRK2 binding to the plasma membrane (Eichmann et al., 2003). The central domain is highly conserved among GRKs and exerts the kinase catalytic function.

The C-terminal domain of GRKs is involved in plasma membrane targeting and membrane binding by means of post-translational modifications or interaction with membrane phospholipids. The C-terminal domains of GRKs are divergent among subfamilies. GRK1 and GRK7 interact with the plasma

membrane via a post-translational modification at their C-termini. The C-terminal domain of GRK2 and GRK3 is composed of a pleckstrin homology domain that includes a phospholipid-binding site and a Gβγ-binding site (Penn et al., 2000; Penela et al., 2003).

We reported in our previous study that GRK2 has a clathrin-binding motif at its C-terminus domain enabling the kinase to cooperate with the heavy chain of clathrin (Shiina et al., 2001), a step necessary for βAR internalization (Mangmool et al., 2006). GRK4 and GRK6 are post-translationally palmitoylated at cysteine residues located in their C-terminal domains, leading to plasma membrane localization and interaction. GRK5 binds to membrane phospholipids through electrostatic interaction of the positively charged amino acid located in its C-terminus. Finally, membrane localization of GRK4, GRK5, and GRK6 is regulated by the interaction with membrane PIP_2 via their PIP_2-binding sites (Penela et al., 2003). GRKs mediate the desensitization and internalization of βARs, including β_1AR and β_2AR. It is likely that subtype specificity of GRKs underlies observed differences in the regulation of βARs. In HEK-293 cells overexpressing GRK2, but not GRK5 and GRK6, the agonist-induced β_2AR phosphorylation was inhibited by the treatment of clathrin heavy chain siRNA. These results suggest an important role for clathrin in GRK2-mediated β_2AR phosphorylation and internalization (Mangmool et al., 2006). In addition, GRK2 phosphorylates four amino acid residues (Thr384, Ser396, Ser401, and Ser407) of the C-terminal tail of β_2AR whereas GRK5 phosphorylates six amino acid residues (Thr384, Thr393, Ser396, Ser401, Ser407, and Ser411) of the C-terminal tail of β_2AR (Fredericks et al., 1996).

More than hundreds of GPCRs have been identified in the human while seven members only of GRK family have been identified. It is not well understood why a limited number of GRKs regulate various GPCRs. The distribution pattern and expression levels of each GRK seem important factors contributing to their specificity and functional roles in many tissues, including the heart.

GRK EXPRESSION IN THE HEART

GRK2 (known as βARK1), GRK3, and GRK5 are highly expressed in the human heart, whereas GRK4, GRK6, and GRK7 are only expressed at minimal levels (Ungerer et al., 1993). The distribution of each GRK isoform is different among heart cells. GRK2 and GRK5 are expressed in almost all cardiac cells, whereas GRK3 is detected only in cardiac myocytes (Vinge et al., 2001; Penela et al., 2006). The functional role of GRK subtypes in the heart under normal and pathological conditions may be influenced by their distribution. The functional role of GRK2 in cardiac fibroblasts was recently identified and GRK2 modulates contractility and remodeling following ischemia/reperfusion injury (Woodall et al., 2016). GRK expression and activity are changed in many cardiovascular diseases, especially HF. Thus, the functional roles of GRKs have been extensively studied in the heart as diagnostic markers and/or therapeutic targets for HF (Hullmann et al., 2016).

ROLE OF GRK IN HF

Dysregulation of βARs is a pathological hallmark of HF; in particular, βARs are significantly downregulated and desensitized because of the upregulation of GRKs, especially GRK2 and GRK5 (Sato et al., 2015). βARs are targets for GRK-mediated phosphorylation and desensitization, and increased expression and activity of GRK2 in the heart are associated with the loss of βAR functions that induces deleterious effects. Although these events lead to the development and progression of HF, the inhibition of GRK2 expression or activity is able to restore cardiac functions (Petrofski and Koch, 2003).

In HF, the increase in catecholamine levels is derived from chronic sympathetic activation, resulting in overstimulation of βARs. The heart adapts to excessive stimulation by blunting the βAR responsiveness to catecholamines (Lymperopoulos et al., 2013; de Lucia et al., 2018). This process called βAR desensitization requires GRKs. However, desensitization and downregulation of βARs are not sufficient to compensate fully for chronic overstimulation of the sympathetic system. Prolongation of excessive stimulation over time becomes harmful to the heart and is responsible for the majority of HF (Lymperopoulos et al., 2013; de Lucia et al., 2018). Indeed, βAR desensitization is involved in the decrease of β_1AR expression (βAR downregulation) and the uncoupling of βAR from G protein occurs during HF.

Because GRKs are involved in desensitization and downregulation of βAR, whether expression and activity of one or more GRKs is increased in HF patients or HF model animals has been extensively investigated in the past several years (**Table 1**). Chronic administration of isoproterenol resulted in βAR desensitization, upregulation of GRK2, and hypertrophy in mouse hearts (Iaccarino et al., 1998b). In addition, several studies have demonstrated that expression and activity of cardiac GRK2 are significantly increased in the failing heart (Ungerer et al., 1993; Harris et al., 2001; Rengo et al., 2011; Sato et al., 2015), indicating that upregulation of GRK2 plays a pivotal role in the HF associated with the dysfunction of βAR-mediated signaling.

Transgenic mice with cardiac-specific overexpression of GRK2 had contractile responses to βAR abolished and displayed physiological alterations (e.g., impairment of βAR functions and signaling, and cardiac hypertrophy), resulting in a failing heart in these mice (Koch et al., 1995; Rockman et al., 1998). Moreover, GRK2 high expression is detected in patients with end-stage dilated HF (Ungerer et al., 1994) and in several conditions related to HF development, including myocardial ischemia (Ungerer et al., 1996) and hypertension (Gros et al., 1997). Taken together, these results show that GRK2 dysfunction plays a pivotal role in heart diseases. However, the exact mechanism responsible for upregulation of GRK2 following βAR overstimulation in the compromised heart is not clearly understood.

Conversely, attenuation of GRK2 activity by expression of the carboxyl terminal domain of GRK2 (βARKct), which inhibits agonist-dependent GRK2 translocation to the membrane, or a reduction in GRK2 expression, enhanced cardiac functions (Koch et al., 1995). In addition, overexpression of βARKct could restore the diminished βAR contractile function and largely reverse the

TABLE 1 | Changes in GRKs and β-arrestins levels and activities in animal models of HF and HF patients.

Experiments/Populations	Results	Reference
Human dilated cardiomyopathy	• Increased GRK2 and GRK5 mRNA levels • Unchanged GRK3 mRNA level	Dzimiri et al., 2004
Human failing heart	• Elevated GRK2 mRNA level and activity in failing heart	Ungerer et al., 1993
Human failing heart	• Increased GRK2 and GRK5 (but not GRK3) protein levels in left ventricles	Agüero et al., 2012
Human failing heart	• Increased GRK2 mRNA level • Slightly increased GRK3 mRNA level • Unchanged β-arrestin1 and β-arrestin2 mRNA levels	Ungerer et al., 1994
Rabbit failing heart	• Elevated GRK2 protein level and activity in post-myocardial infarction (post-MI) heart	Maurice et al., 1999
Isolated perfused rat heart	• Increased GRK2 mRNA level and activity during myocardial ischemia	Ungerer et al., 1996
Rat model of congestive heart failure (CHF)	• Increased GRK2, GRK5, β-arrestin1, and β-arrestin2 mRNA levels in failing heart • Increased GRK2, GRK5, and β-arrestin1 in post-infarction failing heart	Vinge et al., 2001
Pacing-induced CHF in pig	• Increased total GRK activity • Increased GRK5 mRNA and protein levels • Unchanged GRK2 mRNA and protein levels	Ping et al., 1997
Pressure-overload cardiac hypertrophy in mice	• Increased GRK activity	Choi et al., 1997

impaired cardiac functions in animal models of HF, such as muscle LIM protein (MLP) knockout (KO) mice (Rockman et al., 1998) and calsequestrin-overexpression mice (Harding et al., 2001). Thus, GRK2 expression in the heart appears to be related with cardiac contractile function. Previous studies have provided strong evidence that GRK2 plays a pivotal role in the βAR-mediated development of HF (Iaccarino et al., 1998b; White et al., 2000; Raake et al., 2008) and the increase of GRK2 expression can be used as an early marker for HF (Rengo et al., 2011; Lymperopoulos et al., 2013). Taken together, GRK2 acts as a central modulator of βAR signaling in the heart and could serve as a HF diagnosis biomarker.

Moreover, GRK2 also plays an important role in the βAR-mediated cardiac insulin resistance. Overstimulation of myocardial β_2ARs and upregulation of GRK2 are associated with insulin resistance in the heart (Mangmool et al., 2017). Chronic stimulation of β_2ARs, but not β_1ARs, resulted in impaired insulin-induced glucose uptake and IRS-1 phosphorylation (Cipolletta et al., 2009) and also significantly reduced the actions of insulin to induce GLUT4 expression and translocation in cardiac myocytes and heart tissues (Mangmool et al., 2016). In addition, upregulation of β_2AR enhances GRK2 expression that is related with βAR-induced insulin resistance in heart tissue (Ciccarelli et al., 2011) and in animal models of insulin resistance (Cipolletta et al., 2009). Thus, inhibition of GRK2 activity leads to enhanced insulin sensitivity in the heart. In animal models of diabetes, inhibition of GRK2 and GRK3 through synthetic peptides rescues glucose tolerance and improves insulin sensitivity (Anis et al., 2004). Other myocardial GRK isoform functions in this context, including GRK5, and the precise mechanism of GRK2 for βAR-mediated cardiac insulin resistance represent interesting areas of future research.

During pathological states of heart conditions, the expression of other GRK isoforms is also altered. GRK3 and GRK5 have been demonstrated to participate in the pathogenesis of HF. GRK5 expression is increased in animal models of HF (Ping et al., 1997; Vinge et al., 2001; Yi et al., 2002). GRK5 expression is also increased in dilated cardiomyopathy and volume-overloaded human left ventricle (Dzimiri et al., 2004), whereas the expression of GRK3 remains stable in dilated cardiomyopathy and is slightly induced in patients with right ventricular volume overload (Ungerer et al., 1993, 1996; Dzimiri et al., 2004). Nevertheless, dynamic alterations of GRK3 and GRK5 related to the development of HF remain to be elucidated.

In a model of transgenic mice overexpressing cardiac-specific GRK5, a considerable decline of βAR signaling and inotropic responsiveness was detected (Rockman et al., 1996). Interestingly, overexpression of GRK3 in mice does not affect βAR signaling and cardiac function (Iaccarino et al., 1998a), indicating that GRK3 does not share functional characteristics of GRK2, even though these two GRKs belong to the same subfamily. GRK3 might play a role desensitization of in α_1AR rather than βAR. Phosphorylation of α_1AR by GRK3 contributed to cardiac hypertrophy and dysfunctions that occur during chronic pressure overload (von Leuder et al., 2012). In addition, inhibition of GRK3 activity using GRK3ct preserves cardiac function and prevents the development of HF after chronic pressure overload (von Leuder et al., 2012).

Although overexpression of GRK5 in the heart results in attenuation of βAR-mediated signaling and function (Rockman et al., 1996), the complete deletion of GRK5 as in GRK5-KO mice does not significantly affect βAR-mediated responses in the heart (Gainetdinov et al., 1999). However, cardiac hypertrophy and failing heart after pressure overload are detected

in transgenic mice with cardiac-specific overexpression of GRK5 (Martini et al., 2008). These detrimental effects of GRK5 are derived from its activity in the nucleus. GRK5 activity in the nucleus may be associated with a progression of a maladaptive cardiac hypertrophy that is independent of βARs (Martini et al., 2008). In addition, cardiac-specific deletion of GRK5 exhibits cardioprotective effects against pathological hypertrophy and HF after pressure overload (Gold et al., 2012). GRK5 is also found to be required for β_1AR-mediated transactivation of epidermal growth factor receptor (EGFR) that confers cardioprotection in mice (Noma et al., 2007). Since GRK5 has been shown to have both detrimental and cardioprotective effects, regulation and compartmentalization of GRK5 in normal and failing hearts represent the most important issues when considering the inhibition of GRK5 as therapeutic target for HF.

The GRK5-Leu41 polymorphism of GRK5, with leucine at position 41 substituted for glutamine, is abundantly found in African American populations. Interestingly, GRK5-Leu41 is a gain-of-function genetic polymorphism that enhances desensitization of βAR (Liggett et al., 2008). GRK5-Leu41 allele decreases the activity of βAR signaling in a similar way to a partial blockade of βAR by β-blockers, promoting cardioprotective effects against experimental catecholamine-induced cardiomyopathy. HF patients with the GRK5-Leu41 allele show improved survival (Liggett et al., 2008), suggesting that modulation of GRK5 remains a powerful target for the treatment of HF. However, the specific contribution of each GRK isoform to the development of a failing heart remains to be determined.

THERAPEUTIC APPROACHES OF GRKs FOR THE TREATMENT OF HF

Even though β-blockers inhibit HF progression and improve the quality of life in HF patients, these drugs show modest effectiveness in improving the contractile functions of the failing heart in animal models. If increased GRK activity and expression are important elements in desensitization and dysregulation of βAR in HF patients, potential therapeutic strategies aimed to modulate GRKs by preventing their expression and activity would consequently boost the ability of cardiac myocytes to respond to adrenergic stimulation (Koch et al., 1995; Korzick et al., 1997). For example, in animal models of HF, expression of βARKct, a GRK2 inhibitor, delays the progression of functional and biochemical modifications of the βAR signaling associated with HF (Rockman et al., 1998). Thus, inhibition of GRK by different strategies might be a novel therapeutic approach for restoring cardiac functions in the failing heart (**Table 2**). We will summarize results and strategies to inhibit GRK activity that could help to design novel therapeutic strategies for HF management.

βARKct (or GRK2ct)

βARKct, a polypeptide of 194 amino acids, consists of the Gβγ binding domain of GRK2 (Hullmann et al., 2016). Inhibition of GRK2 using βARKct dramatically improves cardiac contractility in animal models of HF (Hata et al., 2004). The mechanism of action proposed for βARKct is to inhibit the activity of endogenous GRK2 by competing with the endogenous GRK2 for Gβγ-binding, thus, attenuating GRK2 membrane translocation and activation, resulting in a reduction of GRK2-mediated βAR desensitization (Koch et al., 1995). Providing inhibitory βARKct to several animal models of HF leads to the delay of cardiac dysfunction and increased survival (**Table 2**). Overexpression of βARKct improves cardiac functions, prevents cardiac remodeling and cardiac hypertrophy, and increases survival rates in several animal models of HF (White et al., 2000; Shah et al., 2001). Moreover, cardiac-specific βARKct overexpression prevents cardiac remodeling and development of HF in MLP KO mice, a model of dilated cardiomyopathy with elevated GRK2 levels in the heart, suggesting that inhibition of GRK2 activity represents an approach to prevent the development of HF (Rockman et al., 1998). Similarly, cardiac-specific overexpression of βARKct results in cardiac contractility improvement, a delay of adverse remodeling, and a prolonged lifespan in HF model mice with calsequestrin overexpression, which show severe cardiomyopathy and markedly reduced survival rate (Harding et al., 2001). The beneficial effects of βARKct were enhanced by co-treatment with metoprolol, suggesting that inhibition of GRK2 provides a better clinical outcome than in HF patients treated only by β-blockers (Harding et al., 2001). When cardiac myocytes isolated from heart tissues from HF patients were infected by adenovirus expressing βARKct, the βARKct-overexpressing myocytes exhibited significant increases of the heart ability to contract and relax in response to adrenergic stimulation (Williams et al., 2004). Thus, inhibition of GRK2 in the failing heart has beneficial effects on cardiac performance. However, it should be noted that βARKct might mediate its beneficial effects via mechanisms distinct from inhibiting GRK2, as βARKct is able to inhibit several Gβγ-mediated signaling pathways.

Even though gene therapy using βARKct represents a promising strategy for the treatment of HF, the large size of βARKct, and the virus requirement for heart-specific expression may represent major obstacles for clinical development. Small peptide or synthetic compounds that specifically inhibit GRK activity (selective GRK inhibitor) may provide a much easier way at a lower cost when searching for therapeutic approaches for HF. These data support the use of GRK2 inhibitor, including βARKct as a promising therapeutic approach for the treatment of chronic HF (Rengo et al., 2011). The effects of GRK2 inhibition might be similar or greater than those of classical β-blocker therapy (Reinkober et al., 2012).

PAROXETINE

Paroxetine is a selective serotonin reuptake inhibitor (SSRI) antidepressant that is found to be a GRK2 inhibitor (Homan et al., 2014). Paroxetine binds to GRK2 and inhibits GRK2 catalytic activity more potently than other GRK isoforms

TABLE 2 | GRKs as the therapeutic targets for HF treatment.

Experiments/Populations	Results	Reference
Transgenic mice with cardiac-specific overexpression of βARKct	• Overexpression of βARKct enhanced cardiac contractility and improved cardiac functions	Koch et al., 1995
Cardiac-specific overexpression of βARKct in HF model mice (MLP KO mice)	• Overexpression of βARKct prevented the progression of cardiomyopathy	Rockman et al., 1998
Cardiac-specific overexpression of βARKct in HF model mice (calsequestrin overexpressed mice)	• Overexpression of βARKct markedly prolonged survival and restored cardiac functions in failing heart	Harding et al., 2001
βARKct was expressed by adenovirus-mediated gene transfer in ventricular myocytes isolated from human failing heart	• Expression of βARKct improved contractile function and βAR-mediated responses in failing human cardiac myocytes	Williams et al., 2004
GRK2 gene ablation in mice of post-MI model	• Deletion of GRK2 before coronary artery ligation delayed maladaptive post-infarction remodeling and restored βAR signaling and functions • GRK2 deletion initiated 10 days after MI enhanced survival, improved contractility, and inhibited cardiac remodeling	Raake et al., 2008
Mice of post-MI HF model	• Paroxetine prevented HF development due to inhibition of GRK2 activity	Schumacher et al., 2015
Cardiac myocytes (*in vitro*) and mice (*in vivo*)	• Paroxetine increased βAR-mediated cardiomyocyte contractility *in vitro* • Paroxetine improved βAR-mediated left ventricular inotropic reserve *in vivo*	Thal et al., 2012

(Thal et al., 2011). From crystallization and structure analysis, paroxetine was reported to occupy the active site of GRK2 leading to the stabilization of the GRK2 kinase domain in a unique inactive conformation (Thal et al., 2011). Paroxetine enhances βAR-mediated shortening and contraction of isolated cardiac myocytes and increases βAR-mediated left ventricular inotropic reserve *in vivo* (Thal et al., 2012), similar to βARKct.

In addition, administration of paroxetine to an HF mouse model demonstrated an improvement of LV structure and function and attenuated the expression of the fetal genes, representing an index of progression to HF (Schumacher et al., 2015). These cardioprotective effects of paroxetine are due to the inhibitory activity of GRK2 but not the SSRI activity of paroxetine, suggesting that paroxetine prevents HF progression in an SSRI-independent manner. Moreover, equivalent doses of fluoxetine, another SSRI, did not show any of these effects. The result emphasizes that the effects of paroxetine are due to GRK2 inhibition (Schumacher et al., 2015). Thus, paroxetine-mediated inhibition of GRK2 enhances cardiac performance, reverses sympathetic overstimulation, normalizes the myocardial βAR functions, and protects the heart after myocardial infarction (MI) (**Table 2**). These data demonstrate that paroxetine-mediated inhibition of GRK2 improves cardiac function after MI and represents a potential repurposing of this drug, as well as starting point for innovative small-molecule GRK2 inhibitor development.

Interestingly, chronic treatment with various SSRIs (e.g., fluoxetine, paroxetine) stimulated serotonin receptor type 4 (5-HT_4R) desensitization in cerebral regions implicated in depression, including basal ganglia and hippocampus (Licht et al., 2009; Vidal et al., 2009). Moreover, upregulation of GRK2 significantly suppressed serotonin-induced cAMP generation in COS-7 cells, suggesting a negative regulatory role for GRK2 at the 5-HT_4R level (Barthet et al., 2005). In addition, serotonin-induced 5-HT_{4A}R internalization was inhibited by expression of either dominant negative (DN) GRK2 and DN β-arrestin1 ($\beta arr_{1319-418}$) in HEK-293 cells, suggesting that GRK2 and β-arrestin are involved in the trafficking of 5-HT_4Rs (Mnie-Filali et al., 2010).

GRK-mediated phosphorylation of 5-HT_4R exhibits high affinity for β-arrestins, which binds to the phosphorylated receptor and inhibit coupling with G proteins. This results in the inhibition of further signaling (Mnie-Filali et al., 2010). The 5-HT_4R/GRK/β-arrestin complex may confer different signaling and regulatory characteristics to the 5-HT_4 receptors, leading perhaps to new functional roles and eventually therapeutic implications. Since paroxetine is an inhibitor of GRK2 that plays an important role in 5-HT_4R desensitization and trafficking, the exact mechanisms by which paroxetine affects the desensitization and the trafficking of various serotonin receptors, especially 5-HT_4R remain to be elucidated.

OTHER SYNTHETIC GRK INHIBITORS

Since GRK2 and GRK5 are upregulated in the failing heart and play major roles in the progression of cardiac dysfunction, including HF, a selective inhibitor of GRK2 and/or GRK5 might represent a promising target for HF treatment. Balanol

is a synthetic compound that was found to inhibit GRK2 activity (Setyawan et al., 1999; Tesmer et al., 2010). However, balanol also inhibits other protein kinases such as protein kinase A (PKA), protein kinase C (PKC), and protein kinase G (PKG). A selective GRK2 inhibitor was developed by a two-step rational drug design process, and compound 10 (Methyl 5-[2-(5-nitro-2-furyl)vinyl]-2-furoate) was found to inhibit GRK2 more selectively than PKA (Iino et al., 2002). This compound was the first inhibitor that was able to distinguish GRK2 from PKA, a protein kinase that has a similar adenine-binding pocket.

A GRK2 inhibitor screening has been performed using a compound library, leading to identify various novel candidates (Homan et al., 2015). Currently, most active compounds can be divided into two chemical classes: indazole/dihydropyrimidine-containing compounds that strongly inhibit GRK2 and pyrrolopyrimidine-containing compounds that are selective for inhibition of GRK1 and GRK5 (Homan et al., 2014, 2015). Several new GRK inhibitors are also synthesized such as GSK180736A and GSK2163632A. These compounds were co-crystallized with GRKs (Homan et al., 2015). GSK180736A is a selective GRK2 inhibitor, which binds to GRK2 in a similar way to paroxetine, whereas GSK2163632A occupies a novel region of the GRK active site that is related to its selectivity (Homan et al., 2015). Further development by in silico screening, using GSK180736A and CCG215022 as templates, identified two new compounds (compounds 33 and 37) as potent GRK2 and GRK5 inhibitors (Waldschmidt et al., 2018). The IC_{50} value of GSK180736A toward GRK2 and GRK5 are 0.77 μM and >100 μM, respectively (Waldschmidt et al., 2018). However, the screening did not identify any compounds that exhibited high GRK5 selectivity.

Overstimulation of βAR is associated with the pathogenesis of insulin resistance in the heart (Mangmool et al., 2017). For instance, chronic βAR stimulation causes the development of insulin resistance through an increase in GRK2 levels (Cipolletta et al., 2009). Moreover, upregulation of GRK2 impairs cardiac glucose uptake and promotes insulin resistance after MI (Ciccarelli et al., 2011). The small peptides (e.g., KRX-683107 and KRK-683124) derived from the catalytic domain of GRK2 and GRK3 have been shown to improve glucose metabolism. By modulating GRK2 and GRK3 activities, these two peptides enhanced GPCR-mediated signal transduction, resulting in an antidiabetic effect (Anis et al., 2004). In animal models of diabetes, inhibition of GRK2 and GRK3 through these synthetic peptides rescues glucose tolerance and enhances insulin sensitivity (Anis et al., 2004). Thus, these small peptides could be useful as GRK inhibitors by interfering with kinase-substrate interactions. However, the efficacies of these small peptides in animal model of HF are not known. To develop specific inhibitors for GRKs, the sequence of the first intracellular loop (ICL1) of the β_2AR was synthesized and found to inhibit GRKs (Winstel et al., 2005). IC_{50} value of a peptide with the sequence AKFERLQTVTNYFITSE for GRK2 is 0.6 μM. This peptide also inhibited GRK3 and GRK5 with an IC_{50} of 2.6 and 1.6 μM, respectively (Winstel et al., 2005). Furthermore, the peptide inhibitor did not suppress PKC and PKA activities because ICL1 of β_2AR presents selectivity GRK2 over PKC and PKA (Benovic et al., 1990). However, the efficacy of these synthetic and peptide inhibitors of GRKs remain to be tested in animal models of HF, which means that another 6 to 10 years are needed before they can be tested in human if they show promising effects in animal models and preliminary safety data are obtained.

β-ARRESTINS

Stimulation of βARs with agonists leads to GRK-mediated receptor phosphorylation that promotes the recruitment of β-arrestins to phosphorylated βARs. Then β-arrestins sterically inhibit further G protein coupling to βARs. After β-arrestin binds to and forms a complex with βAR and GRK, β-arrestin promotes internalization of βAR into the cytosol, which can lead to receptor degradation (downregulation) and receptor recycling back to the plasma membrane (Moore et al., 2007). Thus, β-arrestins play essential roles in βAR internalization and trafficking (Lefkowitz and Shenoy, 2005; Lefkowitz et al., 2006).

Although both β_1ARs and β_2ARs are Gs protein-coupled receptors, their functional properties differ and lead to subtype-specificity interaction with β-arrestins. For instance, the binding of β-arrestin-1 and -2 to the third intracellular loop (ICL3) and the C-terminal tail of β_1AR is lower than that for β_2AR (Shiina et al., 2000). A chimeric β_2AR containing the C-terminal tail of β_1AR lost its ability to promote β-arrestin2-mediated ERK nuclear translocation (Kobayashi et al., 2005). Moreover, stimulation of β_1AR, but not β_2AR, induces a conformational change in β-arrestin that promotes a stable β-arrestin/Epac/Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) complex (Mangmool et al., 2010). The association of β-arrestin with β_1AR stabilizes this complex and promotes CaMKII signaling (Mangmool et al., 2010). It is interesting that β-arrestin deletion results in differential ERK activation in a βAR subtype-selective manner (O'Hayre et al., 2017; Grundmann et al., 2018). Deletion of β-arrestin results in enhancement of β_2AR-mediated ERK activation, but decrease of β_1AR-mediated ERK activation. Enhancement of β_2AR-mediated ERK activation is due to the impaired desensitization of β_2AR and decrease of β_1AR-mediated ERK activation is due to the lack of the scaffolding function of β-arrestin.

In addition to regulate GPCR endocytosis and trafficking, β-arrestins themselves function as scaffolding proteins and signal transducers to stimulate various downstream effectors, including MAPK cascades induction (e.g., ERK1/2 and JNK3), Src activation (Lefkowitz and Shenoy, 2005; Lefkowitz et al., 2006), and EGFR transactivation (Noma et al., 2007). Furthermore, β-arrestins can interact and form a complex with CaMKII (Mangmool et al., 2010). Complex formation facilitates CaMKII activation, which plays a key role in cardiac hypertrophy and apoptosis that cause HF (Mollova et al., 2015). The ability of β-arrestins to desensitize βARs is well established. In the present paper, we will mainly review the roles of β-arrestins in βAR-mediated signaling in normal and HF conditions, their function

in the heart, and their potential as therapeutic target for HF treatment.

β-ARRESTIN FAMILY AND STRUCTURE

The arrestin family consists of four members. Arrestin1 and arrestin4 (known as visual arrestin, and cone arrestin, respectively) are expressed in the rods and cones of eyes, respectively. The β-arrestin1 and β-arrestin2 (known as arrestin2 and arrestin3, respectively) are abundantly expressed throughout mammalian tissues (Gurevich and Gurevich, 2004). Arrestin contains an N-domain and a C-domain, each consisting of seven stranded β-sheets, linked through a short linker region (Graznin et al., 1998; Hirsch et al., 1999).

The N-domain of arrestins contains a recognition region for activated receptors and the C-domain contains a secondary receptor recognition region (Gurevich et al., 1995). The phosphate sensor region is located in the linker between N-domain and C-domain and forms part of the hydrophilic core of arrestins. The interaction between the end of C-domain and the phosphate sensor region maintains the arrestin structure in the resting state. In the active state, this interaction is disrupted upon receptor binding, allowing arrestin to bind the phosphorylated receptor with a high affinity (Gurevich and Gurevich, 2004).

ROLE OF β-ARRESTIN IN βAR DESENSITIZATION

Agonist binding to βAR promotes receptor coupling with heterotrimeric G proteins and triggers the dissociation into activated $G\alpha_s$ and the Gβγ subunits, which stimulates adenylyl cyclase (AC) and increases cAMP levels. cAMP binds to and interacts with its downstream effectors, resulting in activation of cAMP signal transduction. Subsequent to agonist binding, activated βARs are phosphorylated by GRKs leading to recruitment of β-arrestins and inhibition of further interaction of receptor with G proteins. This process is known as "receptor desensitization" as described previously (Ferguson, 2001; Luttrell and Lefkowitz, 2002).

ROLE OF β-ARRESTIN IN βAR TRAFFICKING

In addition to their regulation of βAR desensitization, β-arrestins are essential for βAR trafficking to intracellular compartments located in the cytosol. This process is called "βAR internalization." Currently, β-arrestins are reported to interact with regulatory proteins required for receptor internalization, including clathrin, AP-2, guanine-nucleotide exchange factors, phosphoinositides, and GTPase activating proteins (reviewed in DeWire et al., 2007). After their internalization, βARs are directed toward two main intracellular pathways either degradation or recycling (Tan et al., 2004). The βARs targeted for recycling are transported to early endosomes where the receptors are dephosphorylated by protein phosphatase 2A (PP2A) (known as βAR dephosphorylation) before being transported back to the plasma membrane. Dephosphorylation of βARs is dependent on the acidification in early endosomes because acidification enhances PP2A catalytic function (Krueger et al., 1997). The βARs targeted for degradation are transferred to lysosomes where they are eventually degraded via ubiquitination mediated degradation (Tan et al., 2004). Both GRK-mediated receptor phosphorylation and β-arrestin binding to β_2ARs are necessary for β_2AR ubiquitination (Shenoy et al., 2001). However, the role of β-arrestin mediated βARs targeting for recycling and degradation is poorly understood.

β-ARRESTIN ACTS AS SCAFFOLDING PROTEINS

In addition to their roles in receptor desensitization and internalization, β-arrestins are recognized as multifunctional scaffolding proteins that work as adaptor proteins linking receptors to several downstream effectors such as ERK1/2 (Luttrell et al., 2001; Shenoy et al., 2006), JNK (McDonald et al., 2000), Src (Luttrell et al., 1999), and calmodulin (Wu et al., 2006). β-Arrestins are also known to bind to and activate CaMKII following β_1AR stimulation (Mangmool et al., 2010). Interestingly, proteomic analysis by mass spectrometry demonstrated that β-arrestins bind to and interact with various types of proteins that play roles in cellular signaling (Xiao et al., 2007). Their abilities to form multifunctional protein complexes are related to functionally selective responses. β-arrestins play an essential role in the CaMKII signaling responsible for cardiac hypertrophy and HF. Stimulation of β_1AR promotes a conformational change in β-arrestin which then induces the formation of a stable complex, including β-arrestin, CaMKII, and cAMP-dependent guanine-nucleotide exchange factor (Epac) (Mangmool et al., 2010). The role of β-arrestin in this multiprotein complex consists of holding Epac and CaMKII in structural proximity to activate CaMKII signaling (Mangmool et al., 2010). Discovering the mechanism of multifunctional complex formation by β-arrestins will help to understand the physiological importance of this scaffolding protein complex in HF.

ROLE OF β-ARRESTINS IN THE FAILING HEART

Downregulation and desensitization of βARs occur in the failing heart, leading to dramatically diminish cardiac functions via reduced contractility (Brodde, 1993; Port and Bristow, 2001). The modulation mechanism of βARs is elucidated at the molecular level and involves GRKs and β-arrestins. Two isoforms of β-arrestin are expressed in the heart, namely β-arrestin1 and β-arrestin2 (Ungerer et al., 1994). The signaling mediated by β-arrestins independently to classical G protein-mediated signaling may be associated with cardioprotective effects (Patel

et al., 2009; Noor et al., 2011). However, the specific roles of each β-arrestin isoform in cardiac βAR dysfunction, leading to the pathophysiology of HF are not fully understood.

CARDIOPROTECTIVE EFFECTS OF β-ARRESTINS

McCrink et al. (2017) have demonstrated that overexpression of β-arrestin2 in mice restores inotropic reserves of β-adrenergic regulation. They have shown that β-arrestin2 directly binds to and activates sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a), a key regulator of β_1AR-dependent cardiac contractility. The association of β-arrestin2 with SERCA2a contributes to SERCA2a SUMO (small ubiquitin-like modifier)-ylation and then increases SERCA2a activity, leading to increased cardiac contractility (McCrink et al., 2017). In contrast, β-arrestin1 had no effect on SERCA2a activation.

Although β-arrestin2 expression is low in the mammalian heart, including humans (Ungerer et al., 1994), β-arrestin2 has cardioprotective roles against HF. Thus, cardiac-specific β-arrestin2 gene transfer is a very attractive approach for gene therapy in HF patients. Another approach to treatment of HF is selective activation of β-arrestin2, although the expression level of β-arrestin2 is low in the heart. TRV120067 is a β-arrestin–biased ligand targeted to the angiotensin II type 1 receptor (AT_1R) that works similar to ARBs for selective blockade of Ang II binding to AT_1R and subsequent G protein coupling, while simultaneously and preferentially activates β-arrestin2-dependent signaling. TRV120067 exhibits cardioprotective effects in a mouse model with dilated cardiomyopathy (Ryba et al., 2017), suggesting that β-arrestin2-dependent signaling confers significant benefits on cardiac function and structure. β-Arrestin2 constitutively localizes in cardiac sarcomeres, and the localization is enhanced by TRV120067 that activates β-arrestin2 and the downstream effector SERCA2a (Ryba et al., 2017).

Many studies have demonstrated that excessive inflammation induces detrimental effects on the heart after MI. Because inflammatory cytokines are reported to induce cardiomyocyte apoptosis (Ing et al., 1999; Li et al., 2007), it is possible that they enhance apoptosis in the infarct area. However, inflammation is also reported to be necessary for recovery of the heart from MI-induced injury. Monocyte subsets phagocytose dead cells and produce anti-inflammatory cytokines such as TGF-β (Frangogiannis, 2008). TGF-β plays a crucial role in cardiac repair by suppressing inflammation and promotes differentiation of fibroblasts into myofibroblasts that produce extracellular matrix. Although many reports on the roles of inflammation in MI, it has not been established whether inhibition or enhancement of inflammation is protective against MI-induced cardiac dysfunction and remodeling in clinical practice.

Among GRKs and β-arrestins, β-arrestin2 is an interesting target for the treatment of HF. β-Arrestin2 delays inflammatory responses by interfering with macrophage recruitment to the infarcted area. β-Arrestin2 is highly expressed in infiltrated macrophages, resulting in the inhibition of excessive inflammation and apoptosis after MI (Watari et al., 2013). The level of many inflammatory cytokines was higher in β-arrestin2 KO mice than in wild-type (WT) mice after MI, showing that β-arrestin2 has a protective role in inflammatory processes induced by MI. Moreover, the mortality rate of β-arrestin2 KO mice was increased (Watari et al., 2013). Furthermore, β-arrestin2 has been shown to prevent cell apoptosis (Ahn et al., 2009; Yang et al., 2012). These results indicate that β-arrestin2 in infiltrated macrophages plays an essential role in the inhibition of excessive inflammation after MI, and inhibition of β-arrestin2 function prevents myocyte apoptosis against ischemic injury. Several studies have shown the importance of β-arrestin2-mediated signal transduction in the heart; in particular, β-arrestin2 protects the heart against overstimulation of βAR in *in vitro* and *in vivo* studies (Noma et al., 2007). Therefore, pharmacological activation of β-arrestin2 might represent a beneficial therapy in HF.

It has been reported that β_1AR (Noma et al., 2007) and β_2AR (Shenoy et al., 2006) mediate ERK1/2 signaling in a β-arrestin-dependent manner. Stimulation of β_1AR results in transactivation of EGFR, which activates ERK signaling (Noma et al., 2007). EGFR transactivation following βAR stimulation is mediated by a GRK-dependent and β-arrestin-dependent pathway, which exhibits cardioprotective effects under conditions of excessive catecholamine stimulation (Noma et al., 2007). These results also suggest that the effects of G protein-mediated signaling may contribute to the detrimental cardiac remodeling observed during β_1AR overactivation.

A model of β_1AR signaling in the heart has been proposed, in which β_1AR mediates two distinct signaling pathways following receptor stimulation (Noma et al., 2007). The G protein-dependent signaling might be harmful and cause detrimental effects under excessive catecholamine stimulation, whereas β-arrestin-mediated signaling that is able to transactivate EGFR to evoke cardioprotective effects in response to the same pathological stimuli in the heart (Noma et al., 2007). Although EGFR activation results in cardiac hypertrophy, whether β-arrestin-mediated transactivation of EGFR is associated with cardiac hypertrophy is unknown.

β-ARRESTIN1 HAS DETRIMENTAL EFFECTS TO THE HEART

The role of β-arrestin1 in remodeling of post-MI has been investigated by using β-arrestin1-knockout (-KO) mice and WT mice under normal conditions and after surgical MI operation (Bathgate-Siryk et al., 2014). Normal (sham-operated) β-arrestin1-KO mice display enhanced βAR-dependent contractility due to impairment of βAR desensitization. After MI, β-arrestin1-KO mice display enhanced overall cardiac function (and βAR-dependent contractility) compared to WT mice. β-Arrestin1-KO mice also show increased survival, and decreased cardiac infarct size, apoptosis, and adverse remodeling, as well as circulating catecholamines and aldosterone, compared to WT mice after MI. The underlying mechanisms are; (1) on one

hand improved cardiac βAR signaling and function, as evidenced by increased βAR density and pro-contractile signaling, via reduced cardiac βAR desensitization due to cardiac β-arrestin1 absence, and (2) on the other hand decreased production leading to lower circulating levels of catecholamines and aldosterone due to adrenal β-arrestin1 absence. Thus, β-arrestin1, via both cardiac and adrenal effects, is detrimental for cardiac structure and function and significantly exacerbates development of HF after MI. Thus, β-arrestin1 might be an important negative regulator of βAR-mediated cardiac signaling and functions through the classical processes of βAR desensitization and downregulation. β-Arrestin1 may be a salient β-arrestin isoform that is responsible for βAR desensitization and downregulation in the heart, leading to progression of cardiac abnormality and dysfunction. In contrast, stimulation of β-arrestin2 through βAR leads to EGFR transactivation and ERK1/2 activation that promotes cell survival and proliferation. Thus, the actions of two isoforms of β-arrestin (β-arrestin1 and β-arrestin2) might counteract each other in certain cells and tissues, including in the heart (Rajagopal et al., 2006; Kim et al., 2008). It also suggests that inhibition of β-arrestin1 function in the heart by either a specific inhibitor or via genetic manipulation has beneficial effects on HF. However, the underlying mechanisms and actions of each β-arrestin in desensitization and downregulation of βAR, and in G protein-independent signaling remain to be clarified.

β-ARRESTIN-BIASED LIGANDS FOR βAR

The βAR antagonist is a ligand that binds to βAR but cannot activate the receptor. It also has the ability to antagonize agonist-stimulated βAR. However, the concept of agonist and antagonist is challenged by the findings that ligands for βARs could be an antagonist for the G protein-mediated signaling and also act as agonists for the β-arrestin-mediated signal transduction (Wisler et al., 2007; Kim et al., 2008; Wisler et al., 2014). When a ligand selectively activates one of the G protein-dependent and β-arrestin-dependent signaling pathway, the ligand is called as "biased ligand." Thus, a β-arrestin-biased ligand is a ligand that can antagonize the receptor-mediated G protein activation and at the same time activate signaling pathways in a G protein-independent but β-arrestin-dependent manner (Violin and Lefkowitz, 2007). β-Arrestin-biased ligands are expected to have beneficial effects on HF because of their selective activation of β-arrestin signaling that mediates favorable physiological responses in the heart (Noor et al., 2011). Discovery of novel biased ligands for βARs that are able to block G protein-mediated signaling but stimulate β-arrestin-mediated signaling represents potential therapeutic treatment for HF.

A β-arrestin-biased ligand is believed to activate an alternative signaling pathway due to the stabilization of the receptor in a particular distinct conformation, resulting in the biased activation of G protein- or β-arrestin-dependent signaling. In contrast, unbiased ligand for βAR (e.g., isoprenaline) binds to and stabilizes the βAR conformation that equally activates G protein and β-arrestin (Rajagopal et al., 2010; Wisler et al., 2014; Bologna et al., 2017). We will mainly summarize here the beneficial effects of β-arrestin-biased ligands for βAR.

Long-term use of β-blockers clinically delays progression of HF by reducing cardiac remodeling and correcting left ventricular contractility in the failing heart (López-Sendón et al., 2004). β-Blockers may regulate the βAR system by modulating β_1AR functions and reversing receptor sensitivity. Moreover, administration of β-blockers has been reported to increase βAR responsiveness and decrease GRK2 expression (Iaccarino et al., 1998b), contributing to the sensitization of βAR functions and signaling. Nevertheless, each β-blocker shows a unique effect on βAR-mediated signaling. β-Blockers differ in terms of βAR subtype selectivity, ability to block αAR, antioxidant activity, and anti-inflammatory activity (Barrese and Taglialatela, 2013). Some β-blockers are β-arrestin-biased ligands, including carvedilol, metoprolol, and nebivolol.

INTERACTION OF β-ARRESTIN-BIASED LIGAND WITH βAR

The binding of agonists and antagonists can evoke differential conformational changes of βARs. Biased ligands (or biased agonist) can induce distinct βAR conformations that selectively activate specific signaling pathways, different from full agonists and antagonists (Thanawala et al., 2014). β-Arrestin-biased ligands induce and stabilize a ligand-dependent unique receptor conformation and then selectively activate the particular signaling pathway (Thanawala et al., 2014; McCorvy et al., 2018). Stimulation of vasopressin type 2 receptor (V2R) by a G protein-biased ligand stabilizes V2R in a conformation different from that stabilized by a β-arrestin-biased ligand (Rahmeh et al., 2012). In particular, the third intracellular loop and transmembrane domain 6 (TM6) regions of V2R are necessary for G protein-mediated signaling, whereas the TM7 and putative helix 8 are required for β-arrestin-mediated signaling (Rahmeh et al., 2012). Thus, the functional outcome of biased ligands depends on which ligands stabilized conformation is favorable for G protein- or β-arrestin-coupling.

In addition, site-specific fluorine-19 nuclear magnetic resonance (19F-NMR) of β_2AR has shown that ligand binding to β_2AR modulates G protein- and β-arrestin-dependent signaling by inducing distinct conformations of the receptor depending on stimulation with either unbiased or biased ligands (Liu et al., 2012). Unbiased ligands bind to β_2AR and induce the conformational change of helix 6 into the active state that specifically leads to activate G protein signaling. In contrast, β-arrestin-biased ligands predominantly induce the conformational change of helix 7 of β_2AR that is necessary for β-arrestin-mediated signal transduction (Liu et al., 2012). Moreover, the tyrosine residue at position 308 (Tyr-308) of β_2AR is found to be essential for Gs protein-biased signaling of β_2AR. The unique interaction between ligand and Tyr-308 of TM7 in β_2AR stabilizes the receptor conformation favoring the β_2AR-Gs protein coupling that

is necessary for G protein-dependent signaling (Woo et al., 2014).

Roth's group has reported that ligand binding to amino acid residues at TM5 and extracellular loop 2 (ECL2) of the receptor are important for Gi/o protein and β-arrestin signaling, respectively (McCorvy et al., 2018). They targeted these residues to develop both G protein- and β-arrestin-biased ligands for aminergic GPCRs that contain similar residues at TM5 and ECL2, including βARs. Ligand contacts with TM5 of GPCR trigger the conformational change to induce a cytoplasmic inward movement of TM5 (Warne and Tate, 2013). This movement change then results in movement of ICL2 and TM6 regions that are involved in G protein coupling and activation (Deupi and Standfuss, 2011; Rasmussen et al., 2011). In contrast, the interaction of biased ligand with ECL2 of β_2AR is key for β-arrestin recruitment. ECL2 is an important region that locks the ligand into the binding site, resulting in an increased period of ligand binding and promotion of β-arrestin recruitment required for β-arrestin-mediated signaling pathway. Thus, TM5 and ECL2 are the regions to be focused on to develop specific biased ligands (G protein-biased and β-arrestin-biased ligands) of βARs that possess desirable therapeutic effects with minimal adverse effects.

Lefkowitz's group has used bioluminescence resonance energy transfer-based biosensor of β-arrestin2 to detect the β-arrestin conformational change upon biased ligand binding to receptor (Shukla et al., 2008). Their study showed that β-arrestin can convert βAR into multiple conformations and each unique β-arrestin-favorable conformation can form a complex with different binding proteins and evoke a corresponding specific signal transduction. Although these studies have demonstrated molecular mechanisms and interactions of biased ligand with specific regions of the receptor, further studies are required to establish the effects of β-arrestin-biased ligands in cellular functions in normal and pathophysiological conditions such as HF.

βARs can modulate the contractile function of the heart. Catecholamines, such as adrenaline and noradrenaline, activate cardiac β_1AR and β_2AR, which then activates the canonical Gs/AC/cAMP signaling cascade. Cyclic AMP binds to and activates its downstream effectors, including PKA (Salazar et al., 2007). PKA phosphorylates a set of regulatory proteins that are essential for cardiac contractility such as the voltage-gated L-type Ca^{2+} channel, the cardiac ryanodine receptor, phospholamban, and some myofilament components (troponin I and troponin C) (Salazar et al., 2007). PKA mediates phosphorylation and activation of L-type Ca^{2+} channel and ryanodine receptor results in significant increase in intracellular Ca^{2+} levels, which is necessary for cardiac muscle contraction. Furthermore, βAR-mediated phosphorylation of phospholamban (Sulakhe and Vo, 1995), a negative modulator of SERCA, accelerates Ca^{2+} reuptake into the sarcoplasmic reticulum (SR), and increases SR Ca^{2+} stores available for the next contraction (Brittsan and Kranias, 2000). In addition, troponin I phosphorylation by PKA reduces myofilament sensitivity to Ca^{2+} following βAR stimulation (Endoh and Blinks, 1988), hence inhibiting contractile signaling and accelerating cardiac relaxation (Zhang et al., 1995). Some β-blockers act as β-arrestin-biased ligands that can inhibit classical Gs protein signaling and stimulate β-arrestin signaling. The ability to activate biased signaling may explain the clinical differences between treatment with classical β-blockers and β-arrestin-biased β-blockers. We summarize below recent advances on β-arrestin-biased β-blockers that are used in clinic for the treatment of HF as presented in **Table 3**.

CARVEDILOL

Carvedilol is a nonselective β-blocker that can antagonize both β_1- and β_2-AR. Blockade of βARs in the heart by carvedilol improves cardiac function, including contractility, and attenuates myocardial remodeling in the failing heart (Iaccarino et al., 1998b; Kukin et al., 1999). In addition to nonselective blockade of βAR, carvedilol has other characteristics, including α_1-adrenergic blockade, antioxidant, anti-proliferative, anti-inflammatory, and vasodilating effects, which may explain why its efficacy is higher than other β-blockers (Metra et al., 2005; Pedersen and Cockcroft, 2007; Barrese and Taglialatela, 2013). Interestingly, carvedilol has been classified as a β-arrestin-biased ligand for βARs (Wisler et al., 2007; Kim et al., 2008).

Among the 16 available β-blockers, carvedilol is the only β-blocker that can activate ERK signaling pathway by a β_2AR-mediated, G protein-independent, and β-arrestin-dependent mechanism (Wisler et al., 2007). Alprenolol and carvedilol can activate the β_1AR-stimulated transactivation of EGFR through β-arrestin-mediated signaling without activation of G proteins (Kim et al., 2008). Therefore, carvedilol is different from other β-blockers, as it acts as a β-arrestin-biased ligand that exhibits cardioprotective effects in *in vitro* and *in vivo* studies (**Table 3**). Carvedilol-mediated β-arrestin-biased signaling might contribute to its clinical profile.

On the basis of studies of receptor structure, carvedilol can stabilize divergent receptor conformations and induce phosphorylation of βAR. The phosphorylation sites of βAR by carvedilol are different from those of unbiased ligands (Nobles et al., 2011; Liu et al., 2012). In a meta-analysis, carvedilol has been reported to have superior beneficial effects compared with other β_1-selective β-blockers (i.e., atenolol and bisoprolol) in post-MI (DiNicolantonio et al., 2013). Carvedilol has more potent effects on the reduction of mortality and morbidity in acute MI and HF compared with other β-blockers in randomized comparison trials (DiNicolantonio et al., 2013). The beneficial effects of carvedilol might be due to the activation of β-arrestin-mediated transactivation of EGFR that exhibits cardioprotective effects (Noma et al., 2007). These findings show that β-arrestin-biased β-blocker may provide an increased therapeutic benefit compared with unbiased β-blockers.

METOPROLOL

Metoprolol is reported as a biased ligand that specifically induces a G protein-independent and GRK5/β-arrestin2-dependent

TABLE 3 | Effects of β-arrestin-biased β-blockers.

β-Arrestin-biased β-blockers	Experiments/Models	Effects	Reference
Alprenolol and Carvedilol	*In vitro* (using HEK-293 cells) and *in vivo* (using mice) studies	• Alprenolol and carvedilol stimulated β-arrestin-mediated EGFR transactivation and ERK1/2 activation	Kim et al., 2008
Carvedilol and Propranolol	Rat hippocampal neurons	• Carvedilol and propranolol that inhibit βAR signaling via G proteins, mediated neuronal calcium signaling through β-arrestin2 and ERK1/2	Tzingounis et al., 2010
Carvedilol	β_2AR-expressing HEK-293 cells	• Carvedilol stimulated β-arrestin-dependent ERK1/2 activity in absence of G protein activation	Wisler et al., 2007
Metoprolol	*In vitro* (using cardiac myocytes) and *in vivo* (using GRK5-KO and β-arrestin2-KO mice) studies	• Metoprolol caused cardiac fibrosis in a G protein-independent and GRK5/β-arrestin2-dependent manner	Nakaya et al., 2012
Nebivolol	*In vitro* (using mouse embryonic fibroblasts and cardiac myocytes)	• Nebivolol-mediated ERK1/2 activation was inhibited by inhibition of GRK2 as well as knockdown of β-arrestin1/2	Erickson et al., 2013

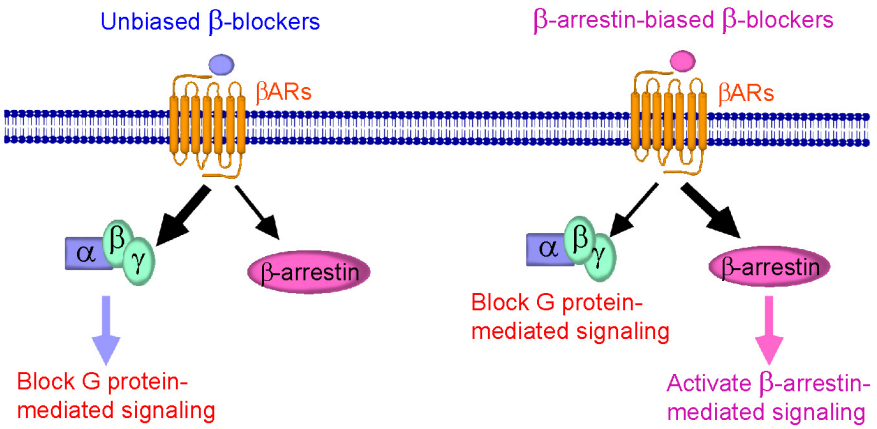

FIGURE 1 | Schematic diagram representing β-blockers mediated β-arrestin-biased signaling. (Left) Binding of unbiased (classical) β-blockers (blue) to βARs resulted in an unbiased response inhibition of G protein-mediated signaling. (Right) Binding of β-arrestin-biased β-blockers (pink) to βARs stabilizes the receptor into a distinct conformation that preferably activates β-arrestin-mediated signaling, resulting in cardioprotection, and is also able to block G protein-mediated signaling.

pathway (Nakaya et al., 2012). Besides receptor regulation, GRKs and β-arrestins have roles in the modulation of cellular signaling via βARs independently of G protein activation. While carvedilol (Wisler et al., 2007; Tzingounis et al., 2010) and alprenolol (Kim et al., 2008) activate intracellular signaling through βARs in the β-arrestin-dependent manner (**Table 3**). In addition, carvedilol and alprenolol are able to transactivate EGFR, resulting in ERK1/2 activation, whereas metoprolol does not (Nakaya et al., 2012). Administration of metoprolol to mice induced cardiac fibrosis, resulting in a decrease of diastolic function (Nakaya et al., 2012). This fibrotic pathway is mediated through the β_1AR, which is dependent on β-arrestin2 and GRK5 and is unrelated to G protein action (**Table 3**). Moreover, metoprolol increases the expression of profibrotic factors, leading to the activation of cardiac fibroblasts, eventually inducing fibrosis (Nakaya et al., 2012).

GRK5, but not GRK6, is necessary for the G protein-independent and β-arrestin2-dependent cardiac fibrosis by metoprolol. Different GRK isoforms phosphorylate distinct sites of βAR to initiate β-arrestin-biased signaling (Violin et al., 2006). For example, inhibition of GRK5 or GRK6 attenuates β-arrestin-mediated ERK1/2 activation following β_1AR stimulation. Phosphorylation of β_1AR is not affected by inhibition of GRK2 or GRK3 (Noma et al., 2007; Kim et al., 2008). Therefore, phosphorylation of βARs by different GRK isoforms together with β-arrestin binding is crucial in initiating receptor signaling toward G protein-dependent and β-arrestin-dependent signal transductions. Although metoprolol causes cardiac fibrosis, it remains useful in the management of HF patients (Kukin et al., 1999) since metoprolol attenuates the effect of catecholamine overstimulation in the patients' hearts and metoprolol-induced fibrosis is neglectable compared with βAR-induced fibrosis (Nuamnaichati et al., 2018) and HF-induced fibrosis.

Understanding of the cellular signaling pathway of β-arrestin-biased β-blockers is important for the development of novel

β-blockers that primarily target β-arrestin-mediated βAR signaling with cardioprotective effects. The discovery of other β-arrestin-biased β-blockers (**Figure 1**) not only provides additional pieces of evidence for their beneficial therapeutic effects, but also helps to design next-generation β-blockers for HF treatment.

CONCLUSION

βAR desensitization and overstimulation is the pathological hallmark of HF. Although GRKs play an important role in βAR desensitization, the involvement of individual GRK isoform in the development of a failing heart is not fully understood. Expression and activity of GRKs, especially GRK2, is significantly increased in the failing heart. Thus, inhibition of GRK activity via βARKct gene therapy, synthetic GRK inhibitors, and small peptide GRK inhibitors, represents promising therapeutic approaches for HF treatment. Although GRK2 inhibitors improve cardiac functions in various animal models of HF, these inhibitors have not yet been tested in clinical studies. β-Arrestins are also involved in the regulation of cardiac functions in normal and failing heart. However, the specific contribution of each β-arrestin isoform that plays a role in the development of a failing heart in patients with HF remains to be clarified. Based on the physiological and pathological functions of GRKs and β-arrestins in the heart, both could be candidates for novel theranostic strategies for HF treatment. Cavedilol, alprenolol, and nebivolol are identified as β-arrestin-biased β-blockers that are able to activate β-arrestin-mediated signaling while blocking G protein-mediated signaling, providing cardioprotection. These β-arrestin-biased β-blockers may exhibit distinct pharmacological effects relative to their unbiased counterparts; however, the clinical outcome of these β-blockers remains to be elucidated. Elucidation of the signaling mechanisms of β-arrestin-biased β-blockers will facilitate our understanding and could lead to the discovery of new β-blockers with fewer side effects while providing effective therapy for HF patients.

AUTHOR CONTRIBUTIONS

SM and WP mainly wrote the manuscript. HK edited this manuscript.

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REVIEW
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β-Arrestin Based Receptor Signaling Paradigms: Potential Therapeutic Targets for Complex Age-Related Disorders

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G protein coupled receptors (GPCRs) were first characterized as signal transducers that elicit downstream effects through modulation of guanine (G) nucleotide-binding proteins. The pharmacotherapeutic exploitation of this signaling paradigm has created a drug-based field covering nearly 50% of the current pharmacopeia. Since the groundbreaking discoveries of the late 1990s to the present day, it is now clear however that GPCRs can also generate productive signaling cascades through the modulation of β-arrestin functionality. β-Arrestins were first thought to only regulate receptor desensitization and internalization – exemplified by the action of visual arrestin with respect to rhodopsin desensitization. Nearly 20 years ago, it was found that rather than controlling GPCR signal termination, productive β-arrestin dependent GPCR signaling paradigms were highly dependent on multi-protein complex formation and generated long-lasting cellular effects, in contrast to G protein signaling which is transient and functions through soluble second messenger systems. β-Arrestin signaling was then first shown to activate mitogen activated protein kinase signaling in a G protein-independent manner and eventually initiate protein transcription – thus controlling expression patterns of downstream proteins. While the possibility of developing β-arrestin biased or functionally selective ligands is now being investigated, no additional research has been performed on its possible contextual specificity in treating age-related disorders. The ability of β-arrestin-dependent signaling to control complex and multidimensional protein expression patterns makes this therapeutic strategy feasible, as treating complex age-related disorders will likely require therapeutics that can exert network-level efficacy profiles. It is our understanding that therapeutically targeting G protein-independent effectors such as β-arrestin will aid in the development of precision medicines with tailored efficacy profiles for disease/age-specific contextualities.

Keywords: β-arrestin signaling, ligand 'bias', GPCRs, age-related disorders, precision, tailored efficacy

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Abbreviations: α_{2A}-AR, α_{2A}-adrenergic receptor; AD, Alzheimer's disease; AT1aR, angiotensin II type 1A receptor; Ang II, angiotensin II; β2AR, β2-adrenergic receptor; cAMP, cyclic adenosine monophosphate; DDR, DNA damage response; GLP-1, glucagon-like peptide 1; GLP-1R, GLP1 receptor; GPCR, G protein coupled receptor; GRK, G protein coupled receptor kinase; GTP, guanosine triphosphate; IRS1, insulin receptor substrate-1; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor-κB; PK, protein kinase; PTH, parathyroid hormone; PTH1R, parathyroid hormone 1 receptor; ROS, reactive oxygen species; SII, Sar1, Ile4, Ile8-AngII; T2DM, type II diabetes mellitus.

INTRODUCTION

The β-arrestin family comprises four members: visual arrestins (arrestin1 and arrestin4) and the non-visual arrestins (β-arrestin1 and β-arrestin2, also referred to as arrestin2 and arrestin3, respectively). Furthermore, an α-arrestin family, structurally related to the β-arrestins has been identified (Chutkow et al., 2010). To provide a more therapeutically-targeted discussion in this review we will focus on the β-arrestins 1 and -2, which are ubiquitously expressed in most mammalian tissues and cell types (Feng et al., 2011). Heptahelical transmembrane GPCRs, were originally thought to be a member of a three-part functional signaling system: (1) the receptor detecting ligands in the extracellular milieu, (2) a heterotrimeric ($\alpha\beta\gamma$) G protein that dissociates into α-subunits bound to guanosine triphosphate (GTP) and βγ-subunits, after interaction with the ligand-bound GPCR, and (3) an effector interacting with the dissociated G protein subunits (α or βγ) to generate soluble small molecule second messengers. This GPCR-G protein interaction is catalytic, i.e., one receptor can sequentially activate multiple G proteins either of the same or of a different type. GPCR signaling through G proteins is terminated after serine-threonine phosphorylation of the receptors, both by members of the GPCR kinase family and second messenger-activated protein kinases such as protein kinase A and C. This is followed by arrestin binding (β-arrestins1 or 2), which sterically inhibits further G protein activation (Freedman and Lefkowitz, 1996). Next, the ligand-bound receptor can be internalized through an arrestin-dependent engagement of clathrin-coated pits (Ferguson et al., 1996; Goodman et al., 1996), after which the receptors are either dephosphorylated and recycled back to the cell surface or targeted for lysosomal degradation if a potent ligand stimulation level persists (Yu et al., 1993).

While this canonical description of GPCR signaling still largely holds true, this is not where GPCR signaling ends. Luttrell et al. (1999) discovered that β-arrestin becomes part of a multi-protein signaling complex (often termed a '*receptorsome*') which functions both to target the receptor-kinase complex to clathrin-coated pits and additionally to recruit activated non-receptor tyrosine kinase c-Src to the plasma membrane. This '*receptorsome*' formation process engenders a receptor-based capacity to induce a novel signaling cascade distinct from the G protein-dependent paradigm. Thus, it was elegantly demonstrated that β-arrestins did not only 'arrest' G protein signaling, but transformed the signaling activity and initiated supplementary signaling cascades where the *'desensitized'* receptor functioned as a part of a mitogenic signaling complex (Luttrell et al., 1999). Additionally, while G protein-dependent signaling is essentially a transient catalytic process which generates soluble small molecule second messenger products, it is evident that β-arrestin-dependent signaling can engender highly characteristic and translatable transcriptomic phenotypes, presumably via the formation of more self-reinforced, complex, higher-order multi-protein signaling interactomes (Maudsley et al., 2015, 2016) similar to the '*encryptome*' complexes proposed for growth factor receptor signaling (Martin et al., 2009). This GPCR interactome capacity for regulating complex transcription patterns, thus revealed the ability for β-arrestin-dependent signaling to create discrete, reinforced coherent patterns of long-term signal transduction. This new signaling paradigm engenders a feasible mechanism for receptor-based cell/tissue engineering independent of traditional GPCR signaling modalities. Since this discovery, considerable research has been undertaken to further investigate the functional role(s) of β-arrestin, beyond its function in receptor desensitization, and has thus rekindled the concept of 'Agonist Trafficking' in the new form of drug-focused *'Biased'* signaling (Kenakin, 1995; Andresen and Luttrell, 2011).

GPCR Biased Signaling

The past two decades of GPCR biology research has reshaped the world of receptor signaling and pharmacotherapeutics via the maturation of two critical concepts: (1) receptor signaling pluridimensionality (Maudsley et al., 2005) and (2) ligand bias (Luttrell and Kenakin, 2011). The effective exploitation of these two facets of receptor functionality will likely facilitate the development in the future of more effective GPCR-based therapeutics (i.e., higher functional specificity and reduced 'off-target' actions) (Luttrell and Kenakin, 2011). Pluridimensional efficacy of GPCRs refers to the discovery that these receptors signal via multiple G protein and non-G protein effectors, and can thus adopt multiple 'active' states. Essentially, this also introduces the concept that a GPCR is never truly quiescent and simply exists in different proportions of various active state complexes in distinct cellular sub-compartments. The stable interactions between GPCRs and β-arrestin enable the formation of receptor-based signal-specific interactomes. Here we can potentially term these structures *'encryptomes'* – the specific protein stoichiometry essentially 'encrypts' the specificity of the downstream intended signal – and potentially the 'choice' of the encryptome-stabilizing ligand. The specific composition of the GPCR encryptome therefore defines its subsequent interactibility with protein- and lipid kinases, phosphatases, ubiquitin ligases, regulators of small G proteins, and other novel effectors (Maudsley et al., 2005; Luttrell and Gesty-Palmer, 2010; Shenoy and Lefkowitz, 2011). The concept of signaling selectivity or ligand bias suggests that distinct ligands may alter the conformational equilibrium of the receptor in a manner distinct from the endogenous ligand, allowing the receptor to couple to a specific subset of its downstream effectors (Wei et al., 2003; Kenakin, 2007; Kenakin and Miller, 2010; Luttrell and Kenakin, 2011; Maudsley et al., 2012). This functional selectivity carries with it the promise of a revolutionary change for drug development: increasing the potential for new, more effective drugs, but also the knowledge that drug efficacy needs to be comprehensively characterized in order to avoid unintended side effects. In the context of the GPCR encryptome, ligand bias results as a function of the protein stoichiometric composition of this. Different protein components chaperoning and interacting with the core GPCR will thus determine the interaction/efficacy profile of the ligand attempting to interact and stabilize the particular encryptome.

While signaling bias using functionally selective xenobiotic ligands has been a topic of ongoing research for nearly two decades, it has become clear that natural ligand bias also occurs for GPCRs and likely represents another physiological level of

receptor signaling complexity. In discussing the natural biased agonism of chemokine receptors (CCRs), Zidar et al. (2009) reinforce the posit that this selectivity is not merely a product of synthetic pharmacology (Kohout et al., 2004). In their research, Zidar et al. (2009) found that the endogenous chemokine ligands CCL19 and CCL21 for the CCR7 receptor, demonstrate a striking difference in activation of the GRK/β-arrestin2 system, despite of their similarity in promoting G protein stimulation and chemotaxis. Only CCL19 promotes desensitization of endogenous CCR7 in the human T cell lymphoma cell line H9 (Kohout et al., 2004), and causes fourfold more ERK1/2 phosphorylation than CCL21, through a β-arrrestin2 dependent mechanism (Kohout et al., 2004; Zidar et al., 2009; Zidar, 2011).

Endogenous ligand bias has also been shown for the Angiotensin II type 1 receptor (AT1R), which is physiologically implicated in the development of hypertension and the natural aging process (Mattson and Maudsley, 2009). Further research in AT1R, indicates that only a subset of AT1R signaling pathways are detrimental, thus by using biased ligands which inhibit these detrimental pathways, it may be possible to promote and enhance beneficial drug-based effects (Galandrin et al., 2016). Angiotensin peptide (1–7) [Ang(1–7)], which lacks the Angiotensin II (Ang II) critical C-terminal phenylalanine residue due to angiotensin-converting enzyme 2 (ACE2)-dependent cleavage is described to cause vasodilatory and cardioprotective effects (Santos et al., 2003, 2013). Ang(1–7) fails to promote G protein activation, behaving as a competitive antagonist for Ang II/$G\alpha_i$ and Ang II/$G\alpha_q$ pathways, it however selectively promotes β-arrestin activation (Galandrin et al., 2016). These researches explain that in nature, ligand bias has likely been exploited even before scientist have begun to uncover the clinical uses of ligand bias, underlining its importance.

β-Arrestin Modulates Downstream Signaling of GPCRs Through Complex Assembly Regulation

As mentioned previously, β-arrestin can affect GPCR signaling in a manner independent of G protein-signaling that involves the physical scaffolding of multiple signal transduction proteins. Thus, it stands to reason that the signaling functionality of β-arrestin1 and 2 can be effectively interpreted via the analysis of the physical interactomes associated with these multidimensional transducers (**Figures 1**, **2**). To assess the current state of the metadata concerning the known functional interactomes of the β-arrestin1 (**Figure 1A**) and β-arrestin2 (**Figure 1B**) we extracted binding partner identities from BioGrid[1], HPRD (Human Protein Reference Database[2]), IntAct[3], MINT (The Molecular INTeraction Database), STRING[4], DIP (Database of Interacting Proteins[5]) and CORUM[6]. The cumulated known interaction partners for β-arrestin1 or β-arrestin2 are detailed in **Supplementary Tables S1**, **S2**, respectively. The diversity of subcellular distribution (**Figure 1C** – β-arrestin1; **Figure 1D** – β-arrestin2) and molecular function (**Figure 1E** – β-arrestin1; **Figure 1F** – β-arrestin2) of these interactors were categorized using Ingenuity Pathway Analysis (IPA)-based annotation. Perhaps the strongest divergence in cellular distribution of β-arrestin-specific binding partner between the two isoforms lies in the stronger plasma membrane representation of β-arrestin1 binding partners compared to β-arrestin2. In a similar regard, the greatest distinction of β-arrestin binding partner functionality resided in the much stronger ion channel function of β-arrestin1-binding partners compared to partners of β-arrestin2. To gain a signaling-based appreciation of the known β-arrestin-based interactomes, an IPA canonical signaling pathway investigation of the known binding partners (**Figure 1G** – β-arrestin1; **Figure 1H** – β-arrestin2) was carried out. Amongst the top 20 most strongly-populated signaling pathways it was evident that the β-arrestin1 binding cohort demonstrated a more profound propensity for G protein-associated functionality and cell cycle regulation compared to β-arrestin2. However, when the degree of potential signaling pathway overlap was assessed for the two curated β-arrestin interactomes (**Figure 1I** – β-arrestin1; **Figure 1J** – β-arrestin2) it was clear that a more interactive and closely associated functional network of activity was predicted for β-arrestin2. Such data suggests perhaps that β-arrestin2 may control a more coherent signaling response away from plasma membrane-associated receptors (see **Figure 1D**). This distinction in interactome coherence was also continued when the two β-arrestin interactome datasets were assessed using the STRING interaction platform (**Figure 2A** – β-arrestin1; **Figure 2B** – β-arrestin2). Hence, with respect to the network statistics of the two interactomes, the β-arrestin2-specific functional network, compared to the β-arrestin1 network, possessed a greater average node degree, a stronger local clustering coefficient and a more significant protein–protein interaction (PPI) enrichment probability. The natural language processing informatic platform GeneIndexer was employed to assess the relative strength of association between proteins β-arrestin1 or β-arrestin2 interactome datasets and gerontological biomedical concept terms. GeneIndexer[7] enables the identification of biomedical text items, i.e., Gene Symbol identifiers of proteins, that possess latent semantic associations with the input interrogator concept term (i.e., Ageing, Aging, Senescence, Senescent, Elderly, Elder, Longevity). Latent semantic analysis is a natural language processing data extraction mechanism (Landauer et al., 2004) that exploits the concept of 'Swanson Linking' (Bekhuis, 2006) to identify cryptic connections between natural language concepts (i.e., Ageing, Aging *etc*. . ..) and specific text entities, in this case Gene Symbol targets identified using the database of gene-word documents assembled in the GeneIndexer database (Cashion et al., 2013). GeneIndexer contains over 1.5 million Medline abstracts corresponding to over 21,000 mammalian genes (Cashion et al., 2013). GeneIndexer extracts both explicit and implicit gene-to-keyword (e.g., Aging) relationships from the literature using Latent Semantic Indexing (LSI:

[1]https://theBioGrid.org/

[2]http://www.hprd.org/

[3]https://www.ebi.ac.uk/intact/

[4]https://string-db.org/

[5]http://dip.mbi.ucla.edu/dip/

[6]http://mips.helmholtz-muenchen.de/corum/

[7]https://geneindexer.com/

Homayouni et al., 2005). GeneIndexer scores individual proteins according to the strength of the association with the keyword query (e.g., Aging), whereby a Cosine Similarity (Landauer et al., 2004) score >0.2 typically indicates an explicit association (i.e., the word actually appears in the protein-related PubMed abstract), while a score between 0.1 and 0.2 typically indicates an implied relationship (Homayouni et al., 2005). Therefore using a basal Cosine Similarity cut-off of 0.1 we were able to assess the group (β-arrestin1 or 2 interactome) strength of correlation between the detailed aging-related interrogator terms (**Figure 3**) and the interactome datasets by summing the various individual protein Cosine Similarity scores extracted for each implicitly and explicitly-associated proteins from the interactome lists for each given gerontological concept input. One of the biggest challenges for the machine-based analysis of human-generated text concerns the vagaries of term use, e.g., authors

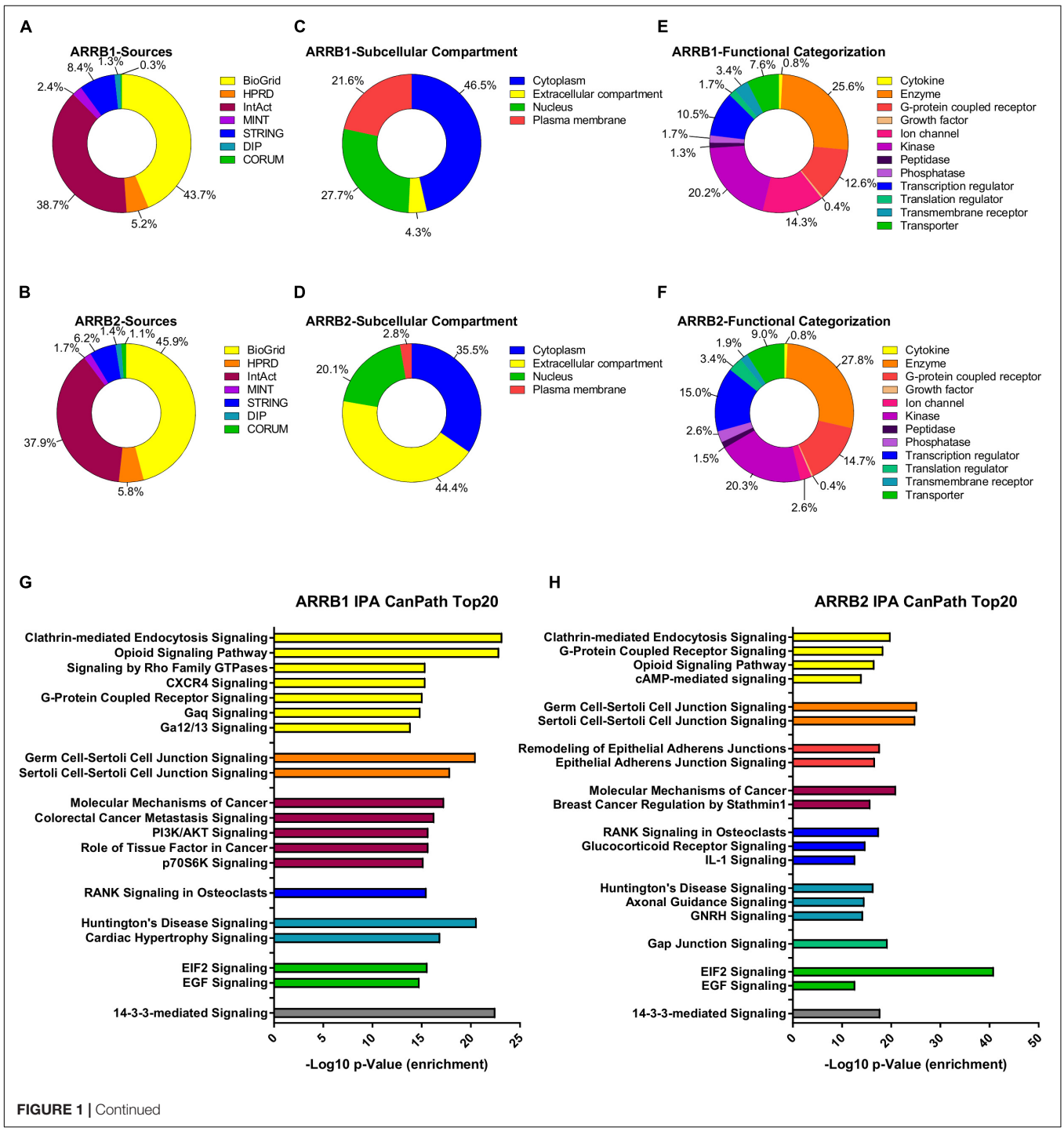
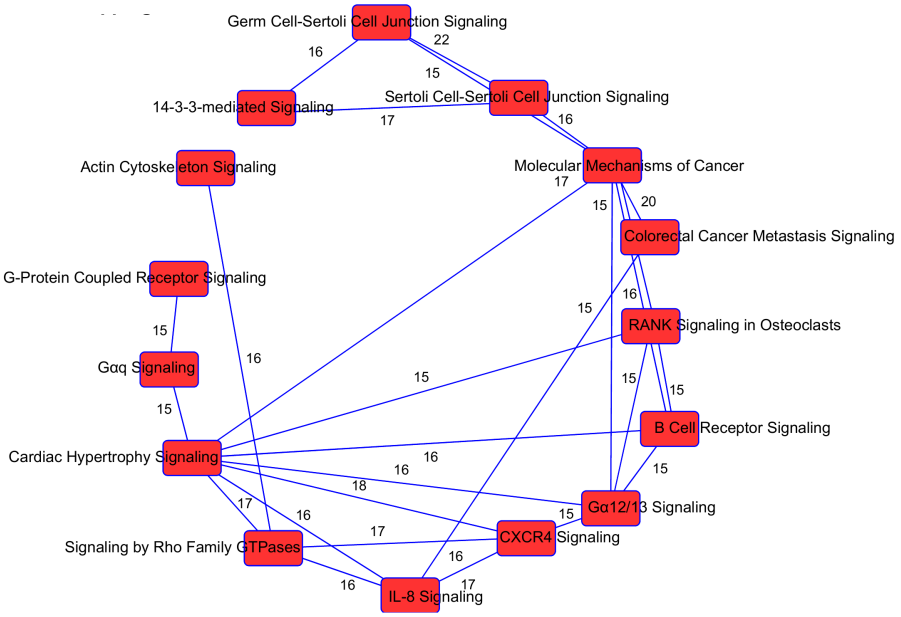


FIGURE 1 | Continued

I ARRB1: IPA Overlapping CanPaths



J ARRB2: IPA Overlapping CanPaths

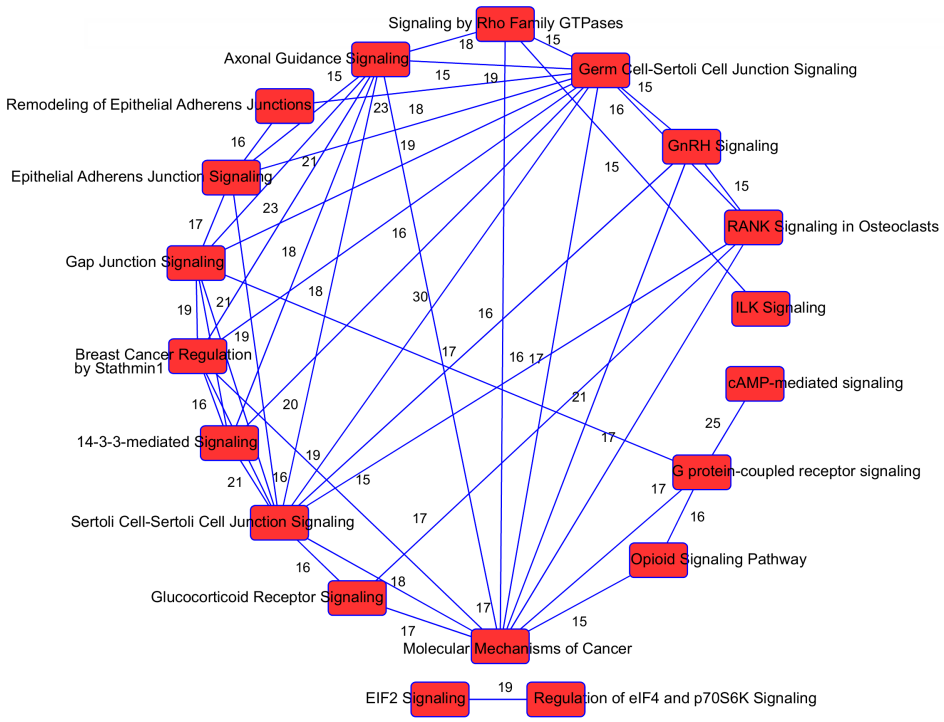


FIGURE 1 | Ingenuity pathway analysis of β-arrestin1 and 2 interactome. The interactors of a protein are indicative of its function. Thus, to further investigate the function of β-arrestin1 we extracted these from seven sources: BioGrid (https://theBioGrid.org/), HPRD (Human Protein Reference Database: http://www.hprd.org/), IntAct (https://www.ebi.ac.uk/intact/), MINT (The Molecular INTeraction Database), STRING (https://string-db.org/), DIP (Database of Interacting Proteins: http://dip.mbi.ucla.edu/dip/), and CORUM (http://mips.helmholtz-muenchen.de/corum/). This resulted in a list of proteins which are proven interactors of β-arrestin1 (445) and 2 (625). **(A,B)** Shows the distribution of the aforementioned databanks for β-arrestin1 and 2 respectively. This dataset was further analyzed using Ingenuity Pathway Analysis (IPA). Where we extracted the following information: **(C,D)** the subcellular distribution – categorized in an unbiased manner using IPA protein annotation – of the interactors: Plasma Membrane, Nucleus, Cytoplasm, and Extracellular space, *i*, for β-arrestin1 and 2 respectively; **(E,F)** the functional annotation – again performed using unbiased IPA-based classification – of the interacting proteins: Cytokine, Enzyme, GPCR, growth factor, ion channel, kinase, peptidase, phosphatase, transcription regulator, translation regulator, transmembrane receptor, and transporter, β-arrestin1 and 2 respectively; and the Top 20 Canonical Pathways, β-arrestin1 and 2 respectively, related to this dataset organized in **(G,H)** a stacked bar chart, and **(I,J)** a network representation with a cut-off of 15 common genes between pathways, to increase the stringency, where the numbers depicted represent the amount of common proteins between the pathways.

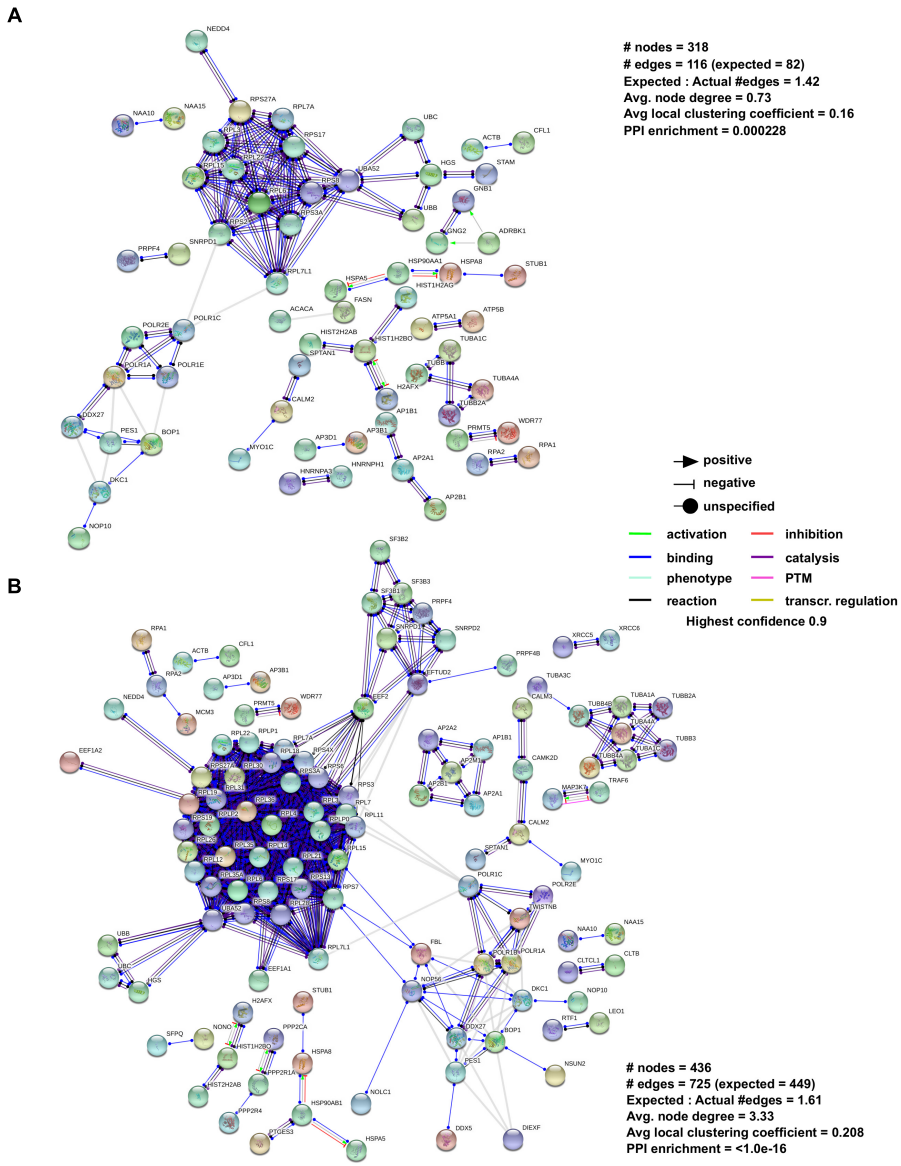


FIGURE 2 | Analysis of β-arrestin1 and 2 interactome using STRING Interaction Network. To further analyze the networks of **(A)** β-arrestin1 and **(B)** β-arrestin2, STRING was used. To increase the stringency of the analysis the following settings were used: for Active Interaction Sources only "Experiments" and "Co-expression" were selected indicating we are only interested in data which is the result of experiments or are known to co-express, this to remove hypothetical data and suggestions. The minimum required interaction score was set to the highest level (0,9), and all unconnected nodes were removed from the network.

may use divergent syntactic entities (words) to describe the same concepts (e.g., Aging or Ageing, Elderly or Elder). Hence to generate the most comprehensive LSI-based appreciation of gene-keyword associations we used a broad range of synonyms to extract the most amount of Gene Symbol data. Using this range of input terms (Aging, Ageing, Senescence, Senescent, Elderly, Elder, Longevity) we found that, using the cumulated Cosine Similarity scores for the interactome proteins associated with these input gerontological concept terms, for the majority of the terms (Aging, Ageing, Senescence, Senescent, and Longevity) a greater total association of the βarrestin2 interactome proteins with these concepts was observed compared to the βarrestin1 interactomes (**Figure 3** and **Supplementary Table S3**). These unbiased results are effectively concordant with our previously derived STRING interactome analyses. This suggests, in line with recent reports (Luttrell et al., 2018b), that highly specific and contextual biological functionalities are likely attributable to either βarrestin1 or 2. Therefore, it seems that indeed a potential bias for β-arrestin2-dependent signaling may occur with respect to the specific context of aging. In the following sections we will discuss how, with specific reference to hypothesis-driven experimentation, the downstream signalers can be influenced by β-arrestin1 or β-arrestin2 interactome activity and subcellular distribution.

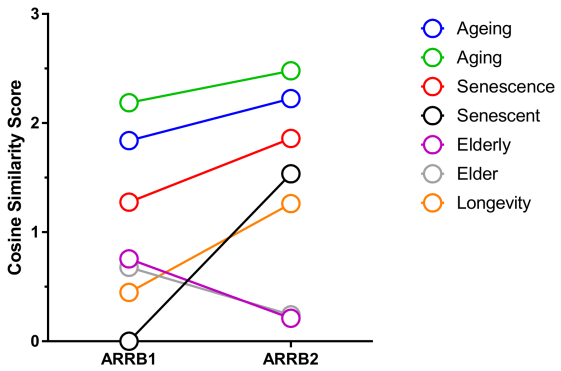


FIGURE 3 | Role of β-arrestins in aging through GeneIndexer analysis of interactome metadata. Reanalysis of the obtained interactomes for β-arrestin1 (ARRB1) and β-arrestin2 (ARRB2), using aging-related interrogation terms with GeneIndexer, in a manner similar to that described previously (Chadwick et al., 2012). The following interrogation terms were used: Ageing, Aging, Senescence, Senescent, Elderly, Elder, and Longevity (i.e., long life). These terms (inclusive of most common spellings and synonyms) were used to obtain as much information as possible as human-generated text descriptions using natural language are often variant between research groups/authors. As seen in previous bioinformatics analyses these interrogation terms all give different results. In this figure, it becomes clear that β-arrestin2 has a stronger connection to aging compared to β-arrestin1, with the exception of the interrogation terms Elderly and Elder. For the generation of this figure, the cosine similarity between the top 10 proteins and every interrogation term was averaged in order to create this table. The cosine similarity scores linking the proteins to each word are listed in **Supplementary Table S3**.

Non-receptor Tyrosine Kinase c-Src

As mentioned previously, β-arrestins also function as downstream signal transducers of GPCRs, allowing the formation of signal/function-specific encryptomes with a wide variety of signaling proteins, including c-Src family tyrosine kinases and components of the ERK1/2 and JNK3 (Jun N-terminal kinases 3) MAP kinase cascades (Luttrell et al., 1999; Luttrell and Lefkowitz, 2002). Through the recruitment of these kinases to GPCRs after agonist-binding, β-arrestins grant distinct signaling activities upon the receptor – discriminating them physically from G protein-dependent functional GPCR forms (Luttrell and Lefkowitz, 2002). This paradigm was illuminated by the initial observation that β-arrestin can directly bind to Src family kinases and recruit them to agonist-occupied GPCRs. Stimulation of β2 adrenergic receptors (β2AR) triggered the co-localization of the receptor with both endogenous β-arrestins and Src kinases in clathrin-coated pits (Luttrell et al., 1999). This reflected the assembly of the prototypic signal-encrypted receptor-protein complex. These GPCR-based encryptomes have also been seen in other research where β-arrestins recruited Src to the neurokinin-1 receptors in KNRK cells (DeFea et al., 2000) and Src family kinases Hck and Fgr to the C-X-C motif chemokine-1 receptor (CXCR1) in neutrophils (Barlic et al., 2000).

Mitogen Activated Protein (MAP) Kinases

The MAP kinases are a family of serine/threonine kinases involved in the transmission of external signals regulating cell division, growth, differentiation, and apoptosis. Mammalian cells contain three major categories of MAP kinases: ERKs, JNKs, and p38/HOG1 MAP kinases (Pearson et al., 2001). In 2000, it became clear that β-arrestins have the ability to function as scaffold proteins for some MAP kinase units. In KNRK cells the stimulation of proteinase-activated receptor 2 (PAR2) promotes encryptome formation containing the internalized receptor, β-arrestin1, Raf-1, and activated ERK1/2, which is required for ERK activation (DeFea et al., 2000). Similar results have been gained for the angiotensin II type 1A receptor (AT1aR) in HEK293 and COS-7 cells, where stimulation resulted in encryptome formation containing the receptor, β-arrestin2, and ERK cascade component kinases: cRaf-1, MEK1 (Mitogen Activated Kinase Kinase 1) and ERK2 (Luttrell et al., 2001). β-Arrestins can sequester ERK in the cytosol, by scaffolding Raf1, MKK1 and ERK (Luttrell et al., 2001). In this manner β-arrestin also scaffolds JNK1/2 through mitogen-activated protein kinase kinase (MKK) 4 and 7 (Kook et al., 2013). Furthermore, the activation of p38 signaling also appears to be dependent on β-arrestin (Sun et al., 2002; Bruchas et al., 2006).

E3 Ubiquitin Ligases

While ubiquitination was long thought to control only protein degradation, it has now become clear it also plays a role in productive signal transduction cascades. β-Arrestins can act as adapters for E3 ubiquitin ligases such as MDM2, which mediates ubiquitination of p53 and as such plays a role in DDR (Shenoy et al., 2001) by controlling p53 expression levels. The complexes that β-arrestin and these E3 ligases create, are essential for mediating ubiquitin-dependent signaling. For instance, β-arrestins are essential in the ubiquitination of receptors, where these scaffolds act on late endosomal population as a lysosomal degradation signal for the receptor. When focusing back on MDM2, ubiquitination of β-arrestin2 is also required for clathrin-mediated β2AR internalization (Shenoy et al., 2001). However, AIP4 E3 ligases are necessary for endosomal sorting of CXCR4, after which it can be sorted to lysosomes (Marchese et al., 2003). For this sorting, an interaction of β-arrestin1 with Signal transducing adapter molecule 1 (STAM1) is necessary, which is part of the endosomal sorting complex (Malik and Marchese, 2010). This connection between β-arrestin1 with CXCR4 also appears in our interactome metadata analysis of the β-arrestin1 interactome specifically (**Figures 1G,J**).

Nuclear Factors

β-arrestin2 has been shown to possess the ability to inhibit nuclear factor κB (NF-κB) signaling through the physical stabilization of IKBα (NFKB inhibitor alpha) (Gao et al., 2004). Next to β-arrestin2, its family co-member β-arrestin1 can directly influence epigenetic modifications through a nuclear interaction with histone acetylases and de-acetylases which influence chromatin structure (Kang et al., 2005). Moreover, β-arrestin1 is also necessary for endothelin-1-induced NF-κB activation in ovarian cancer cells since Cianfrocca et al. (2014)

discovered a previously unrecognized pathway dependent on β-arrestin1 to sustain NF-κB signaling in ovarian cancer.

β-Arrestin can also control NF-κB-mediated signaling through AT1aR activation, which recruits this scaffold protein. This was analyzed in rat vascular smooth muscle cells. Genetic knockdown of β-arrestin1 and 2 inhibited Ang II-induced p64 NF-κB nuclear localization. The internalization of the activated AT1AR mediated by β-arrestin, stimulated the NF-κB signaling pathway, engendered a nuclear localization of the transcription factor and resulted in the initiation of COX-2 (Cytochrome c oxidase subunit 2) protein synthesis. As such, this research line linked receptor internalization with the NF-κB pathway (Morinelli et al., 2013).

In addition, β-arrestin2 exerts anti-inflammatory action through the prevention of NF-κB activation. This action is mediated through the direct inhibition of p38 MAPK. On the other hand, a simultaneous anti-inflammatory effect can also be initiated through cAMP and PKA activation via G protein signaling which exerts an inhibitory effect on NF-κB (Campo et al., 2015). Such output signaling complexity reinforces the need for a more in-depth understanding of the spatiotemporal dynamics of GPCR signaling before rational design of engineered efficacy drugs is feasible.

Receptors and G Protein Subunits

The epidermal growth factor receptor (EGFRs) can be functionally transactivated by GPCRs as well as being functionally regulated by β-arrestins, through the stimulation of a transmembrane matrix metalloprotease which causes cleaving of membrane bound EGF ligand (Noma et al., 2007). In addition to this, it has become clear that β-arrestin can mediate non-canonical G protein signaling, where they promote this novel form of cell signaling by both the Parathyroid hormone 1 (PTH1) (Wehbi et al., 2013) and V2 vasopressin receptor (Feinstein et al., 2013) from cytoplasmic endosome structures. This effect was lost after knockdown of β-arrestin (Feinstein et al., 2013).

The β2AR has been proposed to maintain an active conformation, even in endosomes distant from the plasma membrane, which has the ability to signal productively through G proteins to generate cAMP (Irannejad et al., 2013). This suggests that trafficking of the receptor to endosomes, via β-arrestins, facilitates the stabilization of a receptor that is not necessarily inactivated and ready to be recycled, but one that is still able to activate G protein signaling. This signaling paradigm appears to be facilitated by a Receptor – G protein – β-arrestin complex (Wehbi et al., 2013), thus completely countering the classic paradigm of β-arrestin as 'arresting' G protein signaling (mentioned in see section "Introduction").

β-Arrestin Dependent Signaling in Therapeutic Development

Type 1 Parathyroid Hormone Receptor

β-Arrestin dependent signaling-selective ligands have been investigating in the attempt to design precision *'tailored efficacy'* therapies that possess a more focused efficacy with fewer off-target side effects. Perhaps one of the best characterized arrestin pathway-selective biased agonist is that mediated through the type 1 human parathyroid hormone receptor (PTH1R). The PTH1 receptor regulates calcium homeostasis and bone metabolism. Gesty-Palmer et al. (2006) reported that [D-Trp^{12},Tyr^{34}]bovine PTH(7-34) showed β-arrestin bias, acting as an inverse agonist for PTH1R-$G\alpha_s$ coupling (Appleton et al., 2013). Intermittent administration of bPTH(7-34) *in vivo* increased bone mass, osteoblast number, in the absence of osteoclast proliferation and bone resorption. These effects are in stark contrast to those engendered by similar treatment with endogenous human PTH(1-34) [hPTH(1-34)], which is the current conventional therapy for osteoporosis (Gesty-Palmer et al., 2009). Research has subsequently demonstrated that this diverse and effective functional activity in the PTH1R is mediated through the creation of a highly-translatable molecular signature of altered protein expression patterns that is dependent on a functional β-arrestin2 signaling capacity (Gesty-Palmer et al., 2013; Maudsley et al., 2015, 2016). This research has thus allowed GPCR-based drug design to enter the phase of multidimensional therapeutic targeting to engender highly selective long-lasting proteomics-based remediation of equally complex disease protein profiles.

μ-Opioid Receptor

The μ-opioid receptor (MOR) can be activated by both endogenous enkaphalin peptides and xenobiotic opiates such as morphine or fentanyl (Darcq and Kieffer, 2018). G protein agonism and receptor internalization have been demonstrated for enkephalins and the opiate etorphine, however a lack of receptor agonist-induced internalization was found for the opiate agonist morphine (Keith et al., 1996; Darcq and Kieffer, 2018). This could be explained by the poor ability of morphine to stimulate receptor phosphorylation and thus its recruitment of β-arrestin (Zhang et al., 1998), and is hence a good example of negative β-arrestin bias. This connection between β-arrestin and opioid signaling was also extracted from our obtained β-arrestin interactome dataset (**Figures 1G–J**). Despite this, mice lacking β-arrestin2 demonstrated a marked increase and prolongation of morphine-induced analgesia, indicating, notwithstanding its bias, that morphine is capable of stimulating β-arrestin-mediated desensitization (Bohn et al., 1999). This discovery indicates that ligands showing strong bias away from β-arrestin, toward G proteins, might offer enhanced analgesia, where it may be possible to separate the therapeutic efficacy from the negative side effects of such agents (Bruns et al., 2006; Schmid et al., 2017). One such compound has been discovered, named herkinorin, where it diverts signaling activities away from side effects, such as tolerance, respiratory suppression and constipation, which are mediated by β-arrestin2 (Bohn et al., 2000; Raehal et al., 2005; Groer et al., 2007; Manglik et al., 2016; Schmid et al., 2017). Furthermore, PZM21, a potent G_i activator showed minimal β-arrestin2 recruitment, causing analgesic effects in absence respiratory depression, and is as such another example of β-arrestin independent therapeutics (Manglik et al., 2016).

Angiotensin II Type 1 Receptor

The AT1R possesses a strong trophic role in the control of blood pressure and electrolyte homeostasis, and thus has been widely

targeted in the strategic development of pharmacotherapeutic treatment for hypertension via the employment of classical 'antagonists' such as valsartan and losartan. Ang II typically signals through the AT1R via $G\alpha_q$-mediated activation of phospholipase C (PLC) and induces receptor phosphorylation by GRKs, thus recruiting β-arrestin to the receptor. This β-arrestin recruitment desensitizes $G\alpha_q$ signaling, causes receptor internalization and induces non-G protein-dependent β-arrestin signaling (Anborgh et al., 2000; Sakmar et al., 2002). Sar1, Ile4, Ile8-AngII (SII) was identified as a perfectly β-arrestin biased ligand (Holloway et al., 2002), stimulating β-arrestin signals such as ERK1/2 activation in the absence of G protein signaling (Wei et al., 2003; Ahn et al., 2004). Such discrete signaling has been elegantly demonstrated both *in vivo* and *in cellulo*. SII has been found to induce contractility via β-arrestin signaling in isolated cardiac myocytes, indicating a significant effect on cardiac function (Rajagopal et al., 2006). Several of the SII-activated signaling pathways appear to be pro-survival, cytoprotective, and/or proliferative, i.e., Src, ERK1/2, Akt, and phosphatidylinositol-3-OH kinase (PI3K) (Lefkowitz and Shenoy, 2005; Lefkowitz et al., 2006; DeWire et al., 2007), and are thus worthy of further investigation for drug-development.

Moreover, a potent, selective β-arrestin biased ligand of the AT1R has been recently discovered, i.e., TRV120027 (Sar-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH), which competitively antagonizes G protein signaling through the AT1R, yet stimulates β-arrestin recruitment, activating several kinase pathways in a β-arrestin-dependent manner. This biased ligand increased cardiomyocyte contractility *in vitro*. *In vivo* characterization of the ligand, in rats, showed a reduction in mean arterial pressure, and most importantly (and unlike other unbiased antagonists) TRV120027 increased cardiac performance and preserved cardiac stroke volume (Violin et al., 2010). Unfortunately, even though this drug showed great promise, TRV120027 failed in the clinical phase 2b. Such an initial setback is unlikely to diminish further interest in refining the efficacy profile of GPCR-targeting biased agents.

β-Adrenergic Receptor

Classical orthosteric βAR antagonists have long been employed as effective pharmacotherapeutics for numerous cardiovascular conditions including hypertension (Williams et al., 2004), angina (Gibbons et al., 2003), and heart failure (Bristow, 2000). They possess the ability to block the harmful G protein-mediated effects of superfluous catecholamine stimulation in the heart and other organs. The widely used βAR 'blocker' Carvedilol (Coreg®) has been demonstrated to possess unique survival benefits in congestive heart failure, through a β-arrestin-selective pathway in absence of the $G\alpha_s$-dependent activation of adenylate cyclase (AC) (Wollert and Drexler, 2002; Wisler et al., 2007). This bias potentially explains its unique clinical effectiveness in heart failure and other cardiovascular disorders.

While for some cardiovascular diseases most so-called 'β-blockers' (βAR antagonists) are effective, in others only a subset of such agents exert favorable actions. The βAR receptor has also been targeted to treat asthma, a chronic inflammation of the airways. The use of glucocorticosteroids and β2AR agonists represent the cornerstones of asthma therapy (Thanawala et al., 2014). Recently however, research has been performed into the possibility of using biased ligands in the treatment of asthma. Studies have shown that nadolol and ICI-118,551 are inverse agonists at βAR for both G protein- and β-arrestin-dependent pathways. Interestingly, while carvedilol and propranolol share this inverse agonistic activity, they can still activate ERK1/2 through β-arrestin signaling (Galandrin and Bouvier, 2006; Wisler et al., 2007). Further investigation into murine models for asthma, showed that nadolol, but not propranolol, reduced airway hyperresponsiveness, a functional subset of the asthma symptomologies. This beneficial effect of nadolol in comparison to propranolol is likely related to the ability of the latter to activate ERK1/2 signaling. Thus, blocking β-arrestin-mediated signaling via βAR might be advantageous in asthma treatment (Walker et al., 2011; Thanawala et al., 2014).

β-ARRESTIN IN AGING

With advancing age, as well as age-related pathophysiology, an increased expression and functional impact has been shown for β-arrestin and several other GPCR scaffolding proteins (e.g., GRKs) (Schutzer et al., 2001; Bychkov et al., 2008; Tsutsui et al., 2008; Lu et al., 2017). This finding poses an interesting pathophysiological question related to the 'complexity theory' of aging. As we age there is proposed to be a global homeostatic loss of complexity (Lipsitz and Goldberger, 1992; Manor and Lipsitz, 2013; Sleimen-Malkoun et al., 2014), mediated by the entropic breakdown of metabolic energy generation and damage repair systems. However, simultaneous with this global reduction homeostatic stability, it is likely that multiple compensatory mechanisms, e.g., switching energy metabolism from the Type II Diabetes mellitus (T2DM)-affected glucometabolic system to less efficient systems such as fatty acid beta-oxidation or ketogenesis, are instigated to attenuate damage due to the loss of optimal signaling system efficiency. This aging-driven increase in metabolic molecular signaling diversity results in a counterintuitive rise in 'allostatic' complexity alongside the loss of global homeostatic complexity (McEwen, 1998; Karlamangla et al., 2002; Sturmberg et al., 2015; Shiels et al., 2017). Therefore, at a receptor-based microcosmic level, do the increasing levels of β-arrestin, with the resultant increase in signaling diversity, represent perhaps the '*tip of the spear*' of aging? Alterations in complexity and diversity of GPCR systems will likely impact nearly all physiological systems implicated in aging, thus an enhanced appreciation of how altered GPCR signaling diversity controls the aging process is vital for the future development of pharmacotherapeutics with augmented efficacy profiles in the specific contexts of stress and aging. As a caveat to this posit however, it is likely that alterations in GPCR β-arrestin bias during age-related pathology are not simply unilateral or unidirectional, as signaling diversity from GPCRs may be vital for *both* allostatic and homeostatic mechanisms. Thus, in addition

to increased expression levels, decreases (reducing perhaps excessive allostatic load engendered by GPCR bias) in β-arrestin expression/functionality may also be important (Grange-Midroit et al., 2002). To dissect this complex temporal relationship between β-arrestin-mediated GPCR signaling diversity and the physiological aging context, it is first important to define the intersections between β-arrestin functionality and the major pathophysiological domains of the natural (as well as aberrant) aging process.

β-Arrestin Intersection With the Hallmarks of Aging

Oxidative Stress

The elevated presence of ROS in nearly every cell in the body needs to be countered to relieve the well-documented phenomenon of age-related oxidative damage (Redman et al., 2018). When the body is unable to balance these ROS with antioxidants, oxidative stress and damage occurs, leading to the deleterious modification of proteins, lipids, and nucleic acids. β-Arrestin has been shown to regulate oxidative stress by controlling NADPH oxidase 4 (NOX4), a major source of ROS in the heart. It was demonstrated that β-arrestin knockdown decreases ROS and NOX4 expression by 50% (Philip et al., 2015). Accumulation of ROS after UV and hydrogen peroxide (H_2O_2) treatment leads to the activation of multiple stress kinase cascades, such as the apoptosis signal-regulating kinase 1 (ASK1) signaling pathways, and then induce cell apoptosis (Tobiume et al., 1997; Chang et al., 1998; Ichijo, 1999; Morita et al., 2001). Through ASK1, β-arrestins prevent down-stream signaling and thus inhibits H_2O_2-induced cell apoptosis (Zhang et al., 2009).

DNA Damage

One of the most scrutinized hallmarks of aging is the accumulation of DNA damage. p53 is a central player in DDRs, where this protein is upregulated and activated by genotoxic stress. In cases of cellular stress, p53 induces an active transcriptional response of effectors promoting apoptosis, cell cycle arrest, senescence, and DNA repair (Speidel, 2015). MDM2 is a ubiquitin E3 ligase that targets p53 for proteasomal degradation (Haupt et al., 1997; Kubbutat et al., 1997). Hara et al. (2011) demonstrated that β-arrestin1-mediated signaling downstream of the β2AR, could trigger DNA damage and suppress p53 expression, which adds up to the accumulation of DNA damage. This by facilitating Akt-mediated activation of MDM2 and promoting MDM2 association with, and degradation of, p53, through its role as a scaffolding protein. Further investigation into the link between β-arrestin1 and p53, demonstrated an interaction between these two proteins *in cellulo* and *in vivo* in the brain, where 50% of the binding between β-arrestin1 and p53 occurs in the nucleus (Hara et al., 2011).

Metabolic Dysfunction

With respect to age-related somatic metabolic dysfunction, perhaps the most prevalent are Metabolic Syndrome and generic insulin resistance leading to T2DM (Bonomini et al., 2015). In both of these dysfunctional conditions there is a disruption of the ability/efficiency of insulin to stimulate insulin receptor signaling and effectively mobilize and use glucose as a primary energy source for mitochondrial oxidative phosphorylation (Feng et al., 2011). β-Arrestin1, can scaffold and recruit PI3K to the insulin growth factor-1 receptor (IGF-1R) in an agonist-dependent manner, thus facilitating the activation of PI3K and Akt. By doing so, β-arrestin1 is capable of weakening insulin-induced degradation of insulin receptor substrate-1 (IRS1) and thus promote downstream insulin signaling. Furthermore, β-arrestin1 binds to MDM2, as mentioned in Section "DNA Damage," and competitively inhibits MDM2-mediated IRS1 degradation, and in doing so improves insulin sensitivity (Usui et al., 2004; Lefkowitz et al., 2006).

β-Arrestin1 has also been implicated in insulin secretion through the Glucagon-like peptide-1 receptor (GLP-1R), which has been the focus for both new anti-diabetic and anti-neurodegenerative therapies (Janssens et al., 2014). Sonoda et al. (2008) reported that β-arrestin1 mediated the ability of GLP-1 to stimulate cAMP and insulin secretion in pancreatic β cells. Moreover, a direct physical interaction between the receptor and β-arrestin was found in cultured INS-1 pancreatic β cells (Dalle et al., 2011). Quoyer et al. (2010) discovered that in addition to this, β-arrestin1 can mediated GLP-1 anti-apoptotic effects by phosphorylation of the pro-apoptotic protein Bad through ERK1/2 activation (Quoyer et al., 2010). Broca et al. (2009) reported that β-arrestin1 could control potentiation of glucose-induced long-lasting ERK1/2 activation controlling IRS2 expression, through the actions of pituitary adenylate cyclase-activating polypeptide (PACAP) (Broca et al., 2009). This year, Jones et al. (2018) indicated that compounds retaining GLP-1R at the plasma membrane through a reduction in β-arrestin recruitment were able to engender greater long-term insulin release patterns. These compounds elicited glycemic benefits in mice without related increases in signs of nausea which often occurs with GLP-1 therapies (Jones et al., 2018).

In addition to alterations of metabolism mediated by glycoregulatory disruption, global metabolic functions in aging are affected by changes in thyroid hormone activity. Thyroid hormones serve to control the generic metabolic rate in the body through the control of glucose/lipid/protein catabolism. With advancing age however there is an increased prevalence of hypothyroidism, which is furthermore associated with coronary heart disease, heart failure and cardiovascular mortality (Gesing et al., 2012). An animal model for hypothyroidism showed unaltered β-arrestin1 expression in the heart, yet a decrease was seen in the lung and an increase in the liver, this in contrast to the expression of GRK5 which shows significant up-regulation in lung and heart and a decrease in the liver shortly after birth. This confirms an independent regulation of the expression of these proteins and thus suggesting the presence of significant 'active' compensatory mechanisms (Penela et al., 2001). The potential functional correlation of these changes in β-arrestin1 are highly nuanced, given the fact that this scaffolding protein acts downstream of GRK's in GPCR regulation (Michel-Reher et al., 1993).

Chronic Inflammation

Long-term uncontrolled inflammatory activity is a key trophic facet in the aging process. A chronic pervasive form of inflammation is so common among pathological aging phenotypes that the generation of the novel *portmanteau* description of this process as 'inflammaging' was warranted. β-Arrestins have been shown to act as scaffold proteins or signal transducers for key inflammatory signaling molecules in receptor tyrosine kinases (RTKs) signal transduction pathways, such as the NF-κB pathway (Gao et al., 2004). NF-κB is a ubiquitously expressed transcription factor that regulated genes involved in inflammation, immunity, and stress (Shoelson et al., 2003). The inflammatory NF-κB system has also been recently implicated in the basic metabolic control of the natural aging mechanism itself (Zhang et al., 2013). Campo and co-workers recently demonstrated that the anti-inflammatory action of β-arrestin2 appears to be mediated partially through the direct inhibition of p38 MAPK which prevents the activation of NF-κB, and partially through cAMP and PKA activation through G protein signaling, which also exerts an inhibitory effect on NF-κB (Campo et al., 2015).

Wang et al. (2015) reported that fenoterol, a βAR agonist used for relieving sudden asthma attacks, by stimulating βARs to relax bronchial smooth muscle, has the ability to inhibit 5-amino-1-b-D-ribofuranosyl-imidazole-4-carboxamide (AICAR)-induced AMPK activation and inflammatory cytokine production in cells. The AMPK pathway is involved in regulating inflammation in several cells lines (Wang et al., 2015). This inhibition, as well as the attenuation of tumor necrosis factor α (TNF)-α release, was abolished with the knockdown of β-arrestin2, indicating that β-arrestin2 likely facilitates the anti-inflammatory effects of fenoterol in AICAR-treated cells (Wang et al., 2016). Du et al. (2014) investigated the pro-inflammatory activity of fluoxetine (Prozac®), where they found that this antidepressant increased β-arrestin2 expression and enhanced the association of β-arrestin2 with transforming growth factor beta-activated kinase 1 (TAK1) binding protein 1 (TAB1) and disrupted the TAK1-TAB1 interaction, which attenuates the IκB (inhibitor of κB) degradation and NF-κB nuclear translocation (Du et al., 2014).

Further research in the role of β-arrestins in inflammation, has indicated that the anti- or pro-inflammatory dimensions of β-arrestin2 activity could be dictated by its ubiquitination status, which is linked to its ability to scaffold and localize activated ERK1/2 to receptorsomes (Jean-Charles et al., 2016). This was hypothesized since β-arrestin2 affects tumor necrosis factor receptor-associated factor 6 (TRAF6) in an anti-inflammatory manner, while physiologic β-arrestin2 promotes inflammation in disorders such as atherosclerosis and neointimal hyperplasia. In this specific context, the constitutive ubiquitination of β-arrestin2 furthermore augmented NFκB activation (Jean-Charles et al., 2016).

β-Arrestin in Age-Related Disorders

Type II Diabetes Mellitus (T2DM)

The occurrence of T2DM increases with age, where the body builds up an insulin resistance (linked to insulin receptor desensitization mechanisms), as well as the presence of a secretory dysfunction of the excess plasma-borne glucose (i.e., microvascular disease) (Matthaei et al., 2000; Taniguchi et al., 2006). Recent studies have shown that the orphan receptor GPR40 might be an attractive target to enhance insulin secretion in T2DM patients. Research into the relationship of β-arrestin and this receptor have shown that β-arrestin2, together with GRK2, play an essential role in the regulation of agonist-mediated internalization, but not of the constitutive GPR40 internalization (Qian et al., 2014). This suggests that perhaps targeting and modulating this GPR40 through β-arrestin2, thus influencing the internalization, might be an interesting road to take, in treating T2DM.

Additionally, there is a crosstalk between the insulin/Insulin-like growth factor 1 receptors and signaling pathways including G proteins and β-arrestin (Blair and Marshall, 1997; Lin et al., 1998; Povsic et al., 2003; Ryall and Lynch, 2008). In diabetic mice, β-arrestin2 expression is severely declined, moreover knockdown of β-arrestin2 worsened insulin resistance, while administration of β-arrestin2 could rescue this phenotype and restore insulin sensitivity in the mice. Lastly, the formation of a new β-arrestin2 signal complex occurs after insulin stimulation. In this complex, β-arrestin2 scaffolds Akt and Src to the insulin receptor directly. Loss or dysfunction of β-arrestin2 disrupted the formation of this novel signal complex and caused a disturbance of insulin signaling *in vivo*, as such aiding in the development of insulin resistance and thus the progression of T2DM (Luan et al., 2009). Also, β-arrestin2 plays a central role in irisin-induced glucose metabolism in T2DM by regulating the p38 MAPK signaling (mentioned in see section "E3 Ubiquitin Ligases"), which according to Pang et al. (2017) might present a novel therapeutic target for diabetes.

Lastly, as mentioned in Section "Metabolic Dysfunction," biased GLP-1R targeted ligands, diverting the signaling away from β-arrestin signaling, and allowing the GPL-1R to be retained at the plasma membrane, have shown promise for the treatment of metabolic dysfunction (Sonoda et al., 2008; Al-Sabah et al., 2014). This was further investigated by Zhang et al. (2015) using GLP-1R G protein-biased agonist called P5. In this research, it was shown that this biased ligand promoted G protein signaling comparable to GLP-1 and Exendin-4, yet demonstrated a significantly reduced β-arrestin response. This bias away from β-arrestin dependent signaling appears to be more effective than Exendin-4 at correcting hyperglycemia and lowering hemoglobin A_{1c} levels in diabetic mice after chronic P5 treatment (Zhang et al., 2015).

Age-Related Neurodegeneration

β-Arrestin2 has been shown to play a crucial role in the regulation of neurotransmitter receptors in the brain that are associated with the generation of age-related neurodegenerative phenotypes (Grange-Midroit et al., 2002). With advancing age, diverse and nuanced changes in β-arrestin expression have been noted. For example, a decrease in β-arrestin2 density has been found, which also occurs for most brain neurotransmitter receptors and specific G coupling proteins, indicating that this decline in expression of both receptor

and receptor regulatory proteins is a common consequence of the senescent brain (Sastre et al., 2001). Alzheimer's disease is a neurodegenerative debilitating disorder, which has been investigated thoroughly – unfortunately however no preventative therapy has been discovered yet despite this research. All efforts of helping AD patients are based on symptomatic treatments, in an attempt to slow down the progression of the disorder. Unfortunately, as AD is a neurodegenerative disorder, once the patients display symptoms, the disease has likely already progressed too far to be therapeutically reversed. Proteins being targeted for drug development are amyloid β, γ-secretase and the amyloid β precursor protein (APP). The aberrant processing of these proteins has been identified as a hallmark of AD development (Martin et al., 2013; Thathiah et al., 2013; Zhang et al., 2017).

Nervous systems functions, such as learning and memory, controlled by βAR are recently hypothesized to be mediated by G protein-independent signaling pathways. Liu et al. (2015) reported that memory retrieval induced the activation of β-arrestin signaling through the β1AR. β-Arrestin allows the stimulation of ERK signaling and protein synthesis, which, in this situation, leads to post-memory retrieval restabilization (Liu et al., 2015). In addition to this, β-arrestin2 knockout mice exhibit impaired memory retrieval in novel object recognition tests and in Morris Water Maze analyses. This reveals the potential therapeutic value of β-arrestin-biased ligands in the treatment of memory-related disorders (Liu et al., 2015). Further investigation into this relationship between β-arrestin and neurodegenerative disorders, has shown that β-arrestin2 expression is elevated in two independent cohorts of AD patients (Thathiah et al., 2013), and genetic variation of β-arrestin2 is associated with late-onset AD (Jiang et al., 2014).

When analyzing the relationship between β-arrestin and the AD biomarker protein amyloid β, β-arrestin2 overexpression leads to an increase in amyloid β peptide generation, the opposite was true for genetically silencing β-arrestin2 *in vitro* and *in vivo* in β-arrestin2 knockout mice (Thathiah et al., 2013). Moreover, β-arrestin2 has been shown to interact with GPR3 and β2AR GPCRs, which have both been implicated in AD pathology and mediate their effects on amyloid β generation. β-Arrestin2 also interacts with the anterior pharynx defective 1 homolog A (APH1A) subunit, part of the γ-secretase complex, which mediates the final catalytic step that liberates amyloid β from its precursor protein APP, and as a such, lies central to many amyloid β therapeutic strategies (De Strooper, 2003; Hur et al., 2012; Thathiah et al., 2013). Further investigating this interaction between β-arrestin2 and APH1A, the tri-cyclic antidepressant amitriptyline (Elavil®) has previously shown to cause strongly neurotrophic pharmacological activities, and is thus a possible treatment of neurodegenerative disorders such as AD. Acute cellular treatment of human neurons showed alteration in the physical interaction between the neurotrophic tyrosine kinase receptor 2 (NTRK2), and APP, and APH1A and β-arrestin2, suggesting that changes in this multi-protein complex may be associated with the beneficial therapeutic actions of amitriptyline (Martin et al., 2013).

Zhang et al. (2017) investigated the association of APP with GPCRs – they found that APP interacted with α_{2A}AR. The association between these two proteins is promoted by agonist stimulation, but competes with β-arrestin2 binding to the receptor and thus negatively affects receptor arrestin-dependent internalization and desensitization (Zhang et al., 2017). The α_{2A}AR plays an important role in controlling norepinephrine release and response. This discovery that APP can affect α_{2A}AR internalization may have an impact on modulation of noradrenergic activity and sympathetic tone, but also affecting pain perception and decreasing epileptogenesis and anxiety (Hein, 2006; Masse et al., 2006; Gyires et al., 2009; Zhang et al., 2017). The noradrenergic system regulates arousal, learning and memory, which has also been implicated in regulating neuroinflammation (Ardestani et al., 2017). This loss of the noradrenergic tone may underlie AD progression at many levels (Ardestani et al., 2017; Zhang et al., 2017), and has further also been investigated by Ardestani et al. (2017) in the 5XFAD mouse model of AD. This research group used a partial agonist of the β1AR, Xamoterol, which restores the behavioral deficits of AD mouse models (Ardestani et al., 2017). Xameterol has been demonstrated to show bias away from the β-arrestin toward the cAMP pathway. Moreover, Ardestani et al. (2017) looked for a potential role of the partial agonist to counter neuroinflammation, where chronic administration reduced mRNA expression of neuroinflammatory markers, but also amyloid beta and tau pathology, which are used as markers for AD development, measured by regional immunohistochemistry (Ardestani et al., 2017). This G protein-dependence was further investigated by Yi and co-workers (Yi et al., 2017) – their work lead to the development of a brain-permeable G protein-biased β1AR ligand for the treatment of neurocognitive disorders (Yi et al., 2017). This receptor is fundamentally involved in the pathological features associated with AD, such as the cognitive deficits, and regulation of neuroinflammatory processes. These data therefore indicate that β1AR may be a promising therapeutic target for AD, where its activation may produce neuroprotective effects in neuroinflammatory disorders. In their recent paper, Yi et al. (2017) identified a possible functionally selective partial agonist for this receptor, namely the molecule STD-101-D1, which shows high brain penetration and inhibits TNFα. However, further research needs to be performed in order to confirm its therapeutic potential (Yi et al., 2017).

The cognitive deficit in AD is thought to be caused by the degeneration of the cholinergic receptor system. This pathology is thought to be linked to a loss of signaling through the cholinergic M_1-muscarinic receptor subtype. Current studies offer an alternative mechanism involving the M_3-muscarinic receptor subtype that is expressed in numerous brain regions including the hippocampus. The M_3-muscarinic receptor knockout mouse demonstrates a deficit in fear conditioning learning and memory, which appears to be dependent on receptor phosphorylation/arrestin signaling. This opens the potential for biased M_3-muscarinic receptor ligands that direct phosphorylation/arrestin-dependent (non-G protein-dependent)

signaling as being beneficial in cognitive disorders (Poulin et al., 2010).

Osteoporosis

Human PTH regulates calcium homeostasis as well as bone formation and resorption via activation of the PTH1R – as such it is a possible target in treating the highly prevalent age-related bone disorder osteoporosis (Gesty-Palmer et al., 2005). Functions of arrestin in bone were first described in UMR 106-H5, an osteoblastic cell line, where β-arrestin2 was found to be involved in PTH1R desensitization (Bliziotes et al., 1996). Activated PTH1Rs recruit both β-arrestin1 and 2, which leads to the clathrin-dependent internalization of this complex, and arrestin-dependent scaffolding of the ERK1/2 cascade (Ferrari et al., 1999; Vilardaga et al., 2002; Gesty-Palmer et al., 2006). This ERK1/2 activation occurs via multiple independent pathways, involving PKA, PKC and/or arrestins (Verheijen and Defize, 1997; Lederer et al., 2000; Gesty-Palmer et al., 2009). Thus, as previously mentioned, β-arrestins have the ability to serve as multifunctional scaffolding proteins, in addition to their desensitizing actions. In this context, β-arrestins link the PTH1R to signaling molecules independently of classic G protein-mediated second messenger-dependent pathways (Bohinc and Gesty-Palmer, 2013). This ability to dissociate arrestin- and G protein-dependent PTH1R signaling in bone will likely influence future therapeutic design for osteoporosis treatment.

We have previously mentioned the role for β-arrestin in PTH signaling and in osteoporosis treatment (Gesty-Palmer et al., 2006; Gesty-Palmer et al., 2009; Luttrell and Gesty-Palmer, 2010). In addition to this, when PTH was administered intermittently to β-arrestin knockout mice the anabolic effects on the trabecular bone compartment were blunted, indicating that the recruitment of arrestins may be required to maintain a positive bone remodeling balance (Ferrari et al., 2005; Kostenuik et al., 2007). Unlike PTH, a β-arrestin biased PTH isoform (bPTH7-34) appears to uncouple the beneficial anabolic effects of the PTH1R activation from its catabolic and calcitropic negative side effects, thus confirming an effective role for β-arrestin in osteoporosis treatment (Gesty-Palmer and Luttrell, 2011; Luttrell et al., 2018a). When we analyze the interacting proteins of β-arrestin1 and 2, this clinically-relevant connection to osteoporosis reappears using the unbiased analysis of interactomic metadata (**Figures 1G–J**).

Cardiovascular Disorders

While both β-arrestin1 and 2 are expressed throughout the cardiovascular system, increases in β-arrestin2 expression have been found in aged aortas (Gao et al., 2004; Aplin et al., 2007). Additional research has indicated that this expression alteration also occurs within aged hearts (Dobson et al., 2003). Several GPCRs regulated by these two β-arrestins play immensely important roles in cardiovascular physiology and homeostasis. Contractility or cardiac function is tightly controlled by β1- and β2AR at the plasma membrane of cardiac myocytes. Cardiac structure and morphology on the other hand are regulated by AT1Rs in the cardiac fibroblast and endothelial cell membranes (Lymperopoulos and Bathgate, 2013). *Ex vivo* studies revealed that SII (mentioned in see section "Angiotensin II Type 1 Receptor") can stimulate the contractility of isolated cardiac myocytes via the AT1R and β-arrestin2 (Rajagopal et al., 2006) and MAPK activation in perfused hearts (Aplin et al., 2007). This data indicates that β-arrestin bias may have a substantial impact in a physiological cardiovascular setting.

Dobson et al. (2003) also observed a concomitant age-dependent decrease in β1AR and adenylyl cyclase mRNAs. Taking these results together, this strongly suggests that these expressional changes in β1AR, adenylyl cyclase and β-arrestin play a causal role in the declined adrenergic signaling seen in aged hearts (Dobson et al., 2003). Moreover, in the context of myocardial infarction in a mouse model of heart failure, the molecular deletion of β-arrestin1 at the genetic level leads to increased survival as well as decreased cardiac infarct size, myocardia apoptosis, and adverse cardiac remodeling (Bathgate-Siryk et al., 2014). β-Arrestin2, however, shows the opposite, where it mediated EGFR transactivation by β1AR and thus plays a protective role against cardiac apoptosis (Noma et al., 2007). Carvedilol preferentially stimulates βAR through β-arrestin. But even more than that, it induces the transition of the β1AR from a classical $G\alpha_s$-coupled receptor to a $G\alpha_i$-coupled receptor, thus stabilizing the distinct β-arrestin-dependent receptor conformation. This $G\alpha_i$ recruitment has not been shown for any other screened βAR ligand so far, nor is it required for β-arrestin activation by the β2AR (Wang et al., 2017). This advocates that the concept of β-arrestin-bias may need to be refined to include the selective bias of receptors toward distinct G protein subtypes.

Additionally, the α1ARs in the cardiovascular system function as stimulatory receptors and regulate vascular smooth muscle contraction. As such they aid in the elevation of systemic blood pressure through coupling to the $G\alpha_{q/11}$ protein – PLC – Ca^{2+} mobilization pathway (Piascik and Perez, 2001). Antagonists of this receptor type, and thus biased ligands, have the ability to lower blood pressure in hypertension, hence these ligands biasing toward G protein can be used to treat hypotension by causing vasoconstriction (Jensen et al., 2011).

β-Arrestins also possess the ability to regulate oxidative stress, one of the prime driving factors in metabolic aging, in a NOX4-dependent manner, instigating an increase fibrosis in heart failure. This was further investigated in cardiac fibroblasts, where β-arrestin overexpression increased mitochondrial superoxide production twofold, which stimulates collagen deposition, thus leading to myocardial fibrosis, a precursor to heart failure (Ichijo, 1999; Philip et al., 2015). The recruitment of β-arrestin to the AT1R possibly engages a pathway where Src phosphorylates and activates Akt, which in turn phosphorylates endothelial nitric oxide synthase (eNOS) (Haynes et al., 2003; Suzuki et al., 2006), which could further provide a connection between AT1R-arrestin function and cardiovascular tone via nitric oxide regulation (Violin

et al., 2010). Consistent with β-arrestin bias of TRV120027, eNOS activation by this ligand is eliminated by β-arrestin2 silencing. However, Ang II-stimulated eNOS phosphorylation is reduced by approximately 50% after β-arrestin2 knockdown, suggesting that the Ang II receptor can activate eNOS by both β-arrestin2-dependent and –independent pathways (Violin et al., 2010).

The endocrine peptide apelin has received considerable recent pharmacotherapeutic attention due to its potential therapeutic abilities for disorders such as pulmonary arterial hypertension and heart failure (Yang et al., 2015). Infusion of apelin leads to vasodilation, as well as cardiac inotropy without hypertrophy (Tatemoto et al., 2001; Szokodi et al., 2002; Berry et al., 2004; Ashley et al., 2005; Jia et al., 2006; Atluri et al., 2007; Maguire et al., 2009; Japp et al., 2010; Perjes et al., 2014; Brame et al., 2015). The predominant isoform in the human cardiovascular system is [Pyr^1] apelin-13 (Maguire et al., 2009). Upon activation with its endogenous ligand, the apelin receptor is rapidly internalized through β-arrestin (Zhou et al., 2003; Lee et al., 2010). This receptor desensitization may therefore limit clinical efficacy of apelin-based agents. By creating a G protein-biased small molecule apelin agonist, Read et al. (2016) hypothesized that they could provide a solution to this limitation. Such apelin receptor modulators were shown to have beneficial cardiovascular action compared to the native peptide in humans *in vivo* (Ceraudo et al., 2014; Chen et al., 2014). One such biased small molecule, CMF-019, has therefore been proposed as an effective future biased receptor therapeutic (Read et al., 2016).

Cancer

Next we will shortly discuss the role for β-arrestins in the development of cancer. As a downstream signaler of GPCRs and activator of tyrosine kinase Src it stands to reason that these scaffolding proteins might play a role in cancer development. The activation of the GPCR endothelin-A receptor by endothelin 1 appears to play an important role in ovarian tumorigeneses and advancement, through the recruitment of β-arrestin. The role of this scaffold protein lies in the formation of trimeric complexes through one of which is through Src-interaction leading to transactivation of EGFR and β-catenin tyrosine phosphorylation (Rosano et al., 2009). Additionally, β-arrestin can contribute to β-catenin stabilization, which is part of the cell invasion program, through a physical association with axin. Silencing of β-arrestin1 and 2 completely nullifies the involvement of β-arrestin in the relationship between endothelin-A receptor and β-catenin pathway in this invasive program. One antagonist, ZD4054, allows this abrogation to take place and as such may open up new therapeutic opportunities for the treatment of ovarian cancer (Rosano et al., 2009).

The relationship between EGFR and β-arrestin1 has further been analyzed by Buchanan et al. (2006) in relation to colorectal carcinoma. The prostaglandin E2-induced transactivation of EGFR in these cancer cells are facilitated through a c-Src-dependent mechanism, which regulates cell migration and differentiation, both of which are important in tumor development. In their research, Buchanan et al. (2006) found that the prostaglandin E/β-arrestin1/c-Src signaling complex formation is crucial in this transactivation of EGFR and thus implicates a functional role for β-arrestin1 as a moderator of cell migration and metastasis.

Furthermore, the involvement of β-arrestin signaling in IGF-1R downstream signaling in Ewing's sarcoma implicates that we should focus on a ligand showing bias away from β-arrestin. This was hypothesized as the association of the IGF-1R and β-arrestin1 due to the anti-IGF-1R antibody figitumumab, which has the following negative results: (i) receptor ubiquitination and degradation, and (ii) a decrease in cell viability and ERK signaling activation through β-arrestin1. As such, this signaling bias, i.e., β-arrestin1 regulation, suggests a possible therapeutic strategy to enhance response to anti-IGF-1R therapies (Zheng et al., 2012).

In addition to the demonstration that the EGFR receptor forms functional complexes with GPCR systems (Della Rocca et al., 1999; Prenzel et al., 1999; Maudsley et al., 2000; Roudabush et al., 2000), further GPCR-RTK associations, e.g., with platelet-derived growth factor receptors (PDGFRs) were subsequently shown to mediate a strong linkage between these signaling domains (Conway et al., 1999; Maudsley et al., 2000; Waters et al., 2003). PDGFR signaling has been strongly linked with gastric cancer (Qian et al., 2018), gliomas (Heldin et al., 2018), soft-tissue sarcomas (Vincenzi et al., 2017) and colorectal cancer (Manzat Saplacan et al., 2017). As β-arrestins form a crucial part of GPCR-based signaling it is not surprising that they possess the ability to modulate PDGFR signaling in concert with c-Src (Waters et al., 2005; Pyne and Pyne, 2017).

DISCUSSION

G protein coupled receptors are the most common molecular targets of clinically-relevant drug therapies – at this moment nearly 50% of all therapies are directed at the heterotrimeric G protein signaling GPCR states (Ayoub, 2018). However, it is now clear that the seven-transmembrane receptors can signal through β-arrestins in a clinically-relevant manner. By targeting GPCRs through their β-arrestin signaling, the number of drugs can perhaps be doubled. In addition to increasing the number of drugs, the specificity of these drug can also increase, thus aiding in the development of true 'precision medicine,' a medical model that proposes the customization of therapeutics and healthcare, with medical treatments being adapted to the individual patient or smaller patient groups clustered by similar molecular signatures. It has also become clear that *'biased ligands'* can selectively activate G protein and/or β-arrestin functions, thus eliciting novel biological effects for even the best studied GPCRs (Violin and Lefkowitz, 2007). In this review, we have focused on specific β-arrestin functions, more specifically its important role in aging, and thus the possibility in treating age-related disorders, such as neurodegeneration, and cardiovascular disorders.

It has become clear that depending on the goal of the therapy, it is important to push the *'bias'* toward β-arrestin, as is the case for osteoporosis discussed in Sections "Type 1 Parathyroid Hormone Receptor" and "Osteoporosis," or away from β-arrestin, as is the case for the μ-opioid receptor (see section "μ-Opioid Receptor"). While they have only been recently identified, biased ligands have actually been in pharmaceutical drug development for a long time, but they were labeled as classical 'antagonists' (Andresen and Luttrell, 2011). The most important question now for biased ligand design is 'how much bias is necessary for a successful therapy?' While 100% of β-arrestin bias will most likely cause adverse effects, 60% bias might be ideal for a specific therapy. Furthermore, should drugs be designed to incorporate, or avoid, activation of the β-arrestin signaling pathways? We believe that completely removing one of the two signaling pathways, could be more harmful than advantageous (Maudsley et al., 2012). The body has been designed to function through both pathways, thus completely removing one or the other, could be catastrophic.

G protein coupled receptors located at the plasma membrane function as information channels running between the external environment and the interior of the cell. This signal transduction is based on the physical interaction of receptors with the intracellular effectors after activation by the productive interaction with the extracellular ligands. Additionally, it has become clear that membrane receptors can assume multiple conformations, which are each possibly capable of interacting with a specific subset of possible effectors (Luttrell and Kenakin, 2011). Thus, it is important to note that GPCRs interact with a broad range of diverse proteins other than heterotrimeric G proteins or β-arrestins. It thus stands to reason that there may be other effectors which could elicit specific signaling effects as well. These factors may likely be scaffolding proteins that may interact with GPCRs in diverse physiological contexts (e.g., oxidatively stressed), as opposed to the general 'background' receptor interactivity of G proteins or β-arrestins. As such, ligand efficacy has truly become pluridimensional, which evokes more possibilities for nuanced therapeutic design, but also increases the complexity of ligand classification and design.

While ligand bias is now being widely explored and the development of biased therapeutics has begun, since the discovery of β-arrestin, no concerted systematic research has been performed with respect to the possibility that there might be more therapeutically-valuable scaffolding proteins which interact with GPCRs. These scaffolders might thus be potent downstream effectors and cause signaling cascades of their own just like β-arrestin, i.e., sodium-hydrogen exchange regulatory factor 1 (NHERF1) (Maudsley et al., 2000), Multi-PDZ domain protein 1 (MUPP1) and GRK interacting transcript 2 (GIT2) (van Gastel et al., 2018). The identification of these proteins might cause an even larger increase in the discovery of more effective and selective therapeutics. This allows us to believe that we are on the verge of a new age of GPCR targeted pharmaceuticals that will exploit the ligand bias to adapt drug efficacy by enhancing therapeutically beneficial signals and blocking harmful ones (Rajagopal et al., 2010).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01369/full#supplementary-material

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REVIEW
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GRK5 – A Functional Bridge Between Cardiovascular and Neurodegenerative Disorders

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Complex aging-triggered disorders are multifactorial programs that comprise a myriad of alterations in interconnected protein networks over a broad range of tissues. It is evident that rather than being randomly organized events, pathophysiologies that possess a strong aging component such as cardiovascular diseases (hypertensions, atherosclerosis, and vascular stiffening) and neurodegenerative conditions (dementia, Alzheimer's disease, mild cognitive impairment, Parkinson's disease), in essence represent a subtly modified version of the intricate molecular programs already in place for normal aging. To control such multidimensional activities there are layers of trophic protein control across these networks mediated by so-called "keystone" proteins. We propose that these "keystones" coordinate and interconnect multiple signaling pathways to control whole somatic activities such as aging-related disease etiology. Given its ability to control multiple receptor sensitivities and its broad protein-protein interactomic nature, we propose that G protein coupled receptor kinase 5 (GRK5) represents one of these key network controllers. Considerable data has emerged, suggesting that GRK5 acts as a bridging factor, allowing signaling regulation in pathophysiological settings to control the connectivity between both the cardiovascular and neurophysiological complications of aging.

Keywords: G-protein coupled receptor kinase 5, aging, cardiovascular disease, neurodegeneration, GRK5 interactors

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INTRODUCTION

GPCR Signaling

Heptahelical G protein-coupled receptors (GPCRs) represent one of the largest superfamilies of transmembrane receptor proteins. Rough estimates suggest the presence of between 800 and 900 somatic (i.e., outside of odorant receptors; Fredriksson et al., 2003; Matthews and Sunde, 2012) GPCR genes, or just over 1% of protein-generating genome. These plasma membrane GPCRs facilitate cell sensitivity to a diverse array of external stimuli including light, small chemical transmitters and large glycoprotein hormones (Takeda et al., 2002). GPCRs undergo a conformational change in response to an impinging regulator, allowing them to serve as guanyl nucleotide exchange factors for heterotrimeric GTP-binding proteins (G-proteins) resulting in their

dissociation (Cabrera-Vera et al., 2003). Dissociated heterotrimeric α and βγ subunits can then productively interact with downstream effectors to generate soluble second messenger molecules (e.g., calcium or inositol trisphosphate) to engender a broad range of biological actions (Blank et al., 1992; Exton, 1996; Maudsley et al., 2004; Hewavitharana and Wedegaertner, 2012). In addition to their capacity to confer sensitivity to stimuli involved in productive cellular signaling, GPCR systems also form a crucial part of stress response pathways linked to disease-propagating pathophysiologies.

G Protein-Coupled Receptor Kinases Modulate GPCR Signaling

GPCR-regulated heterotrimeric G protein signaling termination, as a specifically organized molecular event, has been described by many research teams and is now considered canon for most GPCRs (Zhang et al., 1999). After the initial ligand induced conformational change of the receptor and G protein complex, there is a recruitment of a class of serine/threonine kinases to the active receptor, i.e., G protein-coupled receptor kinases (GRKs). This association is initiated by interaction between the free G protein βγ subunits and a pleckstrin homology (PH) domain present within the GRK protein. Upon association, the GRK typically phosphorylates the receptor on available serine or threonine residues within an acidic amino acid context (Aspartate or Glutamate) found in the receptors three intracellular loops or the carboxyl-terminus (C-terminus). This phosphorylation both attenuates subsequent association with GDP-bound Gαβγ G protein heterotrimers, while promoting the association of β-arrestin molecules with the activated and phosphorylated receptor. This stable β-arrestin association serves to further inhibit subsequent G protein heterotrimer association and simultaneously increase the ability of the receptor to interact with components of endocytic systems such as clathrin and the AP-2 adaptor protein (Laporte et al., 1999). Therefore, via both GRK phosphorylation and subsequent β-arrestin association, the ligand-induced receptor activity is considered to be quenched and eventually terminated – this process has been classically referred to as agonist-induced receptor desensitization (Maudsley et al., 1998; Zhang et al., 1999). The desensitization process is vital to maintain continued physiological responsivity to stimuli and to protect the responding cell/tissue against protracted deleterious exposure to ligands. GRKs are not the only kinase type that can phosphorylate GPCRs after ligand activation, so-called heterologous (as opposed to GRK homologous) receptor phosphorylation can be mediated by second messenger-dependent protein kinases, e.g., protein kinase A or C (Steele et al., 2002). Subsequent research, however, has demonstrated that this generic desensitization process is not simply a signal termination event, as more of a signal modulation event. Hence, this canonical GRK-β-arrestin cascade actually transmutes the G protein signaling identity of the receptor to a non-G protein signaling mode, involving GRKs, β-arrestins and other GPCR-associated signaling molecules (Hall et al., 1998; Luttrell et al., 1999; Cant and Pitcher, 2005; van Gastel et al., 2018b). With specific respect to the interactions between GRK and β-arrestin signaling dynamics, there are further levels of nuance in this paradigm. Hence, it has been shown that GPCRs can be regulated by a variety of interacting GRKs, where some display a relative selectivity of interaction, while others demonstrate a promiscuity of association. In this scenario, differential phosphorylation patterns upon the receptor loops or C-terminus can engender a specific downstream effect on subsequent β-arrestin-based signaling cascades (Nobles et al., 2011; Lefkowitz, 2013).

Due to their ability to control a wide array of biological functions, GPCR-based signaling activity has become perhaps the primary target for effective therapeutic research. GPCR-based regulation of cellular pathological mechanisms has shown tremendous clinical relevance for neurodegenerative (Maudsley et al., 2007; Huang et al., 2017), metabolic (Martin et al., 2017; Riddy et al., 2018), neoplastic (Liu et al., 2016), respiratory (Douthwaite et al., 2017), and cardiovascular (Conrad, 2016) disorders. As studies continue to uncover further nuances in GPCR regulatory behavior, new roles, aside from simple receptor phosphorylation, for GRKs in GPCR systems have emerged. It is now apparent from many excellent studies that GRKs also control intermediary cellular metabolic signaling pathways, independent of GPCR functionality. It is in this regard that our review will focus, especially with respect to the body's capacity to control complex multi-tissue processes – such as aging – through coordination via systemic "keystone" proteins that possess a trophic level of control over coherent signaling networks.

Keystone Control of Cardiovascular Disease and Neurodegeneration Communication

At the present time there is considerable evidence emerging that suggests a functional connectivity between blood vessel damage due to arterial stiffness (Cefalu, 2011; Vlachopoulos et al., 2015; Costantino et al., 2016) and resultant dementia (van Sloten et al., 2015; Cooper and Mitchell, 2016; Pase et al., 2016; de Roos et al., 2017). Multiple molecular mechanisms that interconnect these two pathophysiological domains have been proposed based on the evidence of both *in cellulo* and *in vivo* animal studies. Arterial stiffness is a condition linked to the age-dependent pathological elevation of pulse wave velocity (PWV). Both murine and human studies have suggested that increases of PWV can lead to an increased incidence of cognitive decline and dementia (O'rourke and Hashimoto, 2007; Avolio, 2013; Marfella and Paolisso, 2016; Ryu et al., 2017). In addition to elevated PWV, arterial stiffness can result in a potentiated pulsatile flow in a broad range of cerebral blood vessels. Increased blood flow pulsatility has been shown to engender the creation of cellular reactive oxygen species (ROS) that, upon interaction with vascular and neuronal proteins, can induce cerebral gliosis and endothelial dysfunction with concomitant dysregulated blood brain-barrier (BBB) permeability. These deleterious effects of ROS can often cause a functional deficit within cerebral perfusion circuits, that both attenuate the distribution of metabolic fuels, while simultaneously reducing the ability to clear neurotoxic chemical products – these two events then conspire to promote cognitive dysfunction and dementia (Sadekova et al., 2013; Iulita et al.,

2017). It is widely accepted that complex age-related disorders represent highly interconnected molecular events spanning multiple tissues. We have demonstrated previously that in order to control these processes "keystone" proteins, that coordinate self-reinforced signaling networks in these processes, can be identified and potentially targeted therapeutically to attempt to mitigate these systems-level disorders (Lu et al., 2015; Martin et al., 2016; van Gastel et al., 2018a).

Aging, as a pathological process represents perhaps the strongest risk factor for both cardiovascular disease (CVDs) and neurodegenerative conditions (Kennedy et al., 2014). Indicative of the strong etiological role of the pathological aging process in the generation of cardiovascular diseases it has been shown that many of the well-characterized "hallmarks of aging" (López-Otín et al., 2013) are prominent in multiple CVD paradigms. Hence CVDs are often strongly associated with telomere attrition (De Meyer et al., 2018), epigenetic alterations (Rando and Chang, 2012), proteostasis alterations (Wiersma et al., 2016), disrupted nutrient sensing (Uryga and Bennett, 2016), mitochondrial dysfunction (Marzetti et al., 2013), and cellular senescence (Donato et al., 2018). Such a profound intersection is also observed between classical neurodegenerative pathways and those typically associated with pathophysiological aging. For example, aging has been correlated with the occurrence of several types of dementia, affecting 5–10% of people over 65, and about 50% of people over 85 years old (Prince et al., 2015). Alzheimer's disease (AD), perhaps one of the most common forms of dementia, shares multiple functional overlaps with canonical brain aging pathways including mitochondrial dysfunction (Müller et al., 2018), oxidative stress (Watts et al., 2018), calcium management alterations (Popugaeva et al., 2018) and impaired proteostasis (Chadwick et al., 2012; Martínez et al., 2017). Given the significant molecular connections between aging pathomechanisms and these two prevalent disease realms (CVD and neurodegeneration), the likelihood that there are physical bridges between these signaling domains is high. In this review, we will particularly discuss an incipient role of GRK5 in the context of a potential role for this GPCR-associated signaling factor as a trophic coordinator of both cardiovascular and neurodegenerative pathophysiologies.

G-Protein Coupled Receptor Kinases

GRKs are found nearly universally in complex organisms ranging from non-metazoans to vertebrate mammals (Mushegian et al., 2012). Currently there are known to be seven different GRK types, i.e., GRK1 to GRK7 (Kohout and Lefkowitz, 2003; Penela et al., 2003). Across these different types there is a shared 60–70% sequence homology. The seven GRK members are divided into three subfamilies based on their sequence homology (Premont and Gainetdinov, 2007). The rhodopsin kinase or visual GRK subfamily comprises GRK1 and GRK7. The β-adrenergic receptor kinases subfamily, comprising GRK2 and GRK3 have been most widely studied due to their strong association with canonical agonist-induced desensitization pathways. Finally, the GRK4 subfamily, comprises the kinases GRK4, 5, and 6. Here in this review we will focus upon the multidimensional functionalities of one of these isoforms, i.e., GRK5. With respect to their somatic distribution, near ubiquitous somatic expression has been shown for GRKs 2, 3, 5, and 6. The expression of GRK1 and GRK7 is restricted the retina (Hisatomi et al., 1998), while GRK4 expression is predominantly found in testicular, renal and cerebellar tissues (Premont et al., 1996; Sallese et al., 1997).

As previously discussed, the primary role conceptualized for GRKs was their ability to actively phosphorylate-signaling cell surface GPCRs (Pitcher et al., 1998; Penela et al., 2003; Lefkowitz and Shenoy, 2005). This GRK-mediated phosphorylation was then considered to simply attract β-arrestins to the active state receptor to further inhibit G protein-mediated signaling, through the so-called process of homologous receptor "desensitization." As a result of this desensitization process, phosphorylated receptors can then be targeted for endocytic removal from the plasma membrane via clathrin- or caveolae mediated processes. The fate of the internalized receptor is then sensitive to the degree of prevailing ligand stimulation – moderate cell surface receptor stimulation allows for recycling of the receptor to the surface for re-engagement with a ligand, while protracted excessive ligand stimulation overloads the recycling machinery and results in GPCR targeting for lysosomal degradation (Pitcher et al., 1998). While agonist-induced desensitization was considered to represent a signaling termination event, groundbreaking research demonstrated that indeed components of the desensitizing molecular machinery actual constitute a further mode of signaling of the "desensitized" receptor (Maudsley et al., 1998; Luttrell et al., 1999). Thus, pro-desensitizing β-arrestin activity represents only a small component of its activity and instead arrestin signaling itself seems to be potentially independent of G protein signaling and facilitates the creation of ligand-induced GPCR-based adaptor protein scaffolds. As well as possessing a potent role in the regulation of GPCR signaling dynamics, both GRKs and β-arrestins are also important signal conditioning factors in receptor tyrosine kinase signaling cascades (Hildreth et al., 2004; Robinson and Pitcher, 2013; Zhang et al., 2015). Therefore, it is clear that our appreciation of GRK functionality may need to include diverse cellular activities and also subcellular localities. For example, while the majority GRKs are predominantly cytosolic or found in proximity to the plasma membrane, GRK5 is also often concentrated in the cellular nucleus (Martini et al., 2008; Gold et al., 2013). Along with this re-appraisal of canonical GRK functionality, it was originally proposed that the GRK only phosphorylates the active ligand-bound GPCR (Li et al., 2015). With particular respect to this, it has been shown that members of the GRK4 subfamily (including GRK5) can also effectively phosphorylate inactive GPCR structures. This ligand-independent GRK phosphorylation still retains some of the aspects of the canonical agonist-induced desensitization paradigm, i.e., subsequent β-arrestin recruitment to the inactive yet GRK-phosphorylated receptor is still evident.

In addition to these non-canonical GRK functions, these multifunctional kinases can also control the ability of GPCRs to demonstrate biased signaling, dependent on the GRK-specific phosphorylation patterns generated in the receptor primary sequence (Choi et al., 2018). These phosphoprotein patterns can effectively determine which β-arrestin isoforms

(β-arrestin1 or 2) are recruited, and which specific super-structure conformations these eventual receptor-arrestin complexes then adopt (Lee et al., 2016). In this context therefore, the GRK interaction with receptors has a profound effect upon subsequent downstream non-G protein-dependent signaling cascades emanating from GPCRs. Therefore, both the qualitative and quantitative aspects of GPCR signaling (at both the G protein-dependent and –independent level) are likely to be a function of the relative expression level, catalytic activity, interactomic associations and subcellular localization of GRKs. In the following sections, the GRK5 protein itself, numerous GRK5 interacting proteins and GRK5's biological role in the context of connecting multiple age-related disease paradigms will be discussed.

Molecular Functionality of GRK5

GRK5 is composed of 500–700 amino acids and shares multiple common features with other members of the GRK superfamily (**Figure 1**). Thus, GRK5 possesses a central catalytic domain (∼270 residues), surrounded by a C-terminal domain of variable length (∼105–230 residues). GRK5, like the other members of the GRK4 subfamily possesses a specific amino-terminal (N-terminal) domain (∼185 residues). GRK5 possesses an amphipathic helix membrane binding domain, located in its C-terminal RH (RGS homology) domain, which is important for its function and proper localization at the plasma membrane (Pitcher et al., 1992; Koch et al., 1993; Premont et al., 1999; Kohout and Lefkowitz, 2003; Thiyagarajan et al., 2004; Penela et al., 2006; Xu et al., 2014). In contrast, the N-terminal domain of GRK5 appears to be important for intracellular membrane localization as well as for receptor recognition (Murga et al., 1996). The N-terminus also contains an RH domain (∼120 residues) (Kohout and Lefkowitz, 2003; Penela et al., 2003) as well as a phosphatidylinositol (4,5) bisphosphate (PIP2) region that can influence the kinases catalytic activity (Pitcher et al., 1998). Recently, GRK5 has been crystalized in two unique monomeric structures with consistent C-terminal structures closely packed to the RH domain. Individual subunits of the GRK5 architecture have been shown to be insufficient for persistent membrane association since disruption of the C-terminus/RH domain interface significantly decreases the GRK5 catalytic activity on GPCRs (Xu et al., 2014). Both the C- and N-terminal motifs predominantly localize GRK5 to the plasma membrane (at the expense of cytosolic concentration), which in turn facilitates its ability to control activation-independent phosphorylation activity at GPCRs (Li et al., 2015). GRK5 is subtly different from the other GRK4 subfamily members due to its possession of a nuclear localization sequence (NLS) motif. This NLS motif enhances the ability of GRK5 to translocate to the nucleus where it can exert non-canonical GRK activities, e.g., it has been shown that nuclear GRK5 can act as a histone deacetylase kinase and thus control gene transcription activity (Johnson et al., 2004; Martini et al., 2008). Binding of Ca^{2+} and calmodulin (CaM) to GRK5 has been shown to inhibit GRK5's membrane association, thus augmenting its nuclear localization (Gold et al., 2013). Hence it is apparent that the nuclear translocation of GRK5 likely exists in competition with its membrane localization. For example, C-terminal protein kinase C (PKC)-mediated phosphorylation attenuates its nuclear functional activity.

In addition to their classical roles in GPCR signaling cascades, work in the past decade has shown that GRKs also productively interact with many signaling factors outside this paradigm (Kurose, 2011; Gurevich et al., 2012; Hullmann et al., 2016). A broad range of GRK5 binding partners have been identified via different molecular biological approaches, e.g., Affinity Purification Mass Spectrometry and yeast-two hybrid screens. With the advent of well curated interactomic metadata for signaling proteins it is now relatively simple to appreciate that for many signaling systems, e.g., the GRK pathway, the extent of protein–protein interactions can significantly expand the potential of such multidimensional molecules to control both health and disease in a manner outside of their canonical activities. GRK5 interacting partners currently include single transmembrane receptors (Freedman et al., 2002; Hildreth et al., 2004) as well as cytosolic (Liu et al., 2005; Barthet et al., 2009; Lafarga et al., 2012) and nuclear proteins (Parameswaran et al., 2006; Martini et al., 2008). The productive interaction of GRKs with intracellular non-GPCR proteins profoundly influences diverse transduction pathways (Ho et al., 2005; Peregrin et al., 2006; Sorriento et al., 2008; Barthet et al., 2009; Wang et al., 2009; Patial et al., 2010) including cell cycle (Penela et al., 2010; Michal et al., 2012), apoptosis (Chen et al., 2010), cell motility (Penela et al., 2008; Lafarga et al., 2012), and inflammation (Sorriento et al., 2008; Patial et al., 2010). The study therefore of the functional GRK5 interactome, will likely help elucidate both novel mechanisms of intrinsic protein regulation as well as to further clarify GRK5-associated physiological signaling properties.

To assess the current state of the metadata concerning the known functional interactome of GRK5 (**Figure 2A** – left panel), we extracted binding partner identities from BioGrid[1], HPRD[2], IntAct[3], MINT[4] (The Molecular INTeraction Database), STRING[5] and DIP (Database of Interacting Proteins)[6]. The cumulated known physical interaction partners for GRK5 are detailed in the annotated **Supplementary Table S1**. The diversity of subcellular distribution (**Figure 2A** – center panel) and molecular function (**Figure 2A** – right panel) of these interactors were categorized using Ingenuity Pathway Analysis (IPA)-based annotation of the extracted GRK5 interactome metadata. The potential functional relationships between the protein factors in the GRK5 interactome were then analyzed with STRING functional network association analysis (**Figure 2B**). Using a high strength reliability cut-off, a strong functional connectivity between 134 of the 183 known GRK5 interactors was found. This GRK5 associated network demonstrated a very high enrichment probability for the represented network (i.e., $p < 10e^{-16}$). This network contained several functional groups

[1]https://thebiogrid.org/
[2]http://www.hprd.org/
[3]https://www.ebi.ac.uk/intact/
[4]https://mint.bio.uniroma2.it/
[5]https://string-db.org/
[6]http://dip.mbi.ucla.edu/dip/

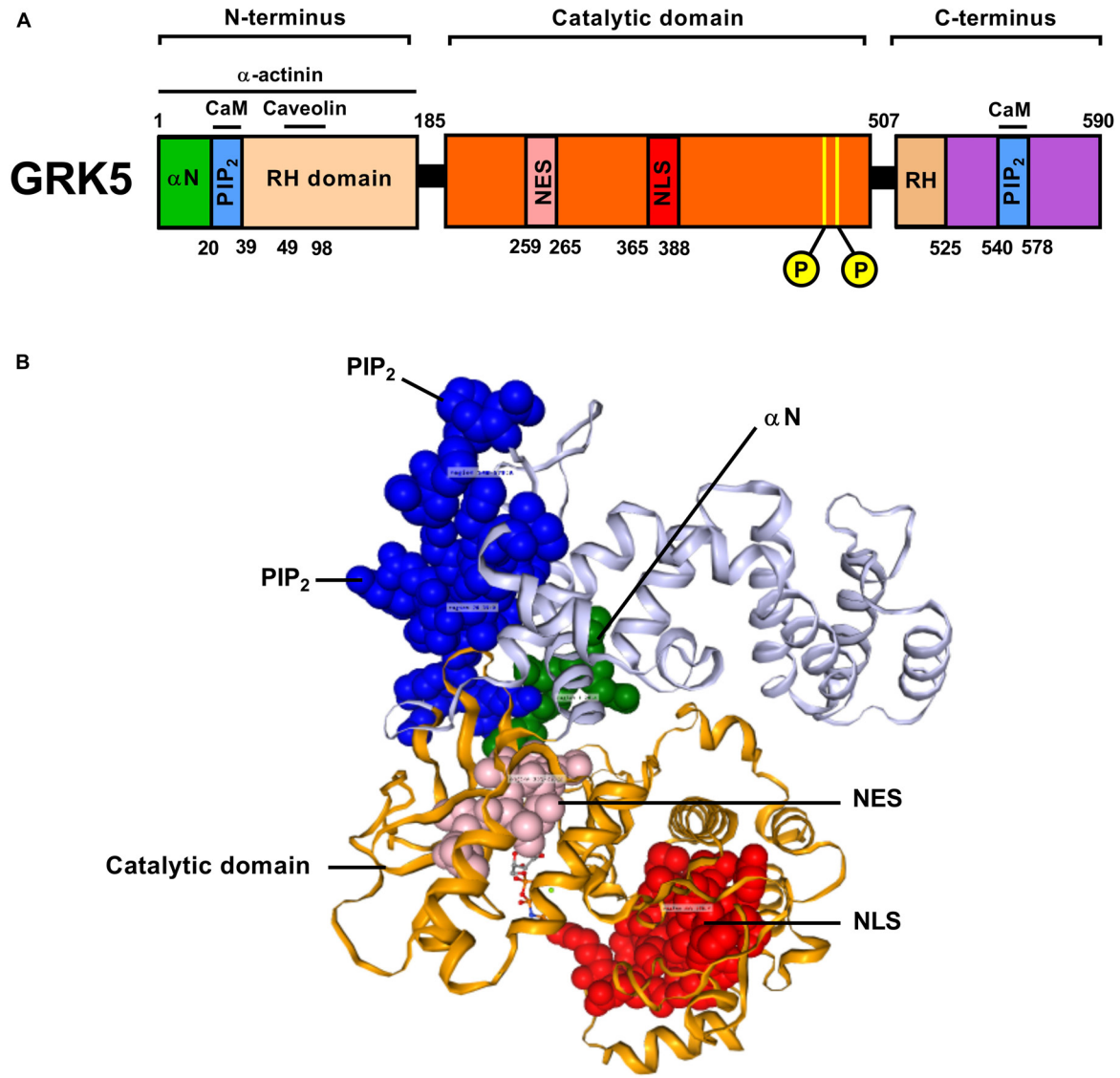

FIGURE 1 | A graphical block depiction **(A)** of the multiple functional regions of human GRK5 indicates the diverse range of activities mediated by this protein (α-N, terminal domain; PIP2, phosphatidylinositol bisphosphate binding domain; RH, Regulator of G protein signaling homology domain; NES, Nuclear Export Sequence; NLS, Nuclear Localization Sequence). In the primary sequence two autophosphorylation sites are indicated within the catalytic domain. A crystal-assisted model of human GRK5 **(B)** was constructed and annotated using 3DBionotes-WS (http://3dbionotes.cnb.csic.es/ws). This model indicates the three-dimensional proximities of the α-N domain (green), PIP2 binding domains (blue), NES (pink), catalytic domain (orange), and the NLS domain (red).

(assessed with unbiased k-means clustering) associated with: (*i*) classical GPCR functionality (group 1-Blue); (*i*) stress- and DNA damage-responsiveness (group 2 – Dark Green); (*iii*) protein chaperoning (group 3 – Mint); (iv) RNA metabolism and transcriptional control (group 4 – Yellow). Reinforcing this unbiased appreciation of the known molecular functionalities of the GRK5 interactome we found, using the network-building suite of the IPA platform, that similar functions (i.e., DNA Replication, Recombination, and Repair, RNA Post-Transcriptional Modification) were enriched in the top three most highly enriched signaling networks created using the metadataset of known GRK5 interactors (**Figure 3**). In addition to these functions reminiscent of the STRING-based predictions, we also noted that physiologically-relevant functions germane to the central hypothesis of this review were also evident related to these networks, i.e., "Cardiovascular System Development and Function" and "Nervous System Development and Function." Merging the top three highest scoring IPA-generated networks and allowing the unbiased IPA-mediated creation of a radial hierarchical super-network, revealed that indeed with simple database cross-analysis, GRK5 was evidently the central nexus of control of this aggregated dataset. Beside the generation of functional insights from protein–protein interaction networks within the GRK5 interactome, we also investigated this metadata

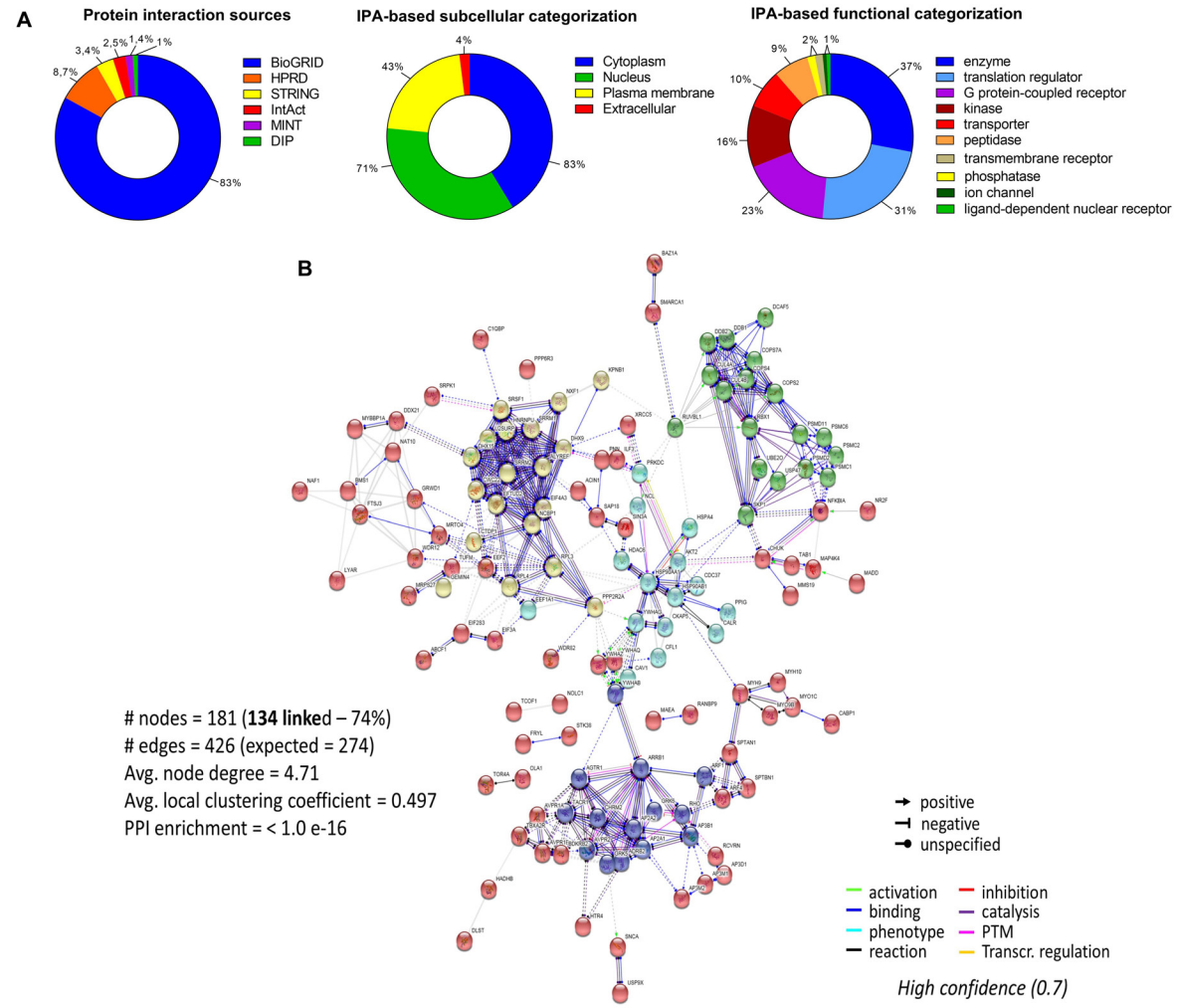

FIGURE 2 | Molecular analysis of the functional GRK5 interactome. The interactors of a protein are indicative of its function. Thus, to further investigate the function of GRK5 we extracted empirically identified GRK5 interacting partners from multiple informatics databases, BioGrid (https://thebiogrid.org/), HPRD (Human Protein Reference Database: http://www.hprd.org/), IntAct (https://www.ebi.ac.uk/intact/), MINT (The Molecular INTeraction Database), STRING (https://string-db.org/) and DIP (Database of Interacting Proteins: http://dip.mbi.ucla.edu/dip/). This curation generated a list of 183 proteins which are proven interactors of GRK5 **(A)**. The left panel indicates the distribution of the curated proteins from the aforementioned databases. This dataset was further analyzed using Ingenuity Pathway Analysis (IPA) to first create an unbiased assessment of the subcellular distribution (center panel) of the interactors and then secondly a functional categorization of the GRK5 interactors (right panel). Using our curated 183 protein input we employed STRING to investigate the strength of interactions between these diverse GRK5 interacting proteins. With this we applied a "strong" cut-off strength of confidence (0.7) and also limited the protein-protein interaction types to those empirically observed in physical interaction of co-expression experiments. In addition, all unconnected nodes were removed from the network **(B)**. K-means clustering was employed to group the interacting proteins in the network into five main clusters (blue, red, yellow, mint, and dark green).

from the aspect of IPA-assisted canonical signaling pathway analysis (**Figure 4A**). Using our standard cut-offs of signaling pathway enrichment (i.e., >2 proteins per pathway, with an enrichment probability <0.05) we found that from a signaling cascade perspective the GRK5 interactome was associated with multiple signaling systems linked to cellular protective activity (e.g., PI3K/AKT Signaling), apoptotic regulation (e.g., Myc Mediated Apoptosis Signaling), DNA damage repair and aging (e.g., Telomerase Signaling), metabolic activity (e.g., PPAR signaling), GPCR regulation and cardiovascular system control (e.g., Nitric Oxide Signaling in the Cardiovascular System). Applying a cut-off of at least three shared proteins between these diverse signaling pathways we found that indeed functional links between all of these cascades were evident (**Figure 4B**). This close connectivity indeed suggests that through a common association with GRK5, the different proteins populating these signaling systems are likely to coordinate these distinct activities in concert across the multiple tissues where GRK5 is expressed. Therefore, using unbiased informatic analyses of our curated GRK5 interactomic metadata, we have been able to demonstrate multiple insights into GRK5 functional biology and also reinforce our central post that GRK5 can act as an age-related bridge between cardiovascular and neurological pathomechanisms. A more detailed appreciation of the GRK5 interactome and its

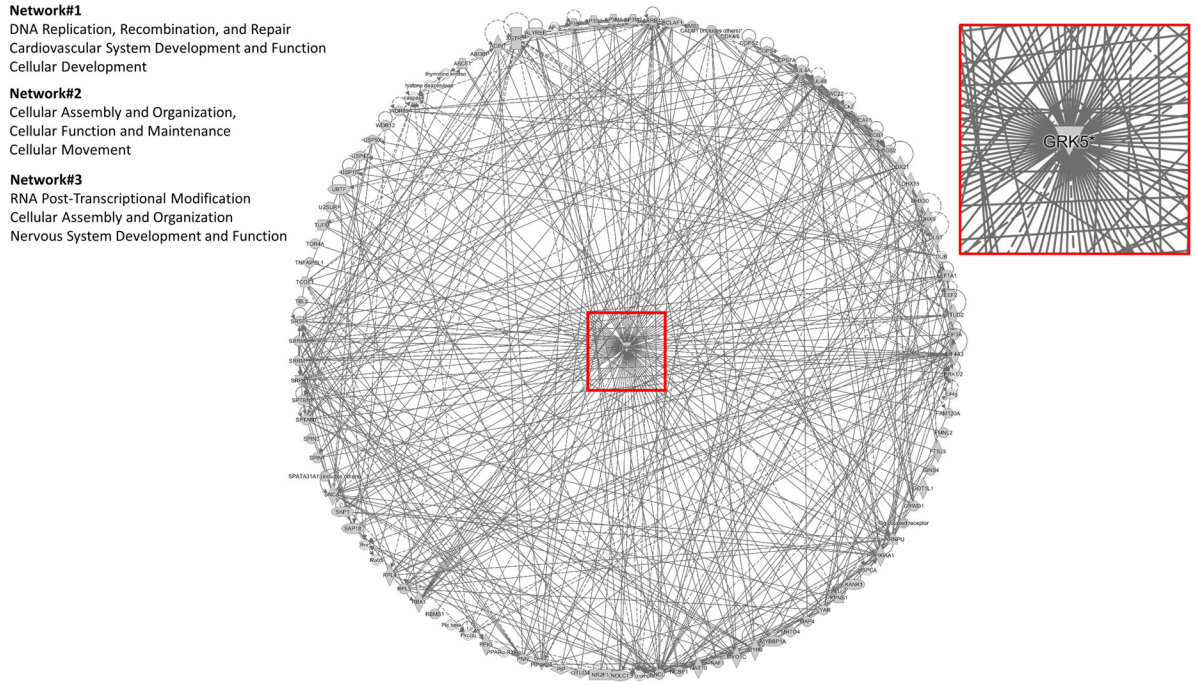

FIGURE 3 | IPA-mediated creation of a radial hierarchical super-network. A radial hierarchical "supernetwork" was constructed using the IPA network construction suite. Through this analysis the three highest scoring networks (based on numbers of interacting proteins from the primary input list of 183 GRK5 interactors) were associated with "*Cellular organization and movement*," "*Cardiovascular System Development and Function*," and "*Nervous System Development and Function*." These networks carry physiologically relevant functions germane to the central hypothesis of this review: GRK5 as a functional bridge between cardiovascular and neurodegenerative disorders. Upon merging these top three scoring protein networks we discovered that they all centrally converged upon GRK5 itself.

functional signaling spectrum will likely assist in the derivation of potentially new signal-specific therapeutics that exploit this signaling paradigm in a beneficial manner. In addition, our expanded understanding of GRK5 interactomics also helps place its comprehensive signaling activity in the context of whole-somatic "programs" of related molecular signatures.

Role(s) of GRK5 in Molecular Aging

GRK activity has been linked with multiple age-associated neoplastic, metabolic, neurodegenerative and cardiovascular ailments (Premont and Gainetdinov, 2007; Gurevich et al., 2012). At the specific disease level, the expressional regulation and activity of GRK5 has been linked with multiple age-related diseases such as type 2 diabetes mellitus (T2DM) (Li et al., 2013), cardiac hypertrophy (Gold et al., 2012), hypertension (Harris et al., 2008), Parkinson's disease (Arawaka et al., 2006; Bychkov et al., 2008), and Alzheimer's pathology in mice and humans (Suo et al., 2007). As we age, a progressive dysfunction of multiple receptor signaling systems, across a broad range of tissues, takes place. In this context of age-related receptor system dysfunction, the loss of signaling system sensitivity has been most intensively studies for the insulinotropic signaling cascade. Disruption of the ability to effectively sense, uptake and eventually metabolize glucose has been identified as a pivotal regulator of the rate of aging in nearly every animal model tested (Ma and Gladyshev, 2017). Many of the first genes identified in lower species that control animal longevity were almost exclusively associated with the insulinotropic/insulin-like growth factor system (Mathew et al., 2016). The glycometabolic system, as well as the somatic sensitivity to insulin receptor functionality, is also strongly controlled through the functional status of adipose tissue in the body, e.g., adiponectin release from white adipose tissue is a potent insulin sensitizing factor (Diez and Iglesias, 2003). Commensurate with a potentially important role of GRK5 in aging, it has been shown to be strongly expressed in adipose tissues, suggesting that its functionality may impact the glycoregulatory system. Wang F. et al. (2012) demonstrated that GRK5 genomic deletion in murine models resulted in the generation of significant insulin resistance. In addition to this, genetic polymorphisms of GRK5 have been strongly associated with the generation of T2DM (Xia et al., 2014) and the efficacy profile of anti-diabetic therapeutic agents (Shang et al., 2018). Furthermore, previous studies performed in GRK5 knock-out mice (GRK5-KO) reinforced the importance of GRK5 in metabolism as these animals displayed a decreased white adipose tissue mass, a lower weight gain, a decreased expression of adipogenic genes and a reduced adipocyte differentiation when fed a high-fat diet (Wang F. et al., 2012; Wang L. et al., 2012). Although human data linking GRK5 to metabolism are sparse, a recent genome-wide association study found a robust association of two single nucleotide polymorphisms (SNPs) in the GRK5 gene with apoB levels and total LDL-cholesterol, highlighting the role of GRK5 in cholesterol metabolism.

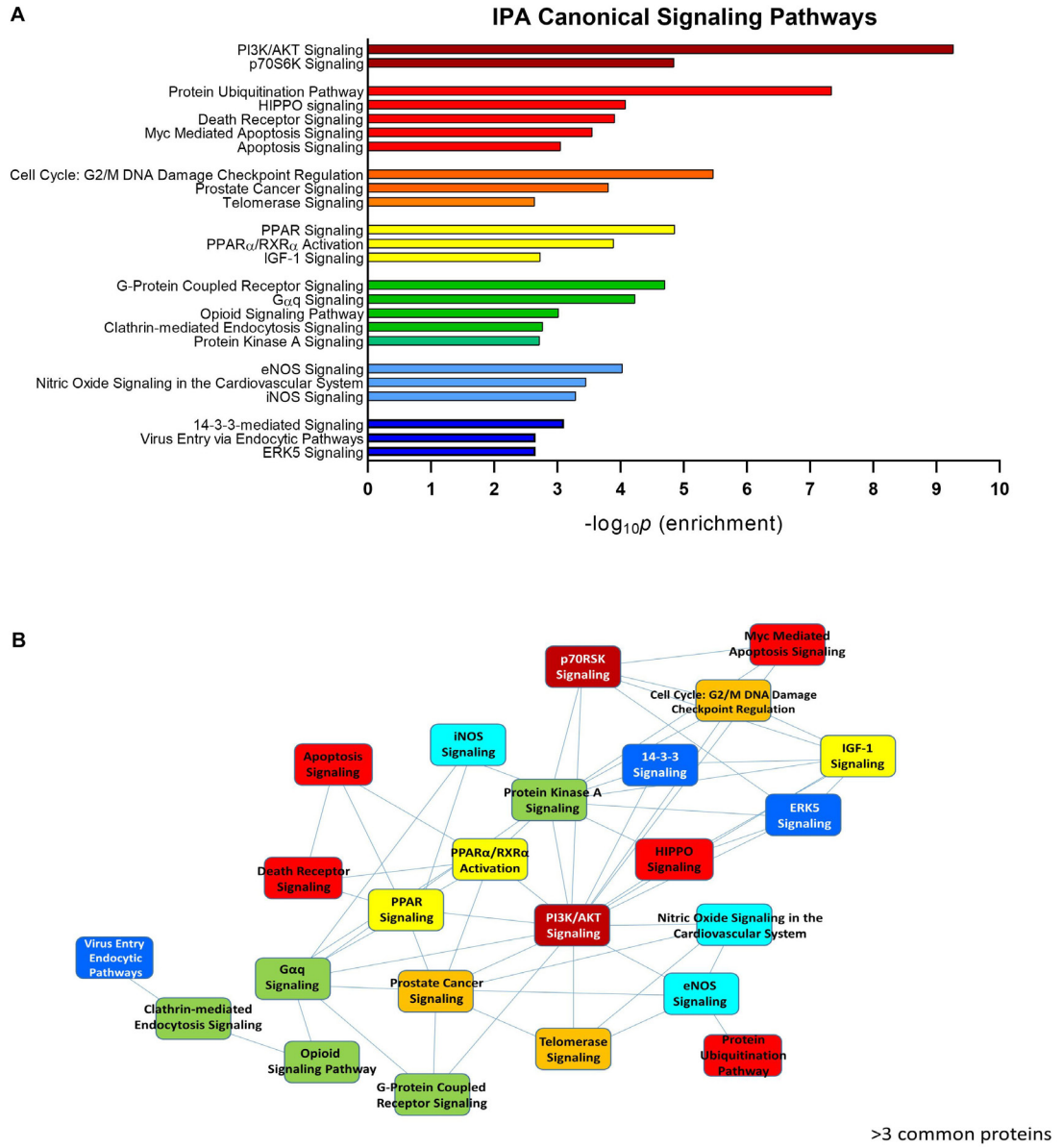

FIGURE 4 | IPA-assisted canonical signaling pathway analysis for the GRK5 interactome. The canonical signaling pathway suite of IPA was employed to generate a signaling cascade appreciation of the functional activity of the GRK5 interactome. **(A)** Indicates the top 20 most significantly ($p < 0.05$) enriched canonical signaling pathways generated with our curated GRK5 interactome. The pathways are color coded and clustered into different functional groups. The close association between these 20 pathways (mediated by common proteins involved in multiple signaling pathways: ≥3 proteins in common) is depicted by the pathway network diagram in **(B)**.

As well as long-term dysfunction of metabolic signaling systems in the aging process, significant disruptions of inflammatory mediator receptor systems are evident. This inflammatory signaling perturbation typically results in the creation of chronic low-grade inflammatory syndrome, recently codified as "inflammaging" (Baylis et al., 2013; Franceschi et al., 2018; Olivieri et al., 2018). Inflammaging, as a process, has been proposed to be functionally independent of exogenous systemic infection (Franceschi et al., 2000; Frasca and Blomberg, 2016). This chronic inflammatory condition has been linked to potentiated circulatory C-reactive peptide and IL-6 (interleukin 6) concentrations. Protracted exposure to these pro-inflammatory agents predisposes patients to an increased incidence of obesity, premature immunological aging, vascular sclerosis and neurodegenerative phenotypes (Tabas, 2010; Barzilai et al., 2012). The inflammaging process itself appears to be closely tied to the mechanisms of whole-somatic aging trajectory control. Hence, inflammaging has been

strongly linked to the potentiation of nuclear factor-κB (NF-κB) activity – a process that at the hypothalamic level seems to act as an arbiter of the aging process (Salminen et al., 2012; Zhang G. et al., 2013).

At a fundamental level NF-κB has been shown possess the ability to regulate the expression of GRK5 (Islam et al., 2013) – therefore these two systems in fact potentiate each other's activity in a feed-forward loop, a mode of signaling highly characteristic of the aging process itself. Demonstrating the intersection of GRK5 with the inflammatory aging process, GRK5 has been shown to antagonize TLR4 (Toll-like receptor 4) mediated phosphorylation of the NF-κB p105 protein. This action inhibits inflammatory mediator (lipopolysaccharide) sensitivity in macrophages (Parameswaran et al., 2006). Subsequent to the discovery of GRK5 regulation of p105, Sorriento et al. (2008) reported that GRK5 binding to IκBα stabilizes this protein and facilitates the nuclear accumulation of IκBα by masking and thus inhibiting its nuclear export signal sequence. This nuclear accumulation of IκBα can then lead to decreased NF-κB activation in vascular endothelial cells. Research from Wu et al. (2012) employing a GRK5 knockout (KO) murine model confirmed that endothelial GRK5 likely stabilizes IκBα in a manner reminiscent to previous studies (Rockman et al., 1996; Sorriento et al., 2008). Using this model, Islam et al. (2013) further demonstrated an NF-κB inhibitory action of GRK5 in cardiac muscle cells.

Taken together, GRK5 is clearly a vital component in both energy metabolism and chronic inflammation paradigms. It is interesting to note that both of these systems are known to strongly control molecular aging pathologies implicated in many different human disorders and consequently in inflammatory pathways (Packiriswamy et al., 2013). These findings therefore make GRK5 a potentially important therapeutic target in the treatment of age-related diseases such as cardiovascular disease, neurological and metabolic disorders. In this review, we discuss the role of GRK5 in the context of cardiovascular and neurodegenerative disease to emphasize its function in inflammaging.

The Role of GRK5 in Cardiovascular Disease Pathology

Cardiovascular pathophysiologies, such as myocardial ischemia, myocardial infarction or hypertension involve the dysregulation of cardiac GPCR responsiveness, which in turn is partly induced by deleterious GRK signaling activity profiles (Dorn, 2009; Cannavo et al., 2013). The first cardiac GRK form identified was GRK2 (Kwatra et al., 1989), while the discovery of cardiac GRK5 came later (Premont et al., 1994). GRK5 was found to be highly expressed in the myocardium through several studies employing genetically engineered mice with altered GRK5 levels (Kunapuli and Benovic, 1993; Premont et al., 1994; Premont and Gainetdinov, 2007). Homozygous GRK5-KO mice are born with a normal basal phenotype, although a loss of both GRK5 and GRK6 in mice results in lethality (Gainetdinov et al., 1999; Burkhalter et al., 2013). Further studies in zebrafish lacking the GRK5 homolog Grk5l, suggested the importance of GRK5 fine tuning capacity in cardiac development through the mTOR pathway. Hence, these Grk5l deficient fish demonstrated altered cardiac tissue generation associated with premature loss of muscle cell progenitors leading to an imbalance of gross structure (Burkhalter et al., 2013; Philipp et al., 2014). Of note, GRK5 is shown to be up-regulated in heart failure (Chen et al., 2001). It has been demonstrated that elevation of GRK5 expression in vascular smooth muscle cells (VSMCs) can also induce the development of high blood pressure (Harris et al., 2008) via altered β1-adrenergic receptor (β1-AR) and angiotensin II (Ang II) receptor signaling dynamics (Eckhart et al., 2002; Keys et al., 2005). GRK5 functionality also appears to be linked to the generation of atherosclerotic vascular pathophysiologies. Hence, the genomic deletion of GRK5 in an ApoE4-deficient murine background significantly accelerated the creation of aortic atherosclerosis compared to control mice (Wu et al., 2012).

Cardiac failure

GRK5 appears to exert a pivotal role in cardiac failure and several cardiomyopathies including cardiac hypertrophy (Dzimiri et al., 2004; Gold et al., 2012). Cardiac hypertrophy refers to the abnormal enlargement, or thickening, of cardiac muscle. This thickening can be caused by increases in cardiomyocyte size themselves or via changes in other cardiac muscular components, such as extracellular matrix. Cardiac hypertrophy can be induced via physiological effects (e.g., elevated cardiovascular exercise) or as the result of pathophysiology (e.g., hypertension or valvular disease) (Tardiff, 2006).

In humans, four non-synonymous SNPs of GRK5 with translational significance have been demonstrated. Of these known SNPs, the RH-domain resident Q41L polymorphism [leucine (L) converted to a glutamine (Q)] is highly enriched amongst African-American (A-A) individuals (Liggett et al., 2008). This divergent form of GRK5 possesses an augmented capacity to desensitize β2-adrenergic receptors (β2ARs) (Wang et al., 2008), thus engendering a population specific cardiovascular effect. The Q41L GRK5 variant appears to afford protection against congestive cardiac failure amongst A-A heart failure patients (Eijgelsheim et al., 2008; Liggett et al., 2008). Reinforcing the potential protective capacity of GRK5 in the cardiac setting, increased GRK5 expression has been shown to attenuate cardiac burden in response to intense adrenergic stimulation (Chen et al., 2001; Brinks et al., 2010). As expected, a GRK5 activity blockade mediates the opposite effect, i.e., increased cardiac performance as well as improved resilience in the context of heart failure (Raake et al., 2008; Vinge et al., 2008). It has also been shown that functional GRK5 inhibition, performed by ectopic expression of an N-terminal GRK5 peptide fragment of GRK5, reduces the extent of cardiac muscle damage and attenuates the risk of heart failure (Sorriento et al., 2010).

During cardiac failure, the expression and activity of GRK5 are reflexively increased to enhance β-adrenergic receptor desensitization and thus attenuate contractility (Chen et al., 2001). Activation of GPCRs by hypertrophic agonists, such as phenylephrine and/or Ang II, engages a number of intracellular signaling pathways, including calcineurin-nuclear factor of activated T cells (NFAT) (Molkentin et al., 1998), Ca^{2+}/CaM – dependent kinase II (CamK II) (Zhang et al., 2007;

Bossuyt et al., 2008), MAPKs (Purcell et al., 2007; Kehat et al., 2011) and the Akt-mechanistic target of rapamycin (mTOR) pathway (Shioi et al., 2002; Sussman et al., 2011) among many others, that are important transducers of the hypertrophic response.

GRK5 can undergo nuclear translocation in a calmodulin-dependent manner following $G\alpha_q$-based signals emanating from α-adrenergic and Ang II receptors. This nuclear translocation of GRK5 has been shown to be mutually exclusive with its interaction with plasma membrane GPCRs – thus distinguishing canonical and non-canonical GRK5 functions (Gold et al., 2012, 2013). This cellular redistribution is proposed to help mitigate the deleterious functions of cardiac hypertrophy (Yi et al., 2002; Johnson et al., 2004, 2013; Martini et al., 2008; Zhang et al., 2011; Gold et al., 2013). Nuclear GRK5 migration is assisted through a productive interaction with calcium sensing proteins (CSP) (Akhter et al., 1998) – thus GRK5 is specifically sensitive to the presence of Ca^{2+}/CaM (Freeman et al., 1998; Haeseleer et al., 2000). Indeed, GRK5, possessing a high affinity for CaM, is rapidly inactivated in cells upon elevations in cytosolic calcium. This aspect of GRK5 biology reinforces its pivotal role in the modulation of calcium-associated muscular contractility (Ikura, 1996; Schafer and Heizmann, 1996).

It has been demonstrated that nuclear GRK5 acts as a class II histone deacetylase kinase (HDAC). In this scenario it has been reported that GRK5 is able to phosphorylate HDAC5 (histone deactylase 5) (Johnson et al., 2013). This GRK5-mediated phosphorylation causes redistribution of HDCA5 out of the nucleus resulting in a function alleviation of its MEF2 (myocyte enhancer factor 2) transcription factor repression – leading to "de-repression" of MEF2. Demonstrating the important role of GRK5 in cardiovascular aging this GRK5-mediated MEF2 activation transcribes multiple genes associated with cardiac hypertrophy (Martini et al., 2008; Johnson et al., 2013). GRK5 activity has further been shown to control hypertrophic responses via its interaction in the nucleus with components of the NFAT pathway (Hullmann et al., 2014). GRK5 interacts with the NFAT-pathway in the nucleus during pathological hypertrophy. In addition, it is clear that GRK5 is strongly connected with the NF-κB signaling cascade (Parameswaran et al., 2006; Sorriento et al., 2008; Patial et al., 2009; Valanne et al., 2010; Islam and Koch, 2012; Wu et al., 2012; Islam et al., 2013) as an NF-κB binding element has been identified within the GRK5 DNA promoter region. This functional signaling region has subsequently been demonstrated to orchestrate the expression pattern of GRK5 in cardiac muscle cells (Islam and Koch, 2012).

Physiological hypertrophy does not only occur naturally in the heart due to augmented exercise regimens but also during pregnancy (Dorn, 2007). Non-pathological cardiac hypertrophy is a process typified by relatively normal and proportionate myocyte growth – this reflexive response increases the capacity for cytoprotective cardiac activity (Huang et al., 2011; van Berlo et al., 2013). In contrast, pathological hypertrophy involves a disruption of the proportions of the new myocytes that causes an eventual diminution of heart chamber volume with a concomitant augmentation of septal wall thickness (van Berlo et al., 2013). Recent research has suggested that GRK5 is only a controller of non-pathological hypertrophy (Traynham et al., 2015). In this study, using TgGRK5 mice, it was shown that physical exercise induced a classical physiological cardiac hypertrophy response. With specific respect to the nuclear functionality of GRK5 in cardiac hypertrophy it was shown in this exercise context that minimal nuclear GRK5 activity was found (Traynham et al., 2015). This corresponds with a study demonstrating that NFAT was not shown to regulate physiological hypertrophy (Wilkins et al., 2004). While elevated levels of intracellular Ca^{2+} levels are common to both physiological and pathological cardiac hypertrophy, it has been proposed that pathological hypertrophic effects are differentially controlled through distinct intracellular calcium stores. Thus differential sources of "activating" calcium may allow the specific stimulation of the GRK5-related hallmarks of pathological hypertrophy, i.e., nuclear GRK5 accumulation, HDAC kinase activity and increased NFAT activity. Reinforcing the concept of differential hypertrophic mechanisms, none of these selective events are routinely found in standard exercise-induced hypertrophic paradigms (Traynham et al., 2015).

While GRK5 is evidently associated with deleterious cardiac signaling and cell growth, GRK5 does appear to possess additional non-pathological roles in heart functionality. For example, GRK5 has been shown to be an important intermediate in the mitogenic and pro-survival signaling cascades emanating from the β1-adrenergic receptor-mediated transactivation (Maudsley et al., 2000) of the epidermal growth factor receptor (EGFR) (Noma et al., 2007). Therefore, it appears that GRK5 possesses a dual functionality with respect to cardiac activity, i.e., GRK5 is involved in both protective and detrimental signaling events that are delineated via differential subcellular compartmentalization between nuclear and non-nuclear sites.

Hypertension

The maintenance of well-controlled vascular blood pressure is imperative for effective and reliable delivery of oxygenated blood to all major life-preserving organs. Significantly and chronically elevated blood pressure, i.e., hypertension, is a prominent risk-determining player in the etiological profile of multiple chronic conditions including ischemic heart disease with associated subsequent cardiac and renal failure (Bath et al., 2003; Chobanian et al., 2003). The major organs and processes that endogenously regulate vascular pressure include the kidney, heart and the contractile state of VSMCs which regulates radial changes of blood vessels, thus modulating peripheral vascular resistance.

High blood pressure, with its associated stressful effects on vascular wall integrity, can result in the potentiation of GRK5 expression within VSMCs. Associated with these observations it has been shown that Ang II stimulation of VSMCs can also increase GRK5 expression levels in a calcium dependent manner (Ishizaka et al., 1997). This association between VSMC-based GRK5 expression and hypertension was again studied by Keys et al. (2005) in which an ectopic increase of GRK5 expression in vessels was genetically engineered. GRK5 overexpression was subsequently found to induce a gender-specific hypertensive response, i.e., blood pressure increases were much more

profound in males compared to females (Keys et al., 2005). Both male and female hypertension in these GRK5-overexpressing mice was ablated upon treatment with the inhibitor of $G\alpha_i$ signaling, pertussis toxin. Further gender-specific effects on the cardiovascular parameters of these GRK5-overexpressing mice were also apparent, e.g., β1-adrenergi receptor signaling in males was altered while Ang II-mediated increased vascular tone was only found in females (Keys et al., 2005). Interestingly, and in contrast to the reported overexpression of GRK2 in VSMCs, the elevation of GRK5 expression failed to induce any significant cardiac hypertrophy (Eckhart et al., 2002).

Atherosclerosis

Atherosclerosis presents as a long-term inflammatory disease found primarily in the major arteries. This condition is typified by the accumulation of oxidized low-density lipoproteins (LDL) within the arterial wall and a progressive inflammatory cell infiltration into the vessel (Ross, 1999; Braunersreuther et al., 2007). The recruitment of inflammatory cells to these lesions is triggered by the production of chemokines within the plaque microenvironment (Braunersreuther et al., 2007). Chemokine-stimulated GPCRs initiate several downstream effectors, promoting actin polarization, shape changes and directed cell movement which ultimately leads to atherosclerotic plaque formation (Kehrl, 1998).

GRK5 possesses the capacity to regulate signaling through multiple heptahelical receptors (Pitcher et al., 1998; Premont and Gainetdinov, 2007) including multiple types that have been strongly linked to etiological activities in the atherosclerotic process (Hayek et al., 1999; Tiruppathi et al., 2000; Fan and Malik, 2003; Kim et al., 2005; Bea et al., 2006; Zernecke et al., 2008). Interestingly GRK5 has also been shown to phosphorylate other signal transduction proteins that can influence the atherosclerotic process too, including p53 (Mercer et al., 2005), IκBα (Patial et al., 2009, 2011), platelet derived growth factor receptor-β (PDGFRβ) (Wu et al., 2006; Cai et al., 2008) and HDCA5, via MEF2 activation (Martini et al., 2008). GRK5 can also stimulate anti-atherogenic signaling activity in model systems. For example, GRK5-KO mice have an increase in lesion area when compared to wildtype mice through two different cell-type regulatory mechanisms in monocyte/macrophages and VSMCs (Wu et al., 2012). In VSMCs, GRK5 is able to promote the degradation of the pro-atherogenic platelet-derived growth factor receptor-β in lysosomes which is thought to reduce platelet-derived growth factor-mediated VSMC proliferation and migration (Wu et al., 2012). GRK5 also regulates monocyte chemotaxis; i.e., *in vitro* GRK5-KO monocytes possess increased migration capacity in response to C-C chemokine ligand 2 (CCL2) (a ligand for the C-C chemokine receptor type 2 (CCR2) receptor) and colony stimulating factor-1 (CSF1) (a ligand for the colony stimulating factor 1 receptor (CSF1R) tyrosine kinase) (Wu et al., 2012). CCL2-mediated leukocyte migration is instrumental in atherosclerotic lesion progression and responsible for the increased macrophage content in lesions from GRK5-KO mice. These findings highlight the potential mechanisms in both monocyte retention and emigration after their migration across the endothelium and present new strategies to limit atherosclerotic lesion progression.

GRK5 in Neurodegeneration

In contrast to its expression profile in cardiovascular organs, central nervous system (CNS) expression of GRK5 is comparatively sparse (Kunapuli and Benovic, 1993; Premont et al., 1994) because of a low GRK5 expression in the majority of cortical areas, except for the limbic system (Erdtmann-Vourliotis et al., 2001). As we have outlined previously there is emerging evidence that demonstrates the multiple non-canonical roles of GRK5 outside of GPCR activity regulation. These novel effects of GRK5 are also associated with multiple important neurophysiological functions. For example, GRK5 deficient mice display a specific and nuanced subtype-specific muscarinic receptor dysfunction while closely-associated adrenergic and opioid receptor activity was not altered (Gainetdinov et al., 1999; Matsui et al., 2004; Liu et al., 2009). CNS muscarinic receptor activity has long been associated with the maintenance of learning and memory behavior (Blokland, 1995). Thus, it is unsurprising that GRK5-KO mice present with cognitive dysfunction shown to correlate with hippocampal neurosynaptic failure (Liu et al., 2009). Again, as with the cardiovascular effects, gender differences in GRK5 activity were seen with respect to neurodegenerative phenotypes, i.e., augmented axonal defects and synaptic degenerative changes, were shown to be greater in female experimental animals as opposed to males. In addition, at the molecular signaling level, hippocampal levels of the synaptosomal-associated protein 25 (SNAP25) and synaptophysin were significantly lower in females compared to males (Liu et al., 2009).

It has also been proposed that the involvement of GRK5 in dementia-related conditions is likely associated with its potent role in regulating neurite outgrowth that is required for optimal learning and memory function (Chen et al., 2011).

Obstructive sleep apnea (OSA) occurs in approximately 2 to 4% of middle-aged women and men, respectively. Among these, OSA is also observed to be more common in obese patients, potentially due to increased tracheal occlusion caused by excessive cervical adipose deposits. While OSA can induce health concerns with respect to lack of effective sleep patterns, it is evident that OSA is also closely associated with intermittent cerebral hypoxia. Considering this deleterious hemodynamic effect it is unsurprising that OSA has been shown to be a potent risk factor for associated cognitive impairment in nearly a quarter of diagnosed OSA patients (Singh et al., 2016). At the molecular level CNS hypoxic episodes can often result in the increased production rate of ROS – these oxygen species can rapidly interact and modify a broad range of CNS lipids, nucleic acids and proteins. Enhanced CNS ROS production has therefore been associated vascular endothelial dysfunction, perturbations of blood-brain barrier integrity and eventual neurosynaptic signal transduction dysfunction. Rodent models of intermittent hypoxia have been developed to effectively replicate the OSA found in human patients (Badran et al., 2014; Singh et al., 2016). Using these, it has been demonstrated that intermittent hypoxia effects upon behavioral rodent activity (anxiety, balance, short-term

memory) are acutely sensitive to, and potently augmented by, the genetic deletion of GRK5 (Badran et al., 2014; Singh et al., 2016). Such research suggests that part of the CNS functionality of GRK5 may be associated with oxygen sensation neurochemistry, potentially via controlling astrocytic functions.

GRK5 and Alzheimer's disease (AD) pathology

For a significant period of time, undue focus on amyloid pathologies and their subsequent association with Alzheimer's disease (AD) has been in effect (Tanzi and Bertram, 2005; Jack et al., 2010; Mawuenyega et al., 2010; Lesne et al., 2013; Selkoe and Hardy, 2016). However, and from a more therapeutically important aspect, there has long been known to be an extant cholinergic receptor (post-synaptic nicotinic and M1 muscarinic acetylcholine) hypofunction evident in AD (Terry and Buccafusco (2003)). In AD it has been demonstrated that augmented presynaptic cholinergic activity results in the reflexive attenuation of synaptic acetylcholine release. This reduced release therefore results in diminished level of activity at the post-synaptic muscarinic M1 GPCRs. Indicating the importance of muscarinic signaling in AD pathophysiology, muscarinic M1 receptor signaling cascades can inhibit β-amyloidogenic (Aβ) amyloid precursor protein (APP) processing, resulting in a decreased level of cytotoxic β-amyloid accumulation (Sadot et al., 1996). From genetic deletion mouse models (i.e., GRK5-KO) it has been shown that GRK5 functionality is associated with severe hippocampal dysfunction (loss of neurosynaptic proteins and axonal swelling) as well as increased amyloidosis (Suo et al., 2007; Li et al., 2009).

When combined with murine AD models (Tg2576) GRK5 deficiency was found to cause increased inflammatory astrogliosis in both hippocampal and cortical brain areas (Li et al., 2008). In addition to this effect, the GRK5 deficiency was also linked with both increased soluble Aβ levels as well as increased insoluble Aβ plaque load (Cheng et al., 2010). These findings were proposed to be due to a GRK5-induced potentiation of presynaptic muscarinic M2 receptor activity that resulted in a significant reduction of synaptic acetylcholine transmission levels (Liu et al., 2009; Cheng et al., 2010). This GRK5-associated alteration of synaptic receptor activity in murine models of AD has been shown to be linked to disruptions in sub-cellular compartmentalization of GRK5. Hence, Zhang et al. (2014) were able to demonstrate that aged AD model mice possess a highly specific plasma membrane deficiency of GRK5 (Zhang et al., 2014). A paucity of pre-synaptic GRK5, with its concomitant detrimental effect on M2-acetylcholine receptor-controlled acetylcholine release, has been subsequently linked to an exacerbation of tau hyperphosphorylation and further neuronal dysfunction. Using chemical blockade of these hyperactivated M2 receptors Zhang et al. (2014) were able to attenuate this tau hyperphosphorylation in a GSK3β-dependent manner.

It is thus apparent that GRK5 may indeed hold the key to the connection between the current major theories of AD, i.e., the amyloid and the cholinergic hypotheses. The cholinergic hypothesis suggests that cholinergic CNS dysfunction is responsible for the cognitive decline (Bartus et al., 1982) while the amyloid hypothesis proposes that Aβ is the AD-causative factor (Bartus et al., 1985; Woolf, 1996; Hardy and Selkoe, 2002; Small and Cappai, 2006; Fisher, 2008). Interestingly, as we have previously outlined, Aβ is thought to be one of the driving forces for alterations of membrane associated GRK5 in AD (Suo et al., 2004). GRK5 plasma membrane deficiencies can mediate presynaptic M2 acetylcholine autoreceptor hyperactivation that, in turn, causes post-synaptic cholinergic hypoactivity through the functional attenuation of cholinergic neurotransmission. This disrupted cholinergic transmission then serves to augment Aβ amyloid production leading to a "feed-forward" process of progressive neurosynaptic dysfunction and amyloid toxicity. In this recursive process both amyloid deposition and cholinergic dysfunction each can serve as a cause and/or consequence of each other, with the extant GRK5 dysfunction as the pivotal mediator. Given the present interest in these hypotheses in AD pharmacotherapy, the importance of GRK5 as a drug target in this system may increase significantly in the future.

As a prelude to our next section, it is intriguing to note that GRK5 can be further connected with AD through its ability to phosphorylate α-synuclein (SNCA) (Pronin et al., 2000; Arawaka et al., 2006; Bychkov et al., 2008), tubulin as well as the AD-associated tau protein (Zhang et al., 2014). This pathological effect has been proposed to occur through GRK5-mediated phosphorylation causing increased SNCA polymerization and eventual aggregation – in a similar manner to that seen with Aβ in the context of AD (Carman et al., 1998).

Parkinson's disease (PD)

Parkinson's disease (PD) is one of the most commonly encountered neurodegenerative diseases at the present time, just behind AD with respect to world prevalence. The pathological effects of PD impact the primary fine motor systems of the body. PD is clinically typified by progressive deterioration of tremor, rigidity, bradykinesia/akinesia, gait disturbance, and postural instability. The major defining neuropathological feature of PD has long been considered to be the loss of neurons in the *substantia nigra* that provide dopaminergic innervation to the striatum, the CNS region most heavily implicated in fine motor control. Since the molecular mechanism causing dopaminergic neuron dysfunction are yet to be comprehensively defined, there are unfortunately no effective current pharmacotherapeutic interventions capable of retarding, or reversing, the disease (Dawson and Dawson, 2002). One of the lesser known aspects of PD is the fact that advancing age is arguably the strongest risk factor for its generation (Rango and Bresolin, 2018). In this light it is unsurprising that PD is typically is presented after the age of 60.

With respect to the functional intersection between GRK5 and PD pathology, it has been demonstrated by multiple research groups that GRK5 represents one of the major kinases that can phosphorylate SNCA. This classical function of GRK5 results in the promotion of the oligomerization of PD (with actual co-localization of GRK5 and SNCA), facilitating the creation of pathological Lewy bodies in the *substantia nigra* and *locus coeruleus* of PD patients (Pronin et al., 2000; Arawaka et al., 2006; Bychkov et al., 2008). The nuclear functionality of GRK5

is one of its defining functional features among GRK proteins – GRK5 activity itself has also been shown to promote the nuclear translocation of SNCA and its associated factors PLK2 and 3 (Polo-like kinase 2 and 3) (Goncalves and Outeiro, 2013; Fares et al., 2014). While the full ramifications of nuclear SNCA remain currently cryptic, it has been proposed that this aspect of SNCA biology may be independent of the classically-pathological SNCA aggregation modality. It is important to note, especially with respect to aging pathomechanisms, that oxidative stress environments promote the enhanced nuclear localization of SNCA (Xu et al., 2006; Monti et al., 2010; Siddiqui et al., 2012). Within the nuclear domain SNCA has been shown to functionally antagonize histone acetylation, resulting in increased neurotoxicity (Goers et al., 2003; Kontopoulos et al., 2006). Nuclear SNCA has also been found to be a transcriptional regulator capable of binding to PGC1-α (Peroxisome proliferator activated receptor gamma coactivator 1-alpha) promoter regions, and in doing so, potentially regulate mitochondrial gene transcription and thus neurometabolic ROS-associated activity (Siddiqui et al., 2012). In addition to these cell signaling-based analyses, genetic association studies have proposed a haplotypic association of GRK5 gene with the clinical presentation of sporadic PD. These pathological haplotypes associated with functional GRK5 SNPs that can control multiple transcription factors (Yin Yang-1 (YY1) and cAMP response element-binding protein (CREB-1)) that together are capable of potentiating SNCA transcription (Arawaka et al., 2006). Unfortunately, and as is quite common with genetic association studies, subsequent studies have failed to reproduce some of these propositions. Hence, studies employing GRK5 deletion in cells have failed to find a resultant attenuation of SNCA phosphorylation (Sakamoto et al., 2009; Liu et al., 2010). In addition, further studies have not observed a strong localization of GRK5 in Lewy bodies (Takahashi et al., 2006) or a firm association of GRK5 SNPs with PD (Tarantino et al., 2011).

Meta-Analysis of Diverse Molecular GRK5 Interactors

In our previous section (Molecular Functionality of GRK5) we applied multiple unbiased informatic pipelines to our extracted GRK5 interactome metadata to demonstrate that these diverse proteins do indeed possess concerted and interconnected molecular functions. Using a latent semantic analysis platform (GeneIndexer) (Cashion et al., 2013; Chen et al., 2013; Maudsley et al., 2018) we were able to prioritize multiple, functionally diverse GRK5 interactome factors that possessed the strongest textual associations with input interrogator terms describing, aging of cardiovascular and nervous systems. These proteins included L-3-hydroxyacylcoenzyme A dehydrogenase type II (HADH), 5-hydroxytryptamine receptor 4 (HTR4), GPCR-kinase interacting protein-1 (GIT1), histone deacetylase 6 (HDAC6), and eukaryotic elongation factor 2 (EEF-2). In the following sections we shall detail how these functionally diverse proteins, informatically prioritized from our unbiased GRK5 interactome metadata, still generate a dimensionally-condensed signature of the greater role of GRK5 in somatic coordination of cardiovascular and neurological deterioration with aging.

Enzyme: HADH – Mitochondrial Trifunctional Enzyme Subunit β

L-3-hydroxyacyl-coenzyme A dehydrogenase type II (HADH) acts as an endoplasmic reticulum (ER) amyloid β-peptide-binding protein (ERAB). It has been proposed that HADH can facilitate amyloid-induced neurodegeneration by enhancing Aβ toxicity and accumulation in neurons of AD patients. Investigations by Frackowiak et al. (2001) showed the absence of HADH in amyloid plaques or vascular amyloid, but did however denote the expression of HADH in VSMCs in both juvenile and aged control subjects as well as in amyloid free blood vessels in AD cases (Frackowiak et al., 2001). In this respect a potential interaction between HADH and Aβ in amyloid-producing cells was further studied in isolated VSMCs from CNS vessels presenting with an Aβ-related angiopathic condition. HADH had a mitochondrial localization (Zhou et al., 2012; Ibdah et al., 2001) and failed to co-localize with classical endoplasmic reticulum marker proteins. Aβ accumulating cells were those with a low HADH expression. However, the association between low HADH expression levels and Aβ depositions by brain VSMCs requires further studies (Frackowiak et al., 2001).

GPCR: HTR4 – 5-Hydroxytryptamine Receptor 4

The 5-hydroxytryptamine receptor type 4 (HTR4) appears to be the predominant cognate receptor responsible for serotonin responsivity within cardioventricular tissue in experimental models of congestive heart failure. HTR4 receptor expression contributes to positive inotropic responses and the productive signaling activity of these receptors is increased upon pathological transitions to heart failure. The HTR4 mediates positive inotropic responses to LV dilatation, as seen in post-infarction congestive heart failure (Brattelid et al., 2007). HTR4 mRNA levels are increased in male Wistar rats with increasing left ventricular hypertrophy and elevated further aging with increasing left ventricular (LV) hypertrophic failure. Therefore, the HTR4 can be differentially induced in LV hypertrophy and failure.

Experimental studies have also demonstrated that the HTR4 serotonin receptor is a prime controller of cognitive activity, depressive conditions and also the etiology of AD. Positron-emission tomographic studies of HTR4 CNS expression patterns across lifespan, using the selective ([11C]SB207145 ligand), found a gender-specific variation in expression profiles, i.e., HTR4 levels showed a profound decline in limbic system areas only in female subjects (Madsen et al., 2011). The deficits of HTR4 receptor expression found in women suggests a role for HTR4 receptors in cognitive and emotional control and may eventually contribute to the higher rate of affective diseases coincident with AD in female patients (Madsen et al., 2011).

Kinase: GPCR-Kinase Interacting Protein-1 (GIT1)

The GPCR-kinase interacting protein (GIT) family of proteins (GIT1 and GIT2) were originally identified as GRK and

GPCR interacting proteins (Premont et al., 1998). GIT1 is a multifunctional scaffold protein that possesses an ADP-ribosylation factor GTPase activating capacity. With respect to the intersection between GIT1 activity and cardiovascular functionality it has been demonstrated using GIT1 knockout (GIT1-KO) mice that loss of this receptor-associated protein caused structural and functional changes in cardiac mitochondria (Pang et al., 2011). In addition, this group found that several mitochondrial regulator genes (PGC-1α, PGC-1β, Tfam) were also profoundly reduced in the hearts of GIT1-KO mice. As expected, these mice subsequently present with reduced ATP-synthetic capacity and a strong increase in cardiac muscle apoptosis (Pang et al., 2013).

GIT1 genomic deficiency also has been shown to profoundly attenuate vascular smooth muscle growth capacity (Pang et al., 2013). Using a specific GIT1 deletion model of aortic smooth muscle cells Cyclin D1, a key cell cycle regulator, was found to be strongly downregulated significantly decreased in GIT1 knockout cells. Pang et al. (2013) continued to demonstrate that GIT1-associated muscle proliferation control occurred in a PLC-γ- and ERK1/2-sensitive manner. Further linking GIT1 functionality to vascular control in an aging paradigm, GIT1 has been shown to be a novel eNOS (endothelial nitric oxide synthase) binding partner. The association of GIT1 with eNOS has been shown to enhance the catalytic activity of this synthase and therefore suggests that GIT1 is an important controller of vascular relaxatory behavior. Interestingly, genomic ablation of GIT1 results in the opposite functional effect upon nitric oxide synthesis. GIT1 expression has also been shown to be reduced in vascular endothelial cells following hepatic damage (Shikata et al., 2003; Zhang et al., 2005; Jones et al., 2009; Pang et al., 2009). In this specific scenario, recovery of the endothelial expression of GIT1 was found to reverse the evident endothelial dysfunction found in hepatic damage cases. Re-expression of GIT1 after liver injury rescued the endothelial phenotype. Hence the GRK5-interacting protein GIT1, appears important for eNOS function and thus such an interaction will likely have tremendous import upon vascular disorders involving dysregulated eNOS such as arterial stiffness (Liu et al., 2012; Avolio, 2013).

In addition to its role in regulating nitric oxide synthesis, GIT1 has been linked to muscle cell proliferation as it can exert potent cardiovascular signaling effects via the control of Ang II-induced angiotensin receptor signaling. In a case of elegant research Pang et al. (2008) were able to demonstrate that Ang II-mediated HDAC5 phosphorylation (implicated in the c-Src-PLCγ-CamK II-HDAC5 signaling cascade that controls VSMC gene transcription) was GIT1-dependent (Pang et al., 2008). Within this paradigm the direct interaction of GIT1 and CamK II was required for effective Ang II-mediated HDAC5 phosphorylation. Finally, Pang et al. (2008) found that GIT1 genetic deletion reduced the transcriptional activity of MEF2 induced by Ang II. As GIT1 was selected as a binding partner of GRK5 in the meta-analysis, these findings reinforce the pivotal role of the non-canonical activity of GRK5 in the cardiovascular system which is largely associated with its nuclear HDAC5 kinase activity.

Transcriptional Regulator: HDAC6

As a cluster of enzymes, the primary role of histone deacetylases (HDACs) is to remove acetyl groups from an N-acetyl lysine amino acid on histone proteins. This histone deacetylation allows for a more compact and efficient packing of DNA with these histones. HDACs, as a protein family, are proteins grouped into four functional classes (I, II, III, IV). Class I, II, and IV possess a zinc dependent active site – these so-called "classic" HDACs are also typified by their enzymatic sensitivity to inhibition by trichostatin A. Class III HDACs comprise a family of trichostatin-insensitive, NAD+-dependent, proteins that are also referred to as "sirtuins" (de Ruijter et al., 2003; Vaquero et al., 2007). With specific respect to the GRK5-interacting HDA6, this protein is classified as a Class IIb HDAC. Demonstrating its close functional association with GRK5 signaling paradigms, HDAC6 expression and activity has been shown to be significantly elevated in stressed cardiac muscle (Lemon et al., 2011), while remaining unchanged in physiological cardiac hypertrophy models. In addition to these *in vivo* analyses, HDAC6 catalytic activity can also be induced by stressful stimuli impinging upon cultured cardiac muscle cells and fibroblasts (Lemon et al., 2011).

In a manner reminiscent to cardiac tissue, HDAC6 levels were found to be potently elevated in CNS regions important for the disease etiology of AD. At the present time research into the role of HDAC6 in AD, it has been proposed that the evident increased HDAC6 expression could be a driving factor in AD-associated neurodegeneration (Zhang L. et al., 2013). While HDAC6 activity has been proposed to be a pro-degenerative factor in the CNS, alternative evidence also points to some potentially neuroprotective functions. For example, molecular targeting of HDAC activity has been shown to be able to directly protect neurons and glia and thus improve physiological outcomes in CNS injury and disease models (Rivieccio et al., 2009).

Translational Regulator – EEF2

Evidence has been generated that directly implicates the transcriptional regulator eukaryotic elongation factor 2 (EEF2) in regulating the functionality of cells in response to myocardial ischemia (Demeulder et al., 2013). EEF2 interaction with mTOR and p70S6K appears to generate a regulatory complex that control protein synthesis in times of cellular stress and metabolic aging. This regulatory activity of EEF2 was subsequently shown to be sensitive to the expression levels of AMPKα2. The AMPKα2 protein was demonstrated in this cardiac ischemic models to control p70S6K and EEF2 in normoxic conditions specifically (Demeulder et al., 2013). Besides the role of EEF2 in the cardiovascular system, this elongation factor is found to be active in neurodegenerative signaling paradigms as well. Arguelles and co-workers investigated the role of EEF2 in the hypothalamic-hypophysis system. In old rats it is observed that during aging a considerable diminution of protein synthesis takes place in several tissues, potentially linked to modifications in EEF2. More specifically, research indicated that oxidative stress could be involved in EEF2 post-translational modification, with the resultant formation of covalent malondialdehyde (MDA) and 4-hydroxynonenal (HNE) EEF2 adducts. These long-lasting

alterations in EEF2 structure have therefore been proposed to effect the age-dependent attenuation of EEF2-controlled protein synthesis and thus dysfunctional hypothalamic control of neurometabolic activity (Arguelles et al., 2011).

DISCUSSION

GPCR signaling is an adaptable and highly dynamic process that forms a major component of the current pharmacopeia. Effective control of GPCR signaling dynamics is strongly dependent on several key proteins that regulate signaling sensitivity and post-activation functional fate (Takeda et al., 2002; Cabrera-Vera et al., 2003; Preininger and Hamm, 2004; Oldham and Hamm, 2008; Ritter and Hall, 2009; Hewavitharana and Wedegaertner, 2012). One such family that is intricately linked to the regulation of the activated receptors are the GRKs (Pitcher et al., 1998; Penela et al., 2003; Lefkowitz and Shenoy, 2005). GRKs demonstrate a broad range of activities within multiple physiologically important processes. Commensurate with this importance, perturbations of GRK systems have been linked to diverse pathologies such as bipolar disease (Barrett et al., 2007), AD (Obrenovich et al., 2009), rheumatoid arthritis (Lombardi et al., 1999), multiple sclerosis (Vroon et al., 2005), and PD (Bychkov et al., 2008). This review has focused on one such GRKs, i.e., GRK5 and in particular, the capacity of GRK5 to mediate a signaling connection between cardiovascular and neurodegenerative disease. GRK5 is one of the main cardiac GRK isoforms which is strongly expressed not only in cardiovascular but also CNS tissues. Moreover, unique non-receptor-dependent regulatory roles of GRK5 have been recently uncovered that may prove important for future therapeutic targeting (Kunapuli and Benovic, 1993; Premont et al., 1994; Premont and Gainetdinov, 2007; Schumacher-Bass et al., 2012). Human studies have suggested that GRK5 is increased in expression and activity in various cardiac diseases (Ishizaka et al., 1997; Tardiff, 2006; Gold et al., 2012; Wu et al., 2012) and GRK5 has recently been designated as a potential therapeutic target in cancer due to its anti-tumor effect when inhibited. Molecular inhibition strategies targeting GRKs have been shown to improve cardiac function in several animal models of cardiomyopathy (Raake et al., 2008; Rengo et al., 2009; Volkers et al., 2011; Homan et al., 2014; Gambardella et al., 2016).

To date, one GRK5 inhibitor, amlexanox, has been reported. This agent directly binds the GRK5 kinase domain and hereby inhibits the MEF2 transcriptional domain significantly (Homan et al., 2014). However, amlexanox is not GRK5 selective and yet has to be tested in cancer-related paradigms. To this end, the synthesis of a GRK5 selective inhibitor would be an effective anti-tumor treatment by promoting apoptosis and cell cycle arrest, especially in tumors with a low pro-apoptotic protein p53 abundancy. On the other hand, stimulation of GRK5 expression would be more effective in GPCR-dependent tumors (Gambardella et al., 2016). Xia et al. (2009) has previously performed work in which they classified 1800 compounds that can stimulate CREB activity, a transcription factor of, among other things, the GRK5 gene (Xia et al., 2009) – hence refining molecular CREB regulators may lead to the development of future GRK5 expression regulators.

Alterations of GRK levels, due to both canonical effects on GPCR sensitivity as well as through non-GPCR effects can lead to changes in signaling pathways that regulate apoptosis (Chen et al., 2010), inflammation (Sorriento et al., 2008; Patial et al., 2010) and hypertrophy (Dzimiri et al., 2004; Gold et al., 2012). While canonical cardiac GRK5 signaling can exacerbate the progression to heart failure, novel, non-canonical nuclear GRK5 molecular mechanisms (Martini et al., 2008; Zhang et al., 2011) suggest tremendous future opportunities for pharmacotherapeutic development (Schumacher and Koch, 2017). With the elucidation of novel therapeutically-tractable GPCR biased signaling mediated via β-arrestins, the importance of GRK signaling has received renewed interest. Hence, it has been shown that the specific coterie of GRKs that phosphorylate a receptor can exert a strong functional effect upon the subsequent nature of both G protein-dependent and β -arrestin-dependent signaling functions (Noma et al., 2007; Nobles et al., 2011; Choi et al., 2018). Thus, GPCR phosphorylation by GRKs is a pivotal cellular event as it desensitizes the active GPCR, but also dictates downstream signaling, functionally selecting for downstream G protein pathways or enabling β-arrestin-mediated pathways.

At the present time, it is widely accepted that complex age-related disorders such as cardiovascular and CNS ailments represent highly interconnected molecular events spanning multiple tissues. Moreover, considerable *in vivo* and *in vitro* research in both humans and animals suggests the functional connectivity of cardiovascular disorders with neurodegeneration. To reinforce this plethora of scientific literature, our review particularly focused on the potential function of GRK5 to both physically and functionally bridge cardiovascular and neurodegenerative disorders. Nowadays, a broad range of GRK5 binding partners, connecting many signaling proteins associated with these disorders, have been identified via multiple molecular biological approaches. The extent of such protein–protein interactions can significantly expand the potential of multidimensional molecules to control both health and disease beyond their canonical activities. More specifically, the interaction of GRK5 with non-GPCR proteins has been shown to profoundly influence transduction pathways controlling both CNS and cardiovascular disease trajectories. Hence, here we have assessed the current state of both literature-based data, as well as functional metadata, concerning the known scientific literature and the GRK5 interactome to better elucidate novel mechanisms of intrinsic protein regulation as well to further clarify GRK5-associated physiological signaling proteins. Our unbiased informatic analysis review of the curated GRK5 interactomic metadata, obtained from previously published material, reveals multiple insights into GRK5 functional biology and thus reinforces our central post that GRK5 can act as an age-related bridge between cardiovascular and neurological pathomechanisms. Likewise, physiologically-relevant functions are noted, when analyzing potential functional relationships in the GRK5 interactome, that are germane to the central hypothesis of this review, i.e., "Cardiovascular System Development and Function" and "Nervous System Development and Function."

An additional radial hierarchical super-network revealed GRK5 was as the central controlling nexus of this aggregated dataset. Moreover, we were able to prioritize multiple, functionally diverse GRK5 interactome factors via a latent semantic indexing platform that possessed the strongest textual associations with input interrogator terms describing, aging of cardiovascular and nervous systems. These proteins included HADH, HTR4, GIT1, HDAC6, and EEF2 which generate a dimensionally-condensed signature of the greater role of GRK5 in somatic coordination of cardiovascular and neurological deterioration with aging. A more nuanced appreciation of the GRK5 interactome and its functional signaling spectrum will likely assist in the derivation of potentially new signal-specific therapeutics in the future that exploit this signaling paradigm in a beneficial manner. In addition, our expanded understanding of GRK5 interactomics also helps place its comprehensive signaling activity in the context of whole-somatic "programs" of related molecular signatures. However, the complete ramifications of the correlation between GRK5 levels to different cardiac and neurodegenerative disease etiologies, across the human lifespan, still remains to be determined. Continued investigation will likely reinforce the importance of GRK5 as a therapeutically-exploitable systems-level controller and coordinator of the cardiovascular-dementia pathological axis.

AUTHOR CONTRIBUTIONS

JH, JvG, HL, PS-O, RP, BM, and SM all contributed to the writing and editing of this review. JH and SM generated the associated Figure and **Supplementary Table S1**.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2018.01484/full#supplementary-material

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Regulatory Role of GRK2 in the TLR Signaling-Mediated iNOS Induction Pathway in Microglial Cells

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G protein-coupled receptor kinase 2 (GRK2) is a ubiquitous member of the GRK family that restrains cellular activation by G protein-coupled receptor (GPCR) phosphorylation leading to receptor desensitization and internalization, but has been identified to regulate a variety of signaling molecules, among which may be associated with inflammation. In this study, we attempted to establish the regulatory role of GRK2 in the Toll-like receptor (TLR) signaling pathway for inducible nitric oxide synthase (iNOS) expression in microglial cells. When mouse MG6 cells were stimulated with the TLR4 ligands lipopolysaccharide (LPS) and paclitaxel, we found that interferon regulatory factor 1 (IRF1) protein expression and activation was upregulated, transcription of interferon-β (IFN-β) was accelerated, induction/activation of STAT1 and activation of STAT3 were promoted, and subsequently iNOS expression was upregulated. The ablation of GRK2 by small interfering RNAs (siRNAs) not only eliminated TLR4-mediated upregulation of IRF1 protein expression and nuclear translocation but also suppressed the activation of the STAT pathway, resulting in negating the iNOS upregulation. The TLR3-mediated changes in IRF1 and STAT1/3, leading to iNOS induction, were also abrogated by siRNA knockdown of GRK2. Furthermore, transfection of GRK2 siRNA blocked the exogenous IFN-β supplementation-induced increases in phosphorylation of STAT1 as well as STAT3 and abrogated the augmentation of iNOS expression in the presence of exogenous IFN-β. Taken together, our results show that GRK2 regulates the activation of IRF1 as well as the activation of the STAT pathway, leading to upregulated transcription of iNOS in activated microglial cells. Modulation of the TLR signaling pathway via GRK2 in microglia may be a novel therapeutic target for treatment of neuroinflammatory disorders.

Keywords: GRK2, iNOS, TLR signaling, interferon-β, IRF1, STAT pathway, microglia

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INTRODUCTION

Lipopolysaccharide (LPS), a glycolipid constituent of the outer membrane of Gram-negative bacteria, initiates inflammatory signaling cascades in cells, including monocytes, macrophages, dendritic cells, and endothelial cells, leading to the upregulation of cytokines, chemokines, and other inflammatory mediators, such as inducible nitric oxide synthase (iNOS) and

cyclooxygenase-2 (COX-2). As critical pattern recognition receptors for the first line of the host defense system against bacteria, viruses, fungi, and parasites, Toll-like receptors (TLRs) are widely found on the surface of those cells and play a key role in the innate immune system (Takeda and Akira, 2003; Beutler, 2009; Kawai and Akira, 2010; Satoh and Akira, 2016). Among these TLRs, TLR4 is activated by LPS, which is primarily associated with the accessory protein MD-2 and the co-receptor CD-14 to recognize LPS, resulting in transducing signals for activation of several transcription factors, such as nuclear factor-κB (NF-κB), in cooperation with myeloid differentiation factor 88 (MyD88) (Beutler, 2009). MyD88 leads to activation of the serine/threonine kinase interleukin-1 receptor-associated kinase 4 (IRAK4), which engages with mitogen-activated protein kinase (MAPK) cascades (extracellular signal-regulated protein kinase [ERK], c-Jun N-terminal kinase [JNK], and p38) and results in NF-κB activation and subsequent upregulation of expression of pro-inflammatory mediators (Byrd-Leifer et al., 2001; Guha and Mackman, 2001; Suzuki and Saito, 2006; Kawai and Akira, 2010; Satoh and Akira, 2016). Alternatively, the phosphoinositide 3-kinase (PI3K)/Akt pathway downstream of TLR4 signaling could also induce NF-κB activation (Li et al., 2003; Rosadini and Kagan, 2017). It should be also noted that the canonical NF-κB pathway responds to diverse stimuli, including TLR activation, is activated with the inducible degradation of IκBα (inhibitor of κBα) triggered through its site-specific phosphorylation by multi-subunit IκB kinase complex, and participates in the induction of type I interferons (IFNs) and pro-inflammatory cytokines (Shin et al., 2015; Liu et al., 2017).

G protein-coupled receptor kinases (GRKs) are serine/threonine kinases that were originally identified to phosphorylate activated G protein-coupled receptors (GPCRs) and cause desensitization of GPCR signaling (Premont and Gainetdinov, 2007; Ribas et al., 2007). The seven mammalian GRKs can be divided into three subfamilies based on sequence and functional similarities: the rhodopsin kinase or visual GRK subfamily (GRK1 and GRK7), the β-adrenergic receptor kinase subfamily (GRK2/GRK3), and the GRK4 subfamily (GRK4, GRK5, and GRK6) (Ribas et al., 2007). Among them, the ubiquitous isoform GRK2, also known as β-adrenergic receptor kinase-1 (βARK1), has been documented to regulate other pathways independently of its role in GPCR phosphorylation (Ribas et al., 2007; Jurado-Pueyo et al., 2008; Penela et al., 2014). Thus, GRK2 can restrain cellular signaling via direct interaction with downstream kinases such as Akt, MAPK kinases 1 and 2 (MEK1/2), PI3K, and p38 MAPK, leading to inhibition of their activities (Reiter and Lefkowitz, 2006; Ribas et al., 2007; Penela et al., 2010; Evron et al., 2012). Incoming evidence suggests a key role of GRK2 in the inflammatory signaling pathways. Intriguingly, GRK2 has been reported to be highly expressed in the immune system being a critical regulator of inflammatory responses (Vroon et al., 2006). Furthermore, mice with GRK2 depletion in cells of myeloid lineage appear to display exaggerated inflammatory cytokine/chemokine production and organ injury as a result of macrophage hyperreaction to endotoxemia (Patial et al., 2011). Besides, it is of interest to note that GRK2 levels are altered in immune cells from human patients with some inflammatory disorders (Lombardi et al., 1999, 2001; Giorelli et al., 2004; Vroon et al., 2005; Arraes et al., 2006; Cruces-Sande et al., 2018).

Our recent work has shown that GRK2 plays a critical role in iNOS gene transcription in microglial cells stimulated with LPS (Kawakami et al., 2018). Based on this result, we postulated that GRK2 may function as TLR signaling to induce iNOS expression. To test this hypothesis, we attempted to delineate the role and mechanisms by which GRK2 regulates the TLR signaling pathway for iNOS induction using cultured mouse MG6 microglial cells.

MATERIALS AND METHODS

Cell Culture and Reagent

Mouse microglial cell line MG6 cells (RCB2403) were obtained from RIKEN BRC (Tsukuba, Japan) and cultured as described previously (Kawakami et al., 2018). Cells were maintained until 70% confluency in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 10 μg/ml insulin, 10 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator. Phosphorothioate-modified oligodeoxynucleotide (ODN) 1668 and control ODN were synthesized by Hokkaido system science (Hokkaido, Japan). The ODN1668 sequence was TCCATGACGTTCCTGATGCT and used as CpG-ODN. The control ODN (CTL-ODN) sequence was TCCATGAGCTTCCTGATGCT. GRK2 inhibitor (GRK2i), methyl 5-[2-(5-nitro-2-furyl)vinyl]-2-furoate was purchased from Calbiochem (San Diego, CA, United States). Stattic was obtained from Abcam (Cambridge, United Kingdom). BAY11-7082 and MG-132 were purchased from Sigma (Sigma, St. Louis, MO, United States).

siRNA Transfection

All small interfering RNAs (siRNAs) were purchased from Sigma-Aldrich (St. Louis, MO, United States). MISSION siRNA universal negative control (SIC-001) was employed as the negative control in this study. GRK2 siRNAs, signal transducers and activators of transcription 1 (STAT1) siRNAs, IFN regulatory factor 1 (IRF1) siRNAs, TIR-domain-containing adaptor-inducing interferon-β (TRIF) siRNAs, STAT3 siRNAs, interferon alpha and beta receptor subunit 1 (IFNAR1) siRNAs and IRF3 siRNAs were transfected at a final concentration of 60, 15, 50, 50, 40, 40, and 50 nM using lipofectamine RNAiMAX (Life Technologies, Carlsbad, CA, United States) according to the manufacturer's protocol, respectively.

Western Blot Analysis

Cells were harvested and lysed in 300 μl of Radio-Immunoprecipitation Assay (RIPA) buffer (Thermo Fisher Scientific, Rockford, IL, United States) containing protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) on ice. The lysates were centrifuged at 18,000 × *g* for 10 min at 4°C and the resulting supernatants were reserved. The supernatant proteins were quantified using BCA Protein Assay Kit (Thermo Fisher Scientific). Samples (20 μg of protein) were run on 10% polyacrylamide gel and electrotransferred

onto polyvinylidene fluoride filter membrane. The membrane was blocked for 60 min at room temperature in 1% bovine serum albumin in Tris-buffered saline containing Tween 20, followed by overnight incubation with primary antibody, anti-iNOS rabbit polyclonal antibody (1:1,000; Cell Signaling, Danvers, MA, United States), anti-STAT1 mouse monoclonal antibody (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, United States), anti-phospho-STAT1 (Tyr-701) mouse monoclonal antibody (1:300; Santa Cruz Biotechnology), anti-GRK2 rabbit polyclonal antibody (1:500; Santa Cruz Biotechnology), anti-STAT3 mouse monoclonal antibody (1:1000; Cell Signaling), anti-phospho-STAT3 (Tyr-705) rabbit monoclonal antibody (1:1000; Cell Signaling), anti-IRF1 rabbit monoclonal antibody (1:1000; Cell Signaling), anti-lamin B1 rabbit polyclonal antibody (1:3000; Proteintech, Rosemont, IL, United States), or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (1:10000; Wako Pure Chemical, Osaka, Japan), at 4°C. Primary antibody detection was performed with horseradish peroxidase-conjugated secondary antibodies. Binding of the antibody was detected by an enhanced chemiluminescence (ECL) Plus chemiluminescent system (GE Healthcare, Tokyo, Japan) and levels of protein expression were quantified by a lumino image LAS-4000 analyzer (Fuji Film, Tokyo, Japan). Additional details are described by our laboratory (Sakata et al., 2015; Abdelzaher et al., 2016; Ohashi et al., 2017; Kawakami et al., 2018).

RNA Extraction and Quantitative Reverse-Transcribed PCR

Total RNA was isolated from cells with the use of Sepazol-RNA I Super G (Nacalai Tesque) according to the manufacturer's manual. ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) was used for the reverse transcription reaction, and quantitative PCR analyses were performed using PowerUp^{TM} SYBR® Green Master Mix (Thermo Fisher Scientific), as described in the manufacturers' instructions. Values were normalized to the housekeeping gene GAPDH according to the manufacturer's protocol (MX3000P real-time PCR system; Agilent Technologies Inc., Santa Clara, CA, United States). The IFN-β primer sequences were 5′-CAGCTCCAAGAAAGGACGAAC-3′ (sense) and 5′- GGCAGTGTAACTCTTCTGCAT-3′ (antisense), the iNOS primer sequences were 5′-TAGGCAGAGATTGGAGGC CTTG-3′ (sense) and 5′-GGGTTGTTGCTGAACTTCCAGT C-3′ (antisense), the IRF1 primer sequences were 5′-ATG CCAATCACTCGAATGCG-3′ (sense) and 5′-TTGTATCGGCC TGTGTGAATG-3′ (antisense), the interferon-γ-inducible 10 kD protein (IP10) primer sequences were 5′-CCAAG

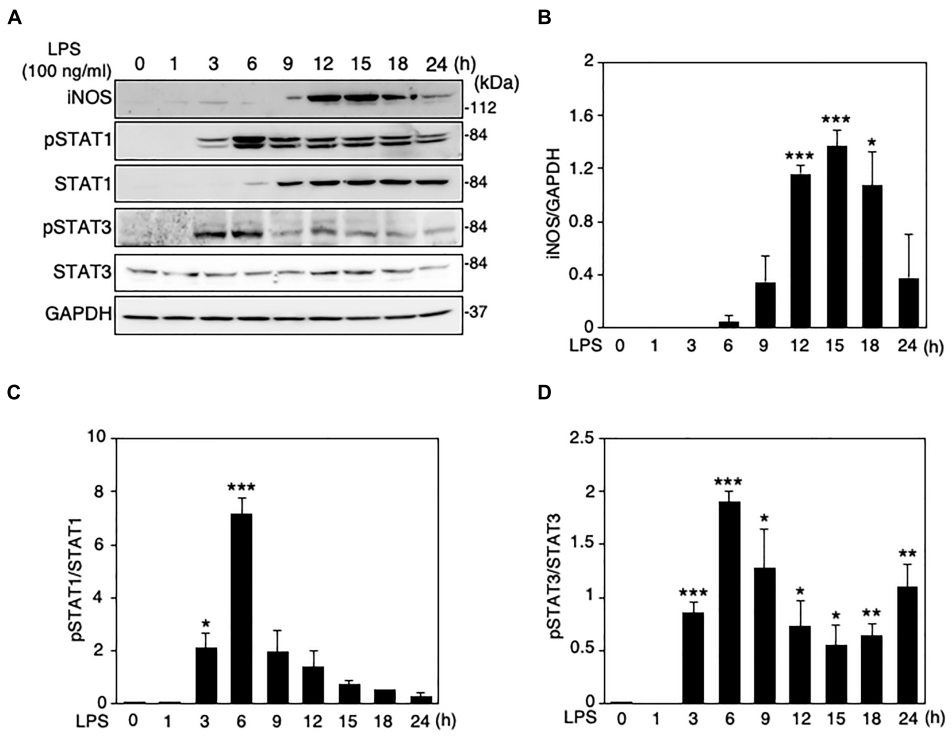

FIGURE 1 | Changes in protein expression levels of iNOS and total and phosphorylation levels of STAT1 and STAT3 in LPS-stimulated MG6 cells. **(A)** Typical Western blots of iNOS, phospho-STAT1 at Tyr-701, total STAT-1, phospho-STAT3 at Tyr-705, and total STAT-3 after challenge with 100 ng/ml LPS. GAPDH served as loading control. **(B)** Time course of changes in iNOS protein expression after LPS application. **(C)** Time course of changes in STAT1 phosphorylation after LPS application. **(D)** Time course of changes in STAT3 phosphorylation after LPS application. The results represent the mean ± SEM for three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. time 0 by *t*-test.

TGCTGCCGTCATTTTC-3′ (sense) and 5′-GGCTCGCAGGGA TGATTTCAA-3′ (antisense), the interleukin (IL) -6 primer sequences were 5′-CCACTTCACAAGTCGGAGGCTTA-3′ (sense) and 5′-GCAAGTGCATCATCGTTGTTCATAC-3′ (antisense), the IL-1β primer sequences were 5′-TCCAGGA TGAGGACATGAGCAC-3′ (sense) and 5′-GAACGTCACACA CCAGCAGGTTA-3′ (antisense), the IRF7 primer sequences were 5′-GAGACTGGCTATTGGGGGAG-3′ (sense) and 5′-GACCGAAATGCTTCCAGGG-3′ (antisense), and the GAPDH primer sequences were 5′-TGTGTCCGTCGTGGATCTGA-3′ (sense) and 5′-TTGCTGTTGAAGTCGCAGGAG-3′ (antisense). Additional details are described elsewhere (Kawakami et al., 2018; Yamashita et al., 2018).

Enzyme Immunoassay for IFN-β

Culture medium levels of IFN-β were measured by the use of commercially available enzyme-linked immunosorbent assay (ELISA) kit (Mouse IFNβ DuoSet ELISA kit, DY8234-05; R&D Systems, Minneapolis, MN, United States) according to the manufacturer's instructions. The plate was read on a microplate reader (Molecular Devices, Menlo Park, CA, United States). Assays were performed in duplicate.

FACS Analysis

Microglial cells were stimulated with 100 ng/ml of LPS for 3 h. The cells were incubated with allophycocyanin (APC)-anti-TLR4 (Clone: SA15-21) for 15 min on ice and then stained with SYTOX-ADDvanced. Fluorescence-activated cell scanning (FACS) analysis by flow cytometry was performed on an Accuri C6 (Becton Dickinson, Franklin Lakes, NJ, United States). The FACS pattern obtained from SYTOX-ADD-negative cells is indicated.

Statistics

Values are presented as mean ± SEM. Data were analyzed by the use of Prism software (version 6; GraphPad Software, San Diego, CA, United States). Statistical significance was calculated by Student's unpaired *t*-test, where differences at $p < 0.05$ was considered statistically significant.

RESULTS

STAT1/3 Activation Is Involved in iNOS Expression in Microglia

When LPS (100 ng/ml) was applied to the mouse microglial cell line MG6 cells, the expression levels of iNOS protein were increased in a time-dependent manner, reaching a peak at 15 h after LPS and declining thereafter (**Figures 1A,B**). The induction of iNOS expression in glial cells and macrophages involves the activation of the Janus tyrosine kinase (JAK)/STAT signaling pathway (Dell'Albani et al., 2001; Ganster et al., 2001). We thus ascertained whether STAT1 and STAT3 can be activated in MG6 cells following LPS challenge. Activation of STAT1 and STAT3

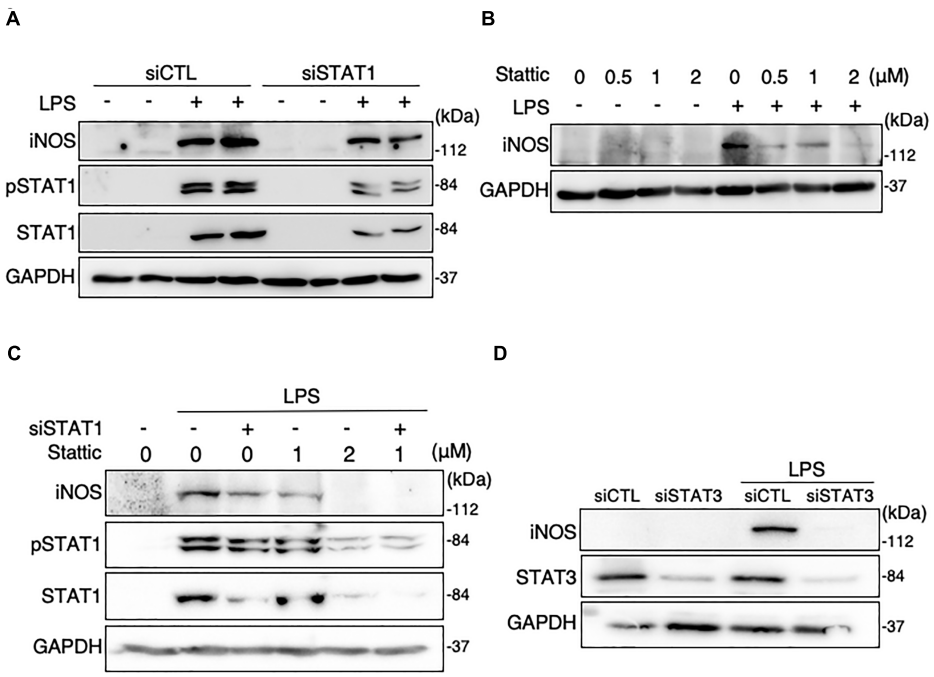

FIGURE 2 | Effects of STAT1 siRNA transfection and stattic on protein expression levels of iNOS and total and phosphorylation levels of STAT1 in LPS-stimulated MG6 cells. **(A)** iNOS, phospho-STAT1 at Tyr-701, and total STAT1 before and 15 h after 100 ng/ml LPS application in the presence of or STAT1 siRNAs (siSTAT1) or the negative control siRNAs (siCTL). **(B)** Concentration-dependent effect of stattic (0.5–2 μM) on iNOS expression before and 15 h after 100 ng/ml LPS application. **(C)** Effects of siSTAT1 alone, stattic (2 μM) alone, and combination of siSTAT1 and stattic (1 μM) on iNOS, phosphorylated STAT1, and total STAT1 before and 15 h after 100 ng/ml LPS application. **(D)** Effect of STAT3 siRNAs (siSTAT3) on iNOS and STAT3 expression before and 15 h after 100 ng/ml LPS application. GAPDH served as loading control. Shown are representative Western blots from three independent experiments in which the same results were obtained.

was assessed by Western blot analysis for phospho-STAT1 at Tyr-701 and phospho-STAT3 at Tyr-705, respectively. STAT1 phosphorylation was transiently but markedly increased in cells at 6 h after LPS, whereas a sustained rise in total STAT1 levels was also observed with LPS stimulation (**Figures 1A,C**). On the other hand, the increase in STAT3 phosphorylation showed a marked peak at 6 h after LPS and a less pronounced sustained response, but total STAT3 levels were unaffected by LPS challenge (**Figures 1A,D**).

The knockdown of STAT1 was conducted in MG6 cells using its specific siRNAs. Our transfection of STAT1 siRNAs effectively silenced STAT1 expression levels in LPS-stimulated microglial cells (**Figure 2A**). Transfection of STAT1 siRNAs evidently but incompletely prevented the LPS-induced increase in iNOS protein expression (**Figure 2A**). Stattic is known to be a small-molecule inhibitor of STAT3 activation, dimerization, and nuclear translocation (Schust et al., 2006). Treatment with stattic reduced iNOS protein expression in LPS-stimulated cells in a concentration-dependent manner (**Figure 2B**). At a concentration of 2 μM, stattic completely eliminated the LPS-induced iNOS upregulation, but also abolished the total and phosphorylation levels of STAT1 following LPS application (**Figures 2B,C**), suggesting that stattic at this concentration appears to act on STAT1 as well as STAT3. Combined treatment with STAT1 siRNAs and stattic at a lower concentration (1 μM) resulted in a complete abolition of iNOS expression in LPS-stimulated cells (**Figure 2C**). We also found that iNOS expression following LPS challenge was inhibited when STAT3 siRNAs were transfected (**Figure 2D**). These results indicate that both STAT1 and STAT3 play a role in iNOS expression triggered by LPS in microglial cells.

GRK2 Regulates STAT1 and STAT3 Phosphorylation in Microglia

To determine whether GRK2 can be involved in STAT1 and STAT3 phosphorylation in LPS-stimulated microglial cells, GRK2 siRNAs were used to knockdown microglial expression

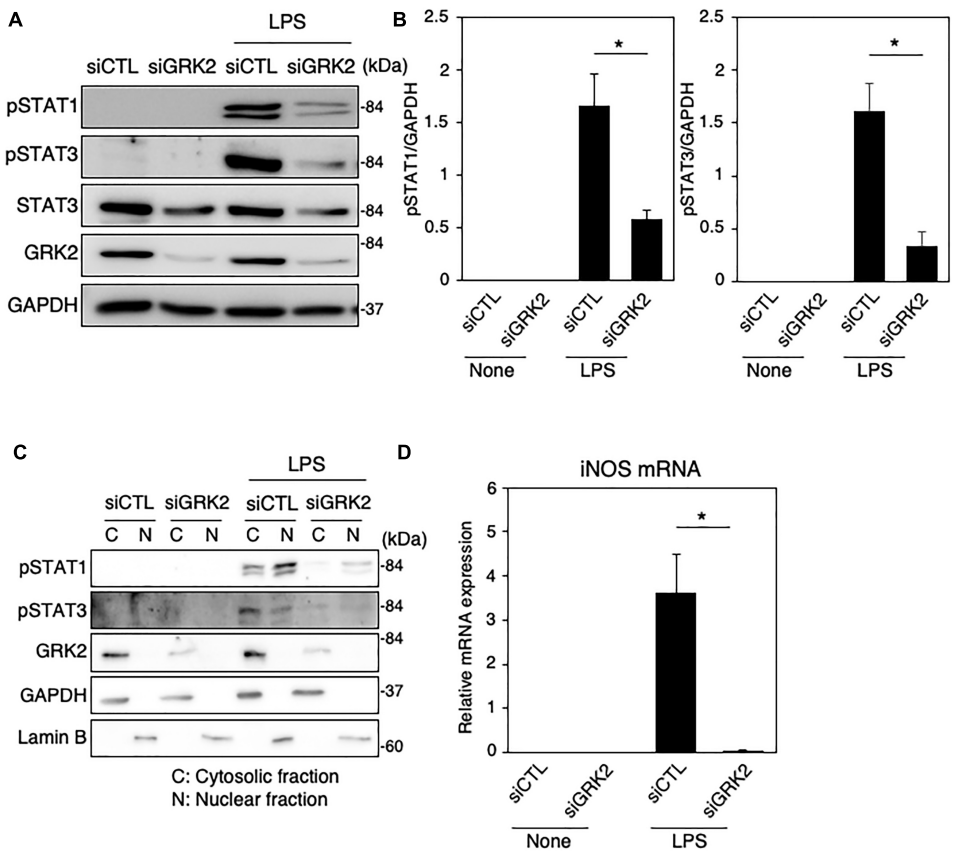

FIGURE 3 | Effect of GRK2 siRNA transfection on total and phosphorylation levels of STAT1 and STAT3 in LPS-stimulated MG6 cells. **(A)** Typical Western blots of phospho-STAT1 at Tyr-701, phospho-STAT3 at Tyr-705, and total STAT3 before and 6 h after 100 ng/ml LPS application in the presence of GRK2 siRNAs (siGRK2) or the negative control siRNAs (siCTL). Transfection of siGRK2, but not of siCTL, effectively decreased GRK2 protein expression, and GAPDH was used as loading control. **(B)** Phosphorylated levels of STAT1 and STAT3 6 h after 100 ng/ml LPS application when siCTL or siGRK2 was transfected. **(C)** Cytoplasmic (C) and nuclear (N) fractions were isolated, and then phospho-STAT1 and phospho-STAT3 6 h after 100 ng/ml LPS were detected by Western blot analysis. GAPDH and lamin B served as a cytoplasmic and a nuclear marker, respectively. Shown are representative Western blots from three independent experiments in which the same results were obtained. **(D)** Effect of siGRK2 transfection on expression of iNOS mRNA 12 h after 100 ng/ml LPS application. The mRNA levels were expressed as a fold increase above control normalized GAPDH. The results represent the mean ± SEM for three independent experiments. $^*P < 0.05$ by *t*-test.

of GRK2. The ablation of GRK2 by siRNAs resulted in a significant inhibition of phosphorylation levels of STAT1 and STAT3 (**Figures 3A,B**). Stimulation of MG6 cells with LPS led to the translocation of phosphorylated STAT1 and STAT3 into the nucleus (**Figure 3C**). The nuclear translocation of phosphorylated STAT1 and STAT3 was dampened when GRK2 siRNAs were transfected. These findings suggest that GRK2 positively regulates activation of STAT1 and STAT3 in microglial cells.

As presented above, both STAT1 and STAT3 are critical regulators of iNOS expression in LPS-stimulated microglial cells. In line with our recent report (Kawakami et al., 2018), the LPS-induced increases in iNOS mRNAs were strongly prevented by transfection of GRK2 siRNAs (**Figure 3D**). In addition, GRK2 siRNA transfection greatly reduced the LPS-induced upregulation of mRNA levels of IL-1β, IL-6, IP-10, and IRF7 (**Supplementary Figure 1**).

GRK2 Does Not Regulate Activation of NF-κB in Microglia

In MG6 cells stimulated with LPS, iNOS protein expression was strongly reduced by the NF-κB-specific inhibitor BAY 11-7082 (**Supplementary Figure 2A**). However, neither phosphorylation nor nuclear translocation of p65 in LPS-stimulated cells was affected when GRK2 siRNAs were transfected (**Supplementary Figures 2B,C**). These findings suggest that GRK2 plays no role in regulating NF-κB activation in LPS-stimulated microglial cells.

GRK2 Regulates LPS-Stimulated Upregulation of IFN-β in Microglia

Type I IFNs, such as IFN-β, is a second major group of cytokines that are produced by LPS-activated immune cells (Noppert et al., 2007), and signal through the JAK/STAT pathway to stimulate nuclear gene expression (Horvath, 2004). Type I IFN can signal through forming a ternary complex with the type I IFN receptor, composed of its two subunits IFNAR1 and IFNAR2 (Lee and Ashkar, 2018). The ablation of IFNAR1 by siRNAs resulted in a disappearance of iNOS expression in LPS-challenged MG6 cells (**Figure 4A**). When LPS was applied to MG6 cells, gene expression levels of IFN-β were greatly upregulated (**Figure 4B**). Furthermore, when the amounts of IFN-β in culture media were measured by ELISA, LPS challenge resulted in a striking increase in IFN-β protein levels (**Figure 4C**). The increased mRNA and protein levels of IFN-β were significantly suppressed by transfection of GRK2 siRNAs (**Figures 4A,B**). These data suggest that GRK2 contributes to the production of IFN-β in LPS-stimulated microglial cells.

GRK2 Regulates Activation of IRF1, Without Affecting TLR4 Endocytosis in LPS-Stimulated Microglia

IRFs, a family of transcription factors, play a central role in controlling the type I IFN induction at the gene transcriptional level (Honda et al., 2006). In microglial cells activated with

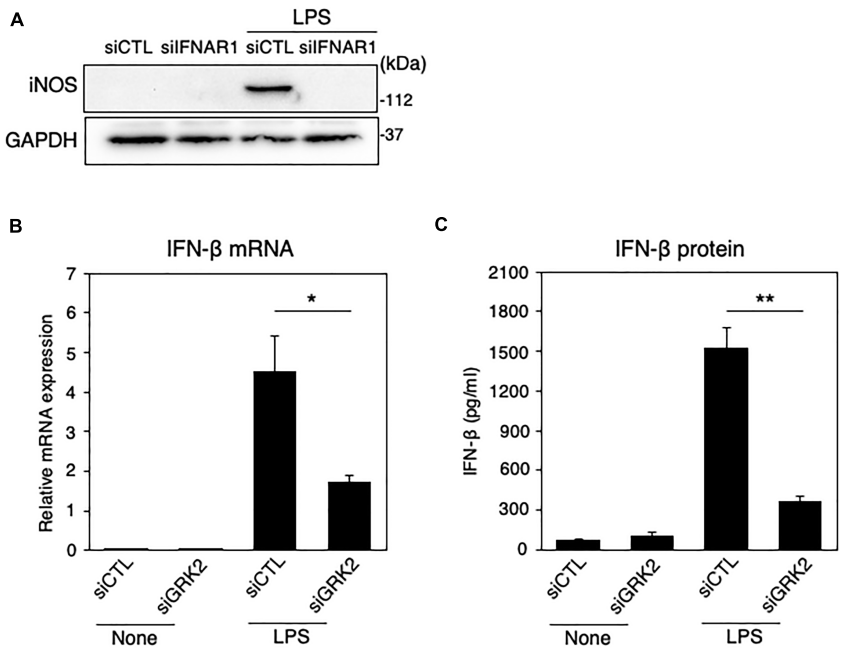

FIGURE 4 | Effect of GRK2 siRNA transfection on IFN-β expression in LPS-stimulated MG6 cells. **(A)** iNOS protein expression before and 15 h after 100 ng/ml LPS application in the presence of IFNAR1 siRNAs (siIFNAR1) or the negative control siRNAs (siCTL). GAPDH served as loading control. Shown are representative Western blots from three independent experiments in which the same results were obtained. **(B)** IFN-β mRNA expression levels. Following transfection of GRK2 siRNAs (siGRK2) or the negative control siRNAs (siCTL), cells were exposed to 100 ng/ml LPS for 3 h. The mRNA levels were expressed as a fold increase above control normalized GAPDH. **(C)** Cell culture media were collected 6 h after application of 100 ng/ml LPS, and the concentrations of IFN-β were measured by the ELISA. The results represent the mean ± SEM for three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ by *t*-test.

LPS and other inflammatory stimuli, IRF1 also appears to be an essential transcription factor for expression of inflammatory mediators, including iNOS (Lee et al., 2001; Jantaratnotai et al., 2013; Matsuda et al., 2015). In MG6 cells, LPS challenge led to the upregulation of IRF1 mRNAs and the translocation of IRF1 proteins into the nucleus (**Figures 5A,B**). Transfection of IRF1 siRNAs strikingly eliminated LPS-induced upregulation of IFN-β mRNA expression (**Figure 5C**). Furthermore, IRF1 siRNA transfection negated LPS-induced iNOS expression (**Figure 5D**). These findings imply that IRF1 plays a crucial role in induction of IFN-β and subsequent production of iNOS in LPS-stimulated microglial cells.

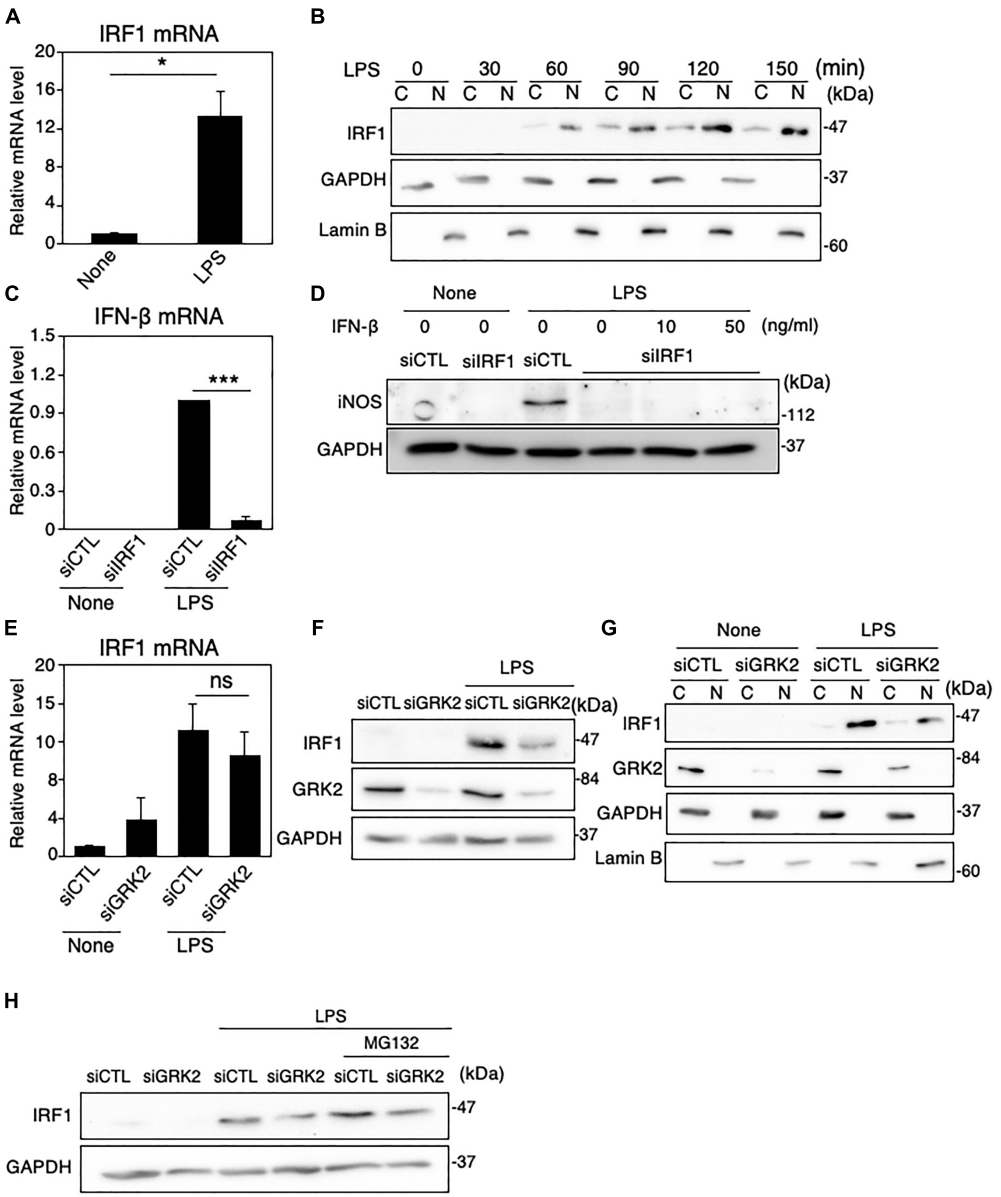

FIGURE 5 | Role of IRF1 in IFN-β-mediated iNOS expression in LPS-stimulated MG6 cells. **(A)** Expression of IRF1 mRNA 3 h after 100 ng/ml LPS application. **(B)** Cytoplasmic (C) and nuclear (N) fractions were isolated, and then the time course of changes in IRF1 levels in each fraction after 100 ng/ml LPS application was tracked by Western blot analysis. **(C)** Effect of IRF1 siRNAs (siIRF1) on expression of IFN-β mRNA 3 h after 100 ng/ml LPS application. **(D)** Effect of transfection of siIRF1 on iNOS protein expression 12 h after challenge with 100 ng/ml LPS in the presence or absence of 10 or 50 ng/ml IFN-β. **(E)** Effect of GRK2 siRNAs (siGRK2) on IRF1 mRNA 3 h after 100 ng/ml LPS application. The mRNA levels were expressed as a fold increase above control normalized GAPDH. **(F)** Effect of siGRK2 on IRF1 protein 1 h after 100 ng/ml LPS. **(G)** Effect of siGRK2 on IRF1 levels in C and N fractions before and 1 h after 100 ng/ml LPS was tracked by Western blot analysis. **(H)** Influence of MG132 on the siGRK2 effect on IRF1 protein expression 90 min after 100 ng/ml LPS. MG132 at a concentration of 2 μM was added 30 min after LPS. All experiments were compared with those when the negative control siRNAs (siCTL) was transfected. GAPDH served as loading control and lamin B was used as a nuclear marker. Shown are representative Western blots from three independent experiments in which the same results were obtained. The bar graph results represent the mean ± SEM for three independent experiments. Ns, not significant. $^{*}P < 0.05$ and $^{***}P < 0.001$ by *t*-test.

A previous study with IRF3-deficient mice has established a key role for IRF3 in LPS-induced IFN-β gene expression (Sakaguchi et al., 2003). We tested whether IRF3 could be involved in induction and/or activation of IRF1 in LPS-stimulated MG6 cells. Transfection of IRF3 siRNAs did not substantially alter the LPS-induced increases in expression and nuclear translocation of IRF1 (**Supplementary Figures 3A,B**).

TRIF is essential for TLR-3 or TLR-4-mediated MyD88-independent pathways and contributes to pro-inflammatory cytokine production and, most importantly, induces type I IFN production, particularly IFN-β (Takeda and Akira,

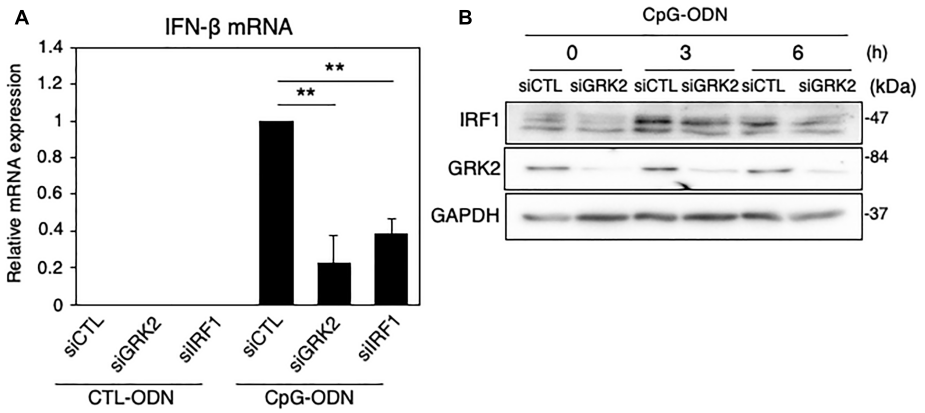

FIGURE 6 | Effect of GRK2 siRNA transfection on TLR9-mediated IRF1 and IFN-β expression in MG6 cells. **(A)** IFN-β mRNA expression 3 h after 1 μM CpG-ODN or control ODN (CTL-ODN) application in the presence of GRK2 siRNAs (siGRK2), IRF1 siRNAs (siRF1) or the negative control siRNAs (siCTL). The mRNA levels were expressed as a fold increase above control normalized GAPDH and the results represent the mean ± SEM for three independent experiments. $^{**}P < 0.01$ by *t*-test. **(B)** Changes in protein expression of IRF1 and GRK2 after 1 μM CpG-ODN challenge in the presence of GRK2 siRNAs (siGRK2) or the negative control siRNAs (siCTL). GAPDH served as loading control. Shown are representative Western blots from three independent experiments in which the same results were obtained.

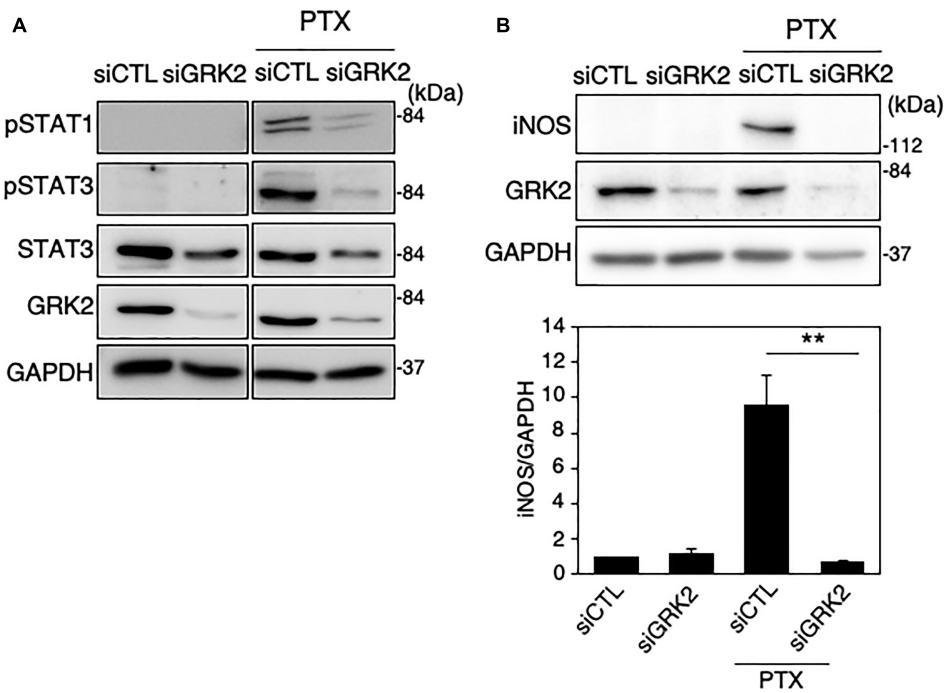

FIGURE 7 | Effect of GRK2 siRNA transfection on paclitaxel-induced TLR4 signaling for iNOS expression in MG6 cells. **(A)** Phospho-STAT1 at Tyr-701, phospho-STAT3 at Tyr-705, and total STAT-3 before and 6 h after 10 μM paclitaxel (PTX) application in the presence of GRK2 siRNAs (siGRK2) or the negative control siRNAs (siCTL). Transfection of siGRK2, but not of siCTL, effectively decreased GRK2 protein expression, and GAPDH was used as loading control. Shown are representative Western blots from three independent experiments in which the same results were obtained. **(B)** Effect of siGRK2 transfection on iNOS protein expression 12 h after 5 μM paclitaxel application. The results represent the mean ± SEM for three independent experiments. $^{**}P < 0.01$ by *t*-test. In the upper side, typical Western blots are shown. GAPDH served as loading control.

2005). However, transfection of TRIF siRNAs had little effect on LPS-induced upregulation of IRF1 mRNA expression (**Supplementary Figure 3C**), indicating that LPS-stimulated induction of IRF1 is independent of the TRIF pathway. On the other hand, BAY 11-7082 greatly inhibited the LPS-induced increase in both mRNA and protein expression (**Supplementary Figures 3D,E**), which suggests that IRF1 is transcriptionally regulated by NF-κB. When GRK2 siRNAs were transfected, the LPS-induced upregulation of IRF1 mRNA was not substantially affected (**Figure 5E**), but that of IRF1 protein was evidently reduced (**Figure 5F**). In addition, the nuclear translocation of IRF1 was strongly reduced by GRK2 siRNA transfection (**Figure 5G**). The ability of GRK2 siRNAs to reduce the LPS-induced upregulation of IRF1 protein was evident regardless of whether the proteasome inhibitor MG132 was present, although the upregulation of IRF1 protein by LPS was markedly increased in the presence of MG132 (**Figure 5H**). These results suggest that GRK2 participates in LPS-induced upregulation and activation of

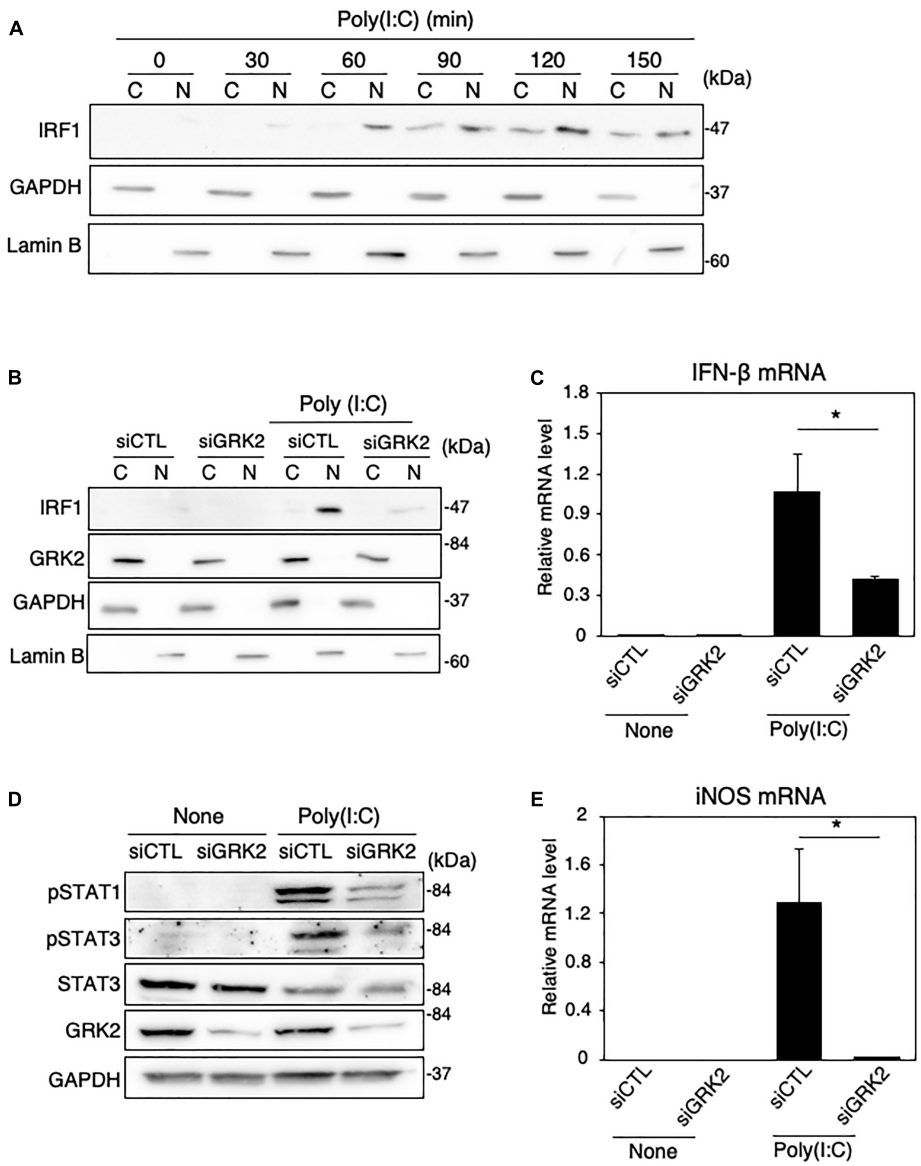

FIGURE 8 | Effect of GRK2 siRNA transfection on TLR3-mediated signaling for iNOS expression in MG6 cells. **(A)** Cytoplasmic (C) and nuclear (N) fractions were isolated, and then the time course of changes in IRF1 levels in each fraction after 50 μg/ml poly(I:C) application was tracked by Western blot analysis. **(B)** Effect of transfection of GRK2 siRNAs (siGRK2) on nuclear translocation of IRF1 60 min after challenge with 50 μg/ml poly(I:C) was compared with that when the negative control siRNAs (siCTL) was transfected. GAPDH and lamin B served as a cytoplasmic and nuclear marker, respectively. **(C)** Effect of siGRK2 transfection on IFN-β mRNA expression levels 3 h after 50 μg/ml poly(I:C) application. **(D)** Effect of siGRK2 transfection on phospho-STAT1 at Tyr-701, phospho-STAT3 at Tyr-705, and total STAT-3 4 h after 50 μg/ml poly(I:C) application. GAPDH served as loading control. Shown are representative Western blots from three independent experiments in which the same results were obtained. **(E)** Effect of siGRK2 transfection on expression of iNOS mRNA 12 h after 50 μg/ml poly(I:C) application. The mRNA levels were expressed as a fold increase above control normalized GAPDH. The results represent the mean ± SEM for three independent experiments. *$P < 0.05$ by *t*-test.

IRF1 protein in microglial cells in a manner independent of the proteasome degradation route.

LPS-induced TLR4 endocytosis is necessary to initiate the TRIF-dependent IFN-β expression (Kagan et al., 2008). We thus examined whether GRK2 can regulate endocytosis of TLR4 in LPS-stimulated MG6 cells. FACS analysis showed much lower surface expression of TLR4 when MG6 cells were stimulated with LPS (**Supplementary Figure 3F**). Transfection of GRK2 siRNAs was without effect on the LPS-induced decrease in TLR4 surface expression, suggesting that GRK2 plays no regulatory role in the endocytosis pathway that delivers TLR4 to endosomes.

GRK2 Regulates TLR9-Mediated IFN-β Expression in Microglia Through IRF1 Upregulation

IRF1 has been known to control TLR9-mediated IFN-β production in myeloid dendritic cells (Schmitz et al., 2007). When TLR9 was activated with CpG-ODN, a synthetic oligodeoxynucleotide containing specific unmethylated CpG motifs, the mRNA levels of IFN-β were strikingly upregulated in MG6 cells (**Figure 6A**). The CpG-ODN-induced increase in IFN-β mRNAs were significantly inhibited by transfection of GRK2 siRNAs or IRF1 siRNAs. Furthermore, the knockdown of GRK2 by siRNAs attenuated CpG-ODN-induced IRF1 protein expression (**Figure 6B**). These findings suggest that GRK2 can positively regulate TLR9-mediated IFN-β production by upregulating expression of IRF1.

GRK2 Regulates Paclitaxel-Derived TLR4 Signaling for iNOS Expression in Microglia

Paclitaxel, an anti-microtubule agent with anti-tumoral activity, is identified as a ligand to activate TLR4 signaling (Byrd-Leifer et al., 2001; Zimmer et al., 2008). We examined whether GRK2 is involved in paclitaxel-derived TLR4 signaling for iNOS expression in microglial cells. MG6 cells were challenged with paclitaxel at concentrations of 5–10 μM. Phosphorylated levels of STAT1 and STAT3 were highly upregulated as seen in LPS-stimulated cells, all of which were hampered by transfection of GRK2 siRNAs (**Figure 7A**). Furthermore, GRK2 siRNA transfection significantly suppressed the paclitaxel-induced increase in iNOS synthesis (**Figure 7B**).

GRK2 Regulates TLR3 Signaling for iNOS Expression in Microglia

Polyinosinic-polycytidylic acid [poly(I:C)] is a synthetic analog of double-stranded RNA recognized by TLR-3 (Matsumoto and Seya, 2008). We examined whether GRK2 plays a regulatory role in TLR3-mediated signaling for iNOS synthesis in microglial cells. When poly(I:C) (50 μg/ml) was applied to MG6 cells, the translocation of IRF1 into the nucleus occurred in a time-dependent manner (**Figure 8A**). Transfection of GRK2 siRNAs blocked the nuclear translocation of IRF1 in poly(I:C)-stimulated cells, as seen in LPS-stimulated cells (**Figure 8B**). In poly(I:C)-challenged cells, GRK2 siRNA transfection also significantly declined the upregulation of IFN-β mRNA levels (**Figure 8C**) and reduced the increases in phosphorylated levels of STAT1 and STAT3 (**Figure 8D**). As expected from the involvement of STAT1/3 in iNOS expression in LPS-stimulated MG6 cells, poly(I:C) administration led to the production of iNOS mRNA, which was blocked by treatment with GRK2 siRNAs (**Figure 8E**). In addition, GRK2 siRNA transfection greatly reduced the poly(I:C)-induced upregulation of mRNA levels of IL-1β, IL-6, IP-10, and IRF7 (**Supplementary Figure 4**). These results suggest that GRK2 serves as a key player for TLR3 signaling to produce iNOS in microglial cells.

GRK2 Regulates STAT1/3 Activation in Microglia Supplemented With IFN-β

We also examined the role of GRK2 in STAT1/3-mediated iNOS production in microglial cells supplemented with exogenous IFN-β. IFN-β supplementation (10 ng/ml) caused time-dependent increases in phosphorylated levels of STAT1 and STAT3 in MG6 cells (**Figure 9A**). These changes were greatly attenuated by transfection of GRK2 siRNAs (**Figure 9A**). Only exposure to IFN-β failed to induce iNOS mRNAs (**Figure 9B**).

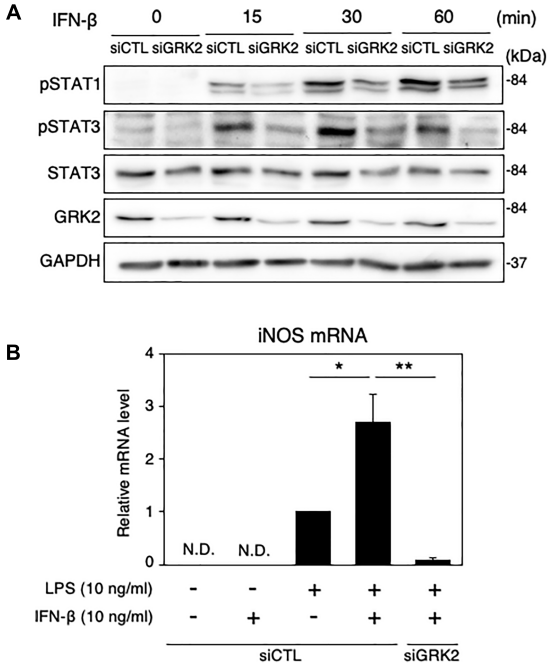

FIGURE 9 | Effect of GRK2 siRNA transfection on phosphorylation levels of STAT1 and STAT3 and mRNA levels of iNOS in MG6 cells supplemented with exogenous IFN-β. **(A)** Time course of changes in phospho-STAT1 at Tyr-701, phospho-STAT3 at Tyr-705, and total STAT3 after supplementation with 10 ng/ml IFN-β. Transfection of siGRK2, but not of siCTL, effectively decreased GRK2 protein expression, and GAPDH was used as loading control. Shown are representative Western blots from three independent experiments in which the same results were obtained. **(B)** Effect of siGRK2 transfection on expression of iNOS mRNA 12 h after 10 ng/ml IFN-β supplementation in the presence of 10 ng/ml LPS. The mRNA levels were expressed as a fold increase above control normalized GAPDH. The results represent the mean ± SEM for four independent experiments. N.D., not detected. $^{*}P < 0.05$, $^{**}P < 0.01$ by *t*-test.

This suggests that IFN-β by itself was not sufficient to stimulate induction of iNOS expression. However, exogenously applied IFN-β markedly augmented iNOS mRNA levels caused by a low dose of LPS (**Figure 9B**). GRK2 siRNA transfection blocked the iNOS mRNA expression in the presence of low-dose LPS and IFN-β (**Figure 9B**), providing evidence to support a role of GRK2 in IFN-β-induced activation of STAT1/STAT3 to augment iNOS production.

GRK2 Inhibitor Reduces STAT1/3 Activation but Not IRF1 Activation in Microglia

Methyl 5-[2-(5-nitro-2-furyl)vinyl]-2-furoate acts as a selective inhibitor of kinase activity of GRK2 (Iino et al., 2002). We examined whether this GRK2 inhibitor can modify the function or expression of STAT1/3 and IRF1 in LPS-stimulated MG6 cells. As demonstrated in our recent report (Kawakami et al., 2018), treatment with the GRK2 inhibitor abrogated the LPS-induced upregulation of iNOS protein (**Figure 10A**). Furthermore, GRK2 inhibitor treatment strongly eliminated phosphorylation of STAT1 and STAT3 after LPS stimulation (**Figure 10B**). The IFN-β-induced increases in STAT1 and STAT3 phosphorylation were also attenuated by the GRK2 inhibitor (**Figure 10C**). However, GRK2 inhibitor treatment did not substantially affect expression and nuclear translocation of IRF1 following LPS challenge (**Figures 10D,E**). These results suggest that STAT1/3, but not IRF1, in activated microglia can be regulated by GRK2 in a kinase-dependent manner.

DISCUSSION

In this work, we delineated the TLR signaling pathway for iNOS expression in microglial cells. When TLR4 is activated by LPS, TRIF-independent signaling activates IRF-1, leading to expression of IFN-β. IFN-β, via activation of its specific receptors, employs STAT1/3 signal transduction for nuclear signaling, which in turn could enhance iNOS expression (**Figure 11**). In this study, we found that LPS challenge led to the induction of IRF1 expression and the translocation of IRF1 into the nucleus in microglial cells. Stimulation of TLR9 with CpG-ODN also increased IRF1 expression and led to upregulate IFN-β transcription. Furthermore, LPS-induced upregulation of IFN-β and iNOS mRNA expression was eliminated by transfection of IRF1 siRNAs, suggesting that, in LPS-stimulated microglia, IRF1 is one of essential transcription factors for expression of IFN-β to augment iNOS production. In accordance with this finding, IRF1 has been implicated in the induction of inflammatory mediators, including iNOS, in microglial cells activated with LPS and other inflammatory stimuli (Lee et al., 2001; Jantaratnotai et al., 2013; Matsuda et al., 2015).

GRK2 belongs to the GRK family to regulate the activity of GPCRs, of which GRK2 is ubiquitously expressed and play multiple roles in cell signaling beyond GPCR desensitization (Penela et al., 2010, 2014; Evron et al., 2012; Mayor et al., 2018; Nogues et al., 2018). Thus, GRK2 is not only capable of interacting with a variety of endocytic proteins, but it can modulate various signaling cascades in a kinase-independent fashion to affect a wide range of cellular processes (Evron et al., 2012; Mayor et al., 2018; Nogues et al., 2018). Our

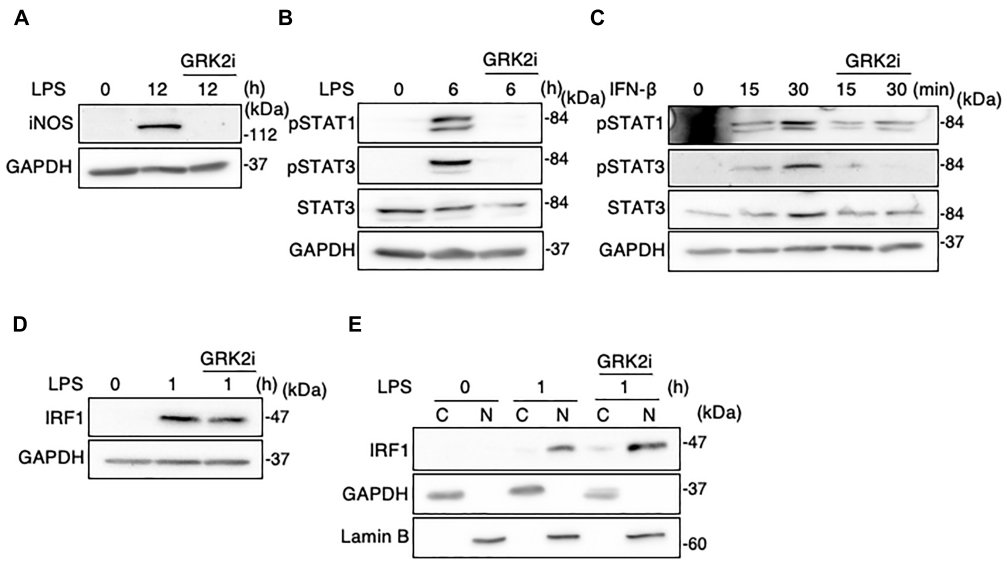

FIGURE 10 | Effect of GRK2 inhibitor treatment on iNOS expression, STAT1/3 activation, and IRF1 activation in microglial cells. GRK2 inhibitor at a concentration of 10 μM was added at 30 min before challenge with 100 ng/ml LPS or 10 ng/ml IFN-β. **(A)** Expression of iNOS protein before and 15 h after LPS application. **(B)** STAT1 and STAT3 phosphorylation before and 6 h after LPS application. **(C)** STAT1 and STAT3 phosphorylation before and 15–30 min after IFN-β supplementation. **(D)** Expression of IRF1 expression before and 1 h after LPS application. **(E)** Cytoplasmic (C) and nuclear (N) fractions were isolated, and then changes in IRF1 levels in each fraction before and 1 h after LPS application was tracked by Western blot analysis. GAPDH served as loading control and lamin B was used as a nuclear marker. Shown are representative Western blots from three independent experiments in which the same results were obtained.

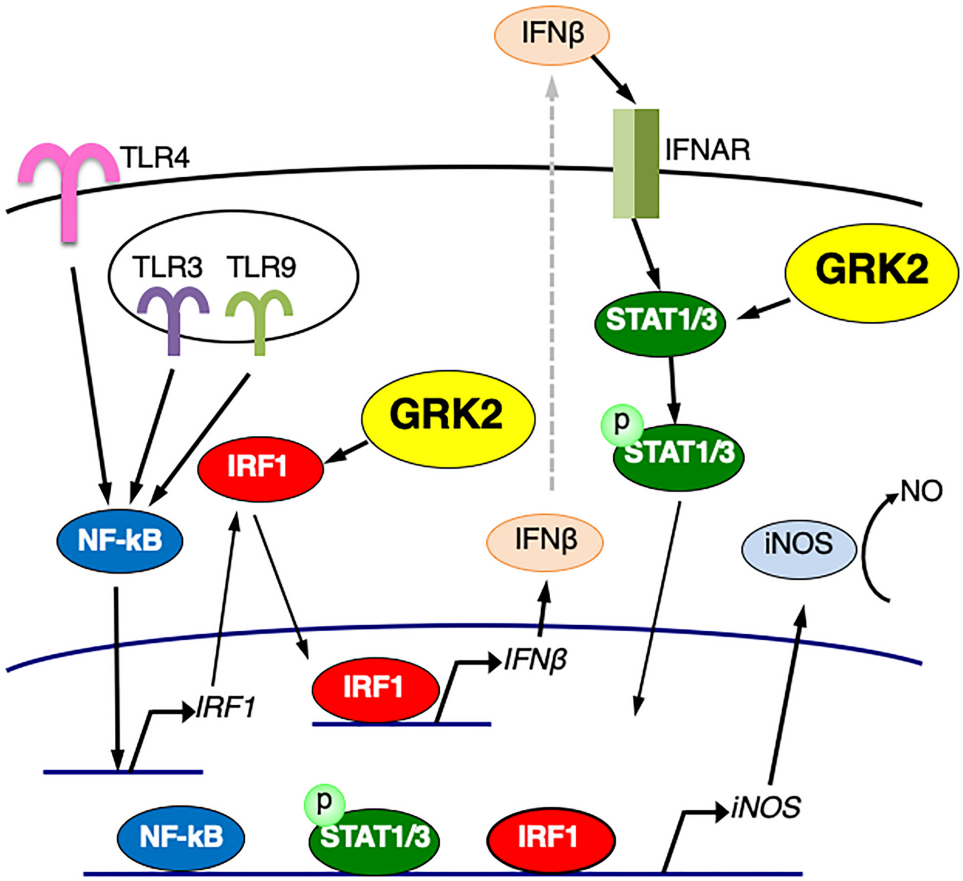

FIGURE 11 | Schematic diagram of the regulatory role of GRK2 in TLRs-mediated signaling pathways for iNOS expression in microglial cells. GRK2 positively regulates not only the expression/activation of IRF1 but the activation of STAT1/3, leading to the induction of iNOS transcription. See text for details.

recent study has shown that GRK2 controls reactive oxygen species production pathway in LPS-stimulated microglial cells (Kawakami et al., 2018). In this study, our results with the use of GRK2 siRNAs demonstrate that the protein expression and nuclear translocation of IRF1 is positively regulated by GRK2 when TLR4 is activated by LPS in microglial cells. It appeared likely that GRK2 is involved in LPS-induced upregulation and activation of IRF1 protein in microglial cells without affecting the proteasome degradation route. TLR4 activates MyD88-dependent signaling mainly at plasma membranes and TRIF-dependent signaling at the endosomal membrane after internalization of TLR4 complex into endosomes whereupon TRIF is recruited (Rajaiah et al., 2015). We showed that GRK2 did not participate in the initiation of events that promote TLR4 endocytosis. These new findings suggest a novel role of GRK2 in TLR4-induced inflammatory signaling in microglial cells and provide promising new insight into the molecular control of GRK2.

TLR3 triggers activation of the MyD88-independent and TRIF-dependent signaling pathway leading to IFN-β production (Yamamoto et al., 2003; Kawai and Akira, 2010; Kawasaki and Kawai, 2014). We found that activation of TLR3 with poly(I:C) promoted the translocation of IRF1 into the nucleus and upregulated transcription of IFN-β in microglial cells. Transfection of GRK2 siRNAs blocked the poly(I:C)-induced nuclear translocation of IRF1, leading to the reduced upregulation of IFN-β expression. We interpret these observations to indicate that the TLR3-mediated induction of IFN-β transcription is highly regulated by GRK2 through its ability to activate IRF1 (**Figure 11**). Alternatively, it seems unlikely that the ability of GRK2 to interfere with MyD88 or TRIF, if any, contributes to its regulatory role in TLRs-mediated iNOS induction.

While LPS activation of TLR4 in immune cells induces expression of multiple pro-inflammatory cytokines, including tumor necrosis factor-α and IL-1β, through the MyD88-dependent signaling pathway, type I IFNs, such as IFN-β, is a second major group of cytokines that are produced by the MyD88-independent and TRIF-dependent signaling pathway in LPS-activated immune cells (Akira and Takeda, 2004; Noppert et al., 2007). We demonstrated that both STAT1 and STAT3 were greatly activated in LPS-stimulated microglial cells, although STAT3 and STAT1 appear to be constitutive and inducible, respectively. The same results were found when microglia were challenged with poly(I:C). In light of the notion that type I IFNs signal through the JAK/STAT pathway to stimulate nuclear

gene expression (Horvath, 2004; Ivashkiv and Donlin, 2014), it would be reasonable to consider that IRF1, which is induced by TRIF-independent fashion, promotes the production of IFN-β downstream of TLR4 signaling as well as TLR3 signaling in microglia, resulting in STAT1/3 activation and thereby iNOS production (**Figure 11**).

We found that transfection of GRK2 siRNAs suppressed the activation of STAT1 and STAT3 in microglial cells when TLR3 and TLR4 were stimulated with poly(I:C) and LPS or paclitaxel, respectively. This cannot be solely the result of the blockade of the IRF1 activation that is positively regulated by GRK2. It should be noted that GRK2 siRNA transfection blocked the exogenous IFN-β supplementation-induced increases in phosphorylated STAT1 levels as well as phosphorylated STAT3 levels. Therefore, we suggest that, in addition to its ability to activate IRF1, GRK2 promotes the activation of the STAT pathway, through which IFN-β signals to stimulate iNOS expression, in activated microglia (**Figure 11**).

The blocking effect of GRK2 siRNA transfection on STAT1 and STAT3 phosphorylation was mimicked by the GRK2 inhibitor which acts on kinase activity of GRK2, suggesting that STAT1 and STAT3 in activated microglia can be regulated by GRK2 in a kinase-dependent manner. However, GRK2 inhibitor treatment exhibited no substantial effect on expression and nuclear translocation of IRF1 following LPS challenge. Whether a direct interaction exists between GRK2 and IRF1 remains the subject of ongoing studies.

CONCLUSION

In conclusion, we present evidence supporting a direct role of GRK2 in regulating TLR3-, TLR4-, and TLR-9-mediated inflammatory signaling in microglial cells. We thus show that GRK2 highly regulates the expression/activation of IRF1 as well as the activation of the STAT pathway, leading to augmented transcription of iNOS. However, the mode of action profiling of GRK2 underlying its interactions with these inflammatory signaling molecules awaits further investigation. Microglial cells are the resident tissue macrophages located in the central nervous system (CNS) and have a role in monitoring the brain for immune insults and invading pathogens (Ohashi et al., 2015). They are the major source of iNOS in the CNS (Saha and Pahan, 2006; Xanthos and Sandkuhler, 2014; Chitnis and Weiner, 2017). The upregulation of iNOS and subsequent excessive NO production are considered to play a contributory role in the pathogenesis of different neuroinflammatory diseases (Smith and Lassmann, 2002; Pannu and Singh, 2006; Ghasemi and Fatemi, 2014). Our findings highlight a novel pathophysiological role of GRK2 in regulating inflammatory signaling in microglia with a potential therapeutic window for CNS disorders in which neuroinflammation plays a critical role.

AUTHOR CONTRIBUTIONS

WO, TA, and YH conceived and designed the experiments. SP, MK, TS, and HY performed the experiments. SP and WO analyzed the data. SP, WO, KH, and YH wrote the article. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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GRKs and β-Arrestins: "Gatekeepers" of Mitochondrial Function in the Failing Heart

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Mitochondrial regulation of energy production, calcium homeostasis, and cell death are critical for cardiac function. Accordingly, the structural and functional abnormalities of these organelles (mitochondrial dysfunction) contribute to developing cardiovascular diseases and heart failure. Therefore the preservation of mitochondrial integrity is essential for cardiac cell survival. Mitochondrial function is regulated by several proteins, including GRK2 and β-arrestins which act in a GPCR independent manner to orchestrate intracellular signaling associated with key mitochondrial processes. It is now ascertained that GRK2 is able to recover mitochondrial function in response to insults. β-arrestins affect several intracellular signaling pathways within the cell which in turn are involved in the regulation of mitochondrial function, but a direct regulation of mitochondria needs further investigations. In this review, we discuss the recent acquisitions on the role of GRK2 and β-arrestins in the regulation of mitochondrial function.

Keywords: G protein-coupled receptor kinase 2, β-arrestins, mitochondria, heart failure, cardiac damage

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MITOCHONDRIAL FUNCTIONS IN DAMAGED HEART

Known as "*powerhouse*" of the cell, mitochondria play essential roles in all human tissues, especially in those that are highly dependent on energy supply such as kidney, skeletal muscles, and myocardium. This latter, in particular, is the most metabolically active organ in the body. Its intense energy demand, needed to generate the contractile force, is supplied through the oxidative metabolism in mitochondria (Schaper et al., 1985; Barth et al., 1992; Hoppel et al., 2009). Therefore, it is not surprising that alterations of mitochondrial functions lead to the development of cardiac pathologies or susceptibility to injury. Indeed, mitochondrial dysfunction has been identified as the cause or a contributing factor in several heart diseases, thus several cardiac disorders, such heart failure, are currently defined "bioenergetic disease" (Murphy et al., 2016). However, beyond the regulation of energetic metabolism, mitochondria are now recognized to orchestrate multiple essential functions within the cell (Dorn, 2015). They sense smooth endoplasmic reticulum (ER) calcium release to modulate their metabolism and increase contractility (Chen et al., 2012; Dorn and Maack, 2013; Dorn, 2015). They are the main source of reactive oxygen species (ROS) to exert both physiological functions and pathological damage (Eisner et al., 2013). Moreover, mitochondria are the "gatekeepers" of programmed cell death (apoptosis and necrosis) (Wei et al., 2001; Whelan et al., 2012; Dorn, 2015). Also, these organelles are involved in the regulation of cardiomyocyte

differentiation and embryonic cardiac development (Kasahara et al., 2013; Cho et al., 2014; Dorn, 2015). Thus, mitochondria exert both "energetic" and "non-energetic" functions (**Figure 1**) whose perturbations contribute to cardiac dysfunction (Rosenberg, 2004).

Energetic Metabolism

Several pieces of evidence support the idea that heart failure is a "bioenergetic diseases" associated with a loss of energetic supply (Neubauer et al., 1997; Ingwall and Weiss, 2004). Indeed, by means of nuclear magnetic resonance (NMR), it has been shown that a significant reduction of cardiac ATP and phosphocreatine (PCr) content occurs in patients with heart failure (Starling et al., 1998; Beer et al., 2002). Experimental models of heart failure show that impaired energetic metabolism leads to heart damage (Ciccarelli et al., 2011). In physiological conditions, the myocardium is characterized by an intensive ATP intake and turnover (Balaban et al., 1986) that is regulated by phosphocreatine levels, produced by mitochondrial creatine kinase (Ingwall et al., 1985; Soboll et al., 1999). In basal conditions, two forms of this enzyme (active and inactive) are in dynamic equilibrium and when this equilibrium is altered, results in impaired control of respiratory chain activity and reduction of mitochondrial ATP production (Ashrafian, 2002). Moreover, a significant reduction of individual electron transport complexes I and IV has been described in humans and animal model of heart failure (Lemieux et al., 2011; Rosca et al., 2011),associated with a reduction of functional super-complexes (Rosca et al., 2008). Defects in the mitochondrial respiratory chain have been observed both in early stage and end-stage of chronic heart failure (Lemieux et al., 2011). All these evidence confirm the involvement of impaired energetic metabolism in the development and progression of cardiac disease.

Mitochondrial ROS Production

Mitochondrial respiration through the electron transport chain (ETC) activity drives ATP synthesis and ROS production. In a healthy heart, the generation of superoxide radical is effectively neutralized by superoxide dismutase (Boveris et al., 1976). MnSOD silencing, the main mitochondrial antioxidant enzyme, is known to produce dilated cardiomyopathy leading to early postnatal death (Fukai and Ushio-Fukai, 2011). When mitochondrial respiration is compromised, decreased ATP production and increased oxidative stress occur (Lesnefsky et al., 2001). Elevated levels of ROS are able to induce oxidative modifications of specific mitochondrial proteins (Complex I and II, and Aconitase) that further stimulate ROS production in a vicious circle (Chen and Zweier, 2014). Moreover, a compromised antioxidant capacity also contributes to mitochondrial-mediated oxidative stress. The high levels of mitochondrial ROS induce alterations of main signaling pathways through oxidative modifications of important proteins such as cardiac ryanodine receptor (RyR2); indeed, the oxidation of this receptor alters its conformation and function contributing to the development of arrhythmias or heart failure (Oda et al., 2015).

Mitochondrial Dependent Apoptosis

It is ascertained that mitochondria, the primary sensor of metabolic stress, activate the programmed cell death. The mitochondria-dependent activation of apoptotic processes occurs in response to irreversible damage induced by intense or perpetuate metabolic stress (i.e., during acute or chronic ischemia) (Chistiakov et al., 2018). Such a condition increases mitochondrial permeability leading to cytochrome c release from mitochondria to the cytosol thus activating apoptosis (Karbowski et al., 2002). Besides this classical pathway, there are also other mechanisms by which mitochondria activates cell death in the failing heart (Dorn and Kirshenbaum, 2008; Dorn, 2010). During cardiac ischemia, a mitochondrial serine protease, known as high-temperature requirement A2 (HtrA2), is also released from mitochondria and promotes caspase activation and apoptosis (Dorn and Kirshenbaum, 2008; Piquereau et al., 2013). Several pieces of evidence suggest that abnormal cardiomyocyte apoptosis also occurs in both animal models and humans with arterial hypertension. Under this pathological stress, a functional cross-talk between death receptors pathways and mitochondrial-dependent apoptosis has been described, that is mediated by the Bcl2 family (Gonzalez et al., 2003). In addition, alterations of the mitochondrial apoptotic pathway have been described in both hypertrophic and dilative cardiomyopathy (Harvey and Leinwand, 2011). Overall the loss of cardiomyocytes due to apoptosis dysregulation is detrimental to cardiac function, and all recent studies support the key role of mitochondria in such phenomenon.

Mitochondrial Dynamics and Turnover

Mitochondrial fusion, fission, and trafficking, collectively called "mitochondrial dynamics", are the regulators of Mitochondrial Quality Control, an essential process that preserves mitochondrial function and ensures cell survival (Ni et al., 2015; Shirihai et al., 2015; Sorriento et al., 2017). This process includes several mechanisms (Shirihai et al., 2015):

(a) fission/fusion, that allows segregation of damaged mitochondria;
(b) mitophagy, that removes the irreversibly damaged mitochondria;
(c) mitochondrial biogenesis, that ensures the generation of new intact mitochondria and new mtDNA molecules.

Perturbations of one or more of these mechanisms culminate in altered mitochondrial architecture and function. In failing heart, structural changes in mitochondria have been frequently observed, including giant mitochondria due to excessive mitochondrial fusion (Tandler et al., 2002). The balanced equilibrium between fusion and fission is switched toward fission during ischemia. Indeed, the up-regulation of dynamin-related protein 1 (Drp1), a key activator of mitochondrial fission, induces prominent mitochondrial fragmentation that in turn leads to loss of mitochondrial potential and apoptosis (Karbowski et al., 2002; Ong et al., 2010). Moreover, alterations in mitochondrial fragmentation or hyperplasia resulting from compromised fission/fusion balance have also been described in human and

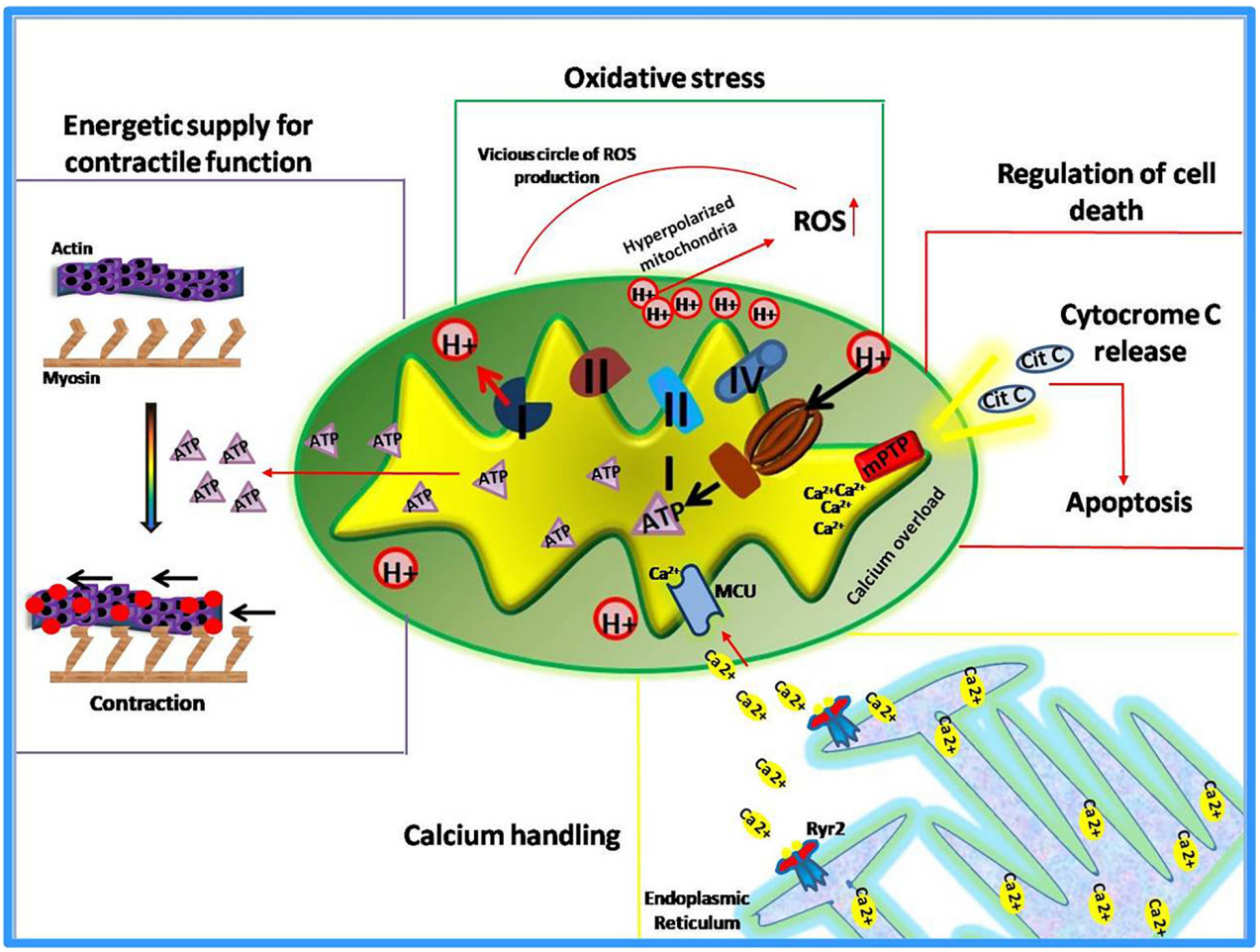

FIGURE 1 | Energetic and non-energetic functions of mitochondria.

animal model of heart failure (Sabbah et al., 1992; Goh et al., 2016). The irreversibly damaged mitochondria are cleared in the cardiac cells by effective mitophagy, a tightly regulated process. In a healthy heart, PINK1 localizes on mitochondrial surface promoting parkin-dependent ubiquitination of MFN2 with the recruitment of several autophagy adaptors and formation of autophagosome around damaged mitochondria (Karbowski et al., 2002). Alterations in this pathway have been described in several cardiac diseases such as ischemic heart disease, cardiac hypertrophy, heart failure and dilated cardiomyopathy (Chistiakov et al., 2018). The mechanisms that lead to alterations in mitochondria clearance are not completely understood. Likely, an excessive autophagy occurs during acute cardiac injury leading also to the loss of functional organelles. On the contrary, mitophagy flux is reduced during the late stages of cardiac diseases thus promoting accumulation of damaged mitochondria, severe oxidative stress and cardiomyocytes apoptosis (Campos et al., 2016). Indeed, a reduction in autophagic activity is associated with poor prognosis of patients with heart disease (Campos et al., 2016). Beside the management of pre-existent mitochondria, also the de-novo synthesis of these organelles seems to be compromised in failing hearts. Replication of mtDNA is significantly impaired in heart failure resulting in depletion of mt-DNA-encoded proteins and in altered mitochondrial biogenesis (Karamanlidis et al., 2014). All these pieces of evidence suggest that mitochondrial turnover is a common compromised process in cardiac dysfunctions, pointing to another aspect of mitochondrial biology with a key role in cardiomyocyte function and survival.

GRKS AND β-ARRESTINS: THE NON-GPCR SIGNALING

G Protein-CoupledReceptor (GPCR) Kinase (GRKs) and β-arrestins are key regulators of GPCR signaling (Ferguson, 2001; Kohout and Lefkowitz, 2003; Santulli and Iaccarino, 2016; Sorriento et al., 2016). Indeed, GRKs are recruited to the plasma membrane when the receptor is activated by agonist binding. Here, GRKs phosphorylate GPCRs favoring the recruitment of β-arrestin which in turn promotes rapid receptors desensitization or their clathrin-mediated endocytosis

and internalization (Ferguson et al., 1996; Goodman et al., 1996; Ferguson, 2001; Pierce et al., 2002; Lefkowitz and Shenoy, 2005). Several evidence endorse the proof of concept that GRKs and β-arrestins also regulate intracellular signaling independently from GPCR by affecting non-GPCR receptors or by direct interaction with target molecules (Kang et al., 2005; Ma and Pei, 2007; Penela et al., 2010; Evron et al., 2012). Among GRKs, GRK2 is emerging as a key node in signal transduction pathways, displaying a very complex "interactome" (Penela et al., 2010). β-arrestins also function as scaffold proteins that interact with several cytoplasmic molecules (DeFea et al., 2000; McDonald et al., 2000) or interact in the cytosol with regulators of transcription factors, such as IκBαand MDM2,to regulate transcription indirectly (Wang et al., 2003; Luan et al., 2005). Thus, these molecules are able to regulate several key processes within the cell in a GPCR-independent manner, affecting cell biology both in physiological and pathological conditions.

The functional cross-talk between mitochondria and other cellular compartments has been shown to regulate mitochondrial function (Hajnoczky et al., 2000; Galganska et al., 2010; Viola and Hool, 2010; Li et al., 2018). This interplay has a key physiological role, placing the mitochondria under the regulation of extracellular stimuli and thus ensuring the adaptation of mitochondrial activity to environmental needs. This functional cross-talk is regulated through the activity of several proteins, among whichGRK2 and β-arrestins are potential candidates. Indeed, these molecules are able to move among different compartments and interact with different partners thus interfering with the mitochondrial signaling transduction pathway. GRK2 and β-arrestins regulate mitochondrial function in cardiac cells through mechanisms which are independent of GPCR signaling. This issue was only recently investigated but several data have already been generated with important translational implications.

GRK2: A Dynamic and Multifunction Protein

GRKs are critical regulators of cardiac function both in physiological and pathological conditions. Among them, GRK2 and GRK5 are the most abundant G protein-coupled receptor kinases in the heart. In particular, GRK2 is essential for cardiac health. Indeed, the ablation of GRK2 gene in myocytes affects cardiac phenotypes in adulthood leading to a prevalent eccentric remodeling after chronic exposure to β adrenergic stimulation (Matkovich et al., 2006; Sorriento et al., 2015). Moreover, the total deletion of the kinase is lethal by preventing the correct development of the cardiovascular system in prenatal life (Raake et al., 2012). This developmental importance of GRK2 also concerns the endothelium. Indeed, the deletion of GRK2 in endothelial cells resulted in alteration of vascular phenotype and integrity, due to an increase of inflammation and oxidative stress (Ciccarelli et al., 2013; Rivas et al., 2013). Conversely, the deletion of GRK5 does not affect heart function but even ameliorates cardiac responses to insults (Hullmann et al., 2014). These data suggest that even though these kinases are both involved in the regulation of cardiac biology, GRK2 is the one fundamental for cardiac cell survival both in physiological and pathological conditions. It is clear that this effect could not be limited to the regulation of GPCR activity on plasma membrane where kinase effects seem, on the contrary, to trigger and sustain the development of heart failure (Lymperopoulos et al., 2012). That's the reason why in the last decade research focused on the identification of other kinase activities within the cell which were independent of GPCR. A previous report from Mayor group clearly summarizes the novel identified GRK2 substrates and their functional roles in several conditions (cardiovascular diseases, inflammation, cancer) (Ribas et al., 2007; Penela et al., 2010). GRK2 has been shown to associate with PI3K, GIT, caveolin, MEK, AKT, α-actinin, clathrin, calmodulin, c-SRC, PKA, PKC, IkBα, and RKIP, regulating different signal transduction pathways within the cell (Ribas et al., 2007; Sorriento et al., 2015). Moreover, GRK2 can also interact with non–GPCR receptors, such as Ins-R, PDGF-R, and EGF-R (Hupfeld and Olefsky, 2007; Cipolletta et al., 2009).

Based on these findings, the idea that GRK2 is a dynamic molecule, that moves within the cell depending on cell requirements, started to take ground (Sorriento et al., 2014; Sorriento et al., 2016) suggesting that the regulation of GRK2 trafficking within the cell could be a potential strategy to regulate the adaptative effects of the kinase on cell functions (Sorriento et al., 2014).

β-Arrestins and the Signalosomes

Besides their cardioprotective role through means of GPCR desensitization, common acquisitions suggest that β-arrestins also function as GPCR signal transducers (Luttrell and Lefkowitz, 2002). They directly initiate signaling through the formation of multiprotein signaling complexes, known as a "signalosomes," in which they act as scaffolds, adaptors, and signal transduction proteins (Lefkowitz et al., 2006). They can form complexes with several signaling proteins, including Src family tyrosine kinases and components of the ERK1/2 and JNK3 MAP kinase cascades (DeWire et al., 2007; Kang et al., 2014). Moreover, β-arrestins can also regulate gene transcription in the nucleus. Indeed, they are able to interact with IkBα and sequester the complex IkBα-NFkB in the cytosol, thus inhibiting NFkB transcription activity (Witherow et al., 2004). More than 300 proteins have been identified that interact with β-arrestins with multiple implications in most key processes within the cell (Xiao et al., 2007). These proteins are mainly localized in the cytosol but they are also distributed in other compartments including mitochondria.

GRK2 DEPENDENT REGULATION OF MITOCHONDRIAL FUNCTION

Energetic Metabolism and Mitochondrial Dynamics

Among the novel functions of GRK2, in the last decade, the potential role of the kinase in the regulation of the metabolic state of the cell emerged (Cipolletta et al., 2009; Sorriento et al., 2014).

The evidence comes from the demonstration that GRK2 accumulation leads to the shut-off of insulin signaling and inhibits glucose extraction (Usui et al., 2004; Cipolletta et al., 2009; Ciccarelli et al., 2011). In the cardiovascular setting, given the key role of mitochondria to supply the energy need of the heart and the importance of GRK2 for cardiac biology, research also focused on the identification of a potential role of GRK2 in the regulation of energy metabolism leading to the discovery of GRK2as key mediator of production and expenditure of energy within the cell (Fusco et al., 2012; Chen et al., 2013). The first evidence of GRK2 localization at mitochondria comes from the study of Obrenovich's group in a rat model of Alzheimer disease (Obrenovich et al., 2006). In the early pathogenesis of Alzheimer Disease and in ischemia-reperfusion brain injury models, GRK2 accumulates in damaged mitochondria, suggesting a role for the kinase in this organelle. However, Fusco and colleagues were the first who effectively identified the kinase role in mitochondria (Fusco et al., 2012). Indeed, they showed that the overexpression of this kinase increased mitochondrial mass, ATP production and mitochondrial biogenesis (Fusco et al., 2012). Ischemia causes acute accumulation of GRK2 in mitochondria both *in vitro* and *in vivo*, and reperfusion reverted such effect. The overexpression of the kinase protects ATP production even after hypoxia/reperfusion damage (Fusco et al., 2012). This suggests a potential role of the kinase in energy production, which is particularly relevant for tissues that need a great amount of energy to work well, such as the heart. Successively, other reports confirmed that GRK2 localizes into mitochondria. Indeed, Chen showed that both in hearts *in vivo* and in cultured myocytes, GRK2 localizes into mitochondria after an ischemia-reperfusion insult. The authors also propose a potential mechanism by which the kinase is able to traffic to mitochondria (Chen et al., 2013). In particular, they demonstrate that phosphorylation at residue Ser670 within the carboxyl-terminus of GRK2 by extracellular signal-regulated kinase (ERK) allows GRK2 to bind the heat shock protein 90 (HSP90), which chaperones the kinase to mitochondria (Chen et al., 2013). Accordingly, the same Authors also show that a mutant form of GRK2, that cannot bind HSP90, does not localize to mitochondria (Sato et al., 2018). Mitochondrial localization of the kinase is not limited to cardiac cells. Indeed, we demonstrated that GRK2 localizes into mitochondria of macrophagic cells in a time-dependent manner and an early translocation supports the cell to better respond to LPS dependent mitochondrial dysfunction (Sorriento et al., 2013). In these cells, the overexpression of the carboxy-terminal domain of GRK2 (βARK-ct), known to displace GRK2 from plasma membranes, induces earlier localization of GRK2 to mitochondria in response to LPS leading to increased mt-DNA transcription and reduced ROS production and cytokine expression (Sorriento et al., 2013). These data confirm that the mitochondrial localization of GRK2 ameliorates mitochondrial function, as shown in other models (Fusco et al., 2012). Accordingly, Franco recently showed that the overexpression of GRK2 protects mitochondria from the damage induced by ionizing radiation. Indeed, GRK2 favors the rescue of mitochondrial mass, morphology, and respiration (Franco et al., 2018). On the opposite, the kinase deletion accelerates degenerative processes induced by the exposure to ionizing radiation. This evidence clearly supports the idea that GRK2 is beneficial for mitochondrial function and is effective to protect mitochondria from insults. The mechanism involves a novel "interactome" of GRK2 which includes HSP90, as also previously demonstrated (Chen et al., 2013), and molecules involved in the regulation of mitochondrial dynamics, mitofusins (MFN-1 and MFN-2) (Franco et al., 2018). GRK2 dynamically binds MFN-1/2 by means of HSP90 and phosphorylates these molecules affecting mitochondrial fusion (Franco et al., 2018). MFN-1 and 2 are key regulators of mitochondrial fusion and fission processes that are critical for cardiac health (Santel, 2006). Recently, it has been demonstrated that these molecules can adopt either a fusion-constrained or a fusion-permissive molecular conformation that allows them to regulate mitochondrial dynamics (Franco et al., 2016). The imbalance between fission and fusion causes mitochondrial dysfunction. The finding that GRK2 is able to phosphorylate and activate these molecules suggest its involvement in mitochondrial dynamics and biology. This is the first finding regarding a phosphorylation-dependent regulation of the activity of the mitofusins. Likely, GRK2 by phosphorylating mitofusins can orchestrate mitochondrial dynamics. However, further data are needed to support this hypothesis.

Apoptosis

Even though all *in vitro* findings strongly support a protective effect of GRK2 in damaged mitochondria, other reports, on the contrary, suggest a pro-death role of the kinase in this organelle. Indeed, in cardiac myocytes, the inhibition of GRK2 increased ATP production whereas the overexpression of the kinase increased oxidative stress and negatively regulated FA oxidation (Sato et al., 2015). Moreover, mitochondrial GRK2 is reported to promote cell death in ischemic myocytes and its inhibition by means of βARKct is reported to be cardioprotective.(Chen et al., 2013; Woodall et al., 2014). Conversely, the same authors previously published that this pro-apoptotic effect of GRK2 was only due to its effects on plasma membrane through the regulation of AKT signaling, also showing that the treatment with βARK-ct inhibited such phenomenon (Brinks et al., 2010). Thus, it is not clear yet whether the effects of βARK-ct could depend on GRK2 reduction in mitochondria or in the plasma membrane. Further studies are needed to better clarify this issue.

Also, the same Authors recently show that a mutant form of GRK2, that cannot bind HSP90, inhibits kinase localization in mitochondria and confers protection in response to ischemia-reperfusion (Sato et al., 2018).

In this context, the disruption of GRK2 binding with HSP90 has been shown to decrease the expression of endogenous GRK2 in a dose- and time-dependent manner (Luo and Benovic, 2003) and this could justify the cardioprotective role of βARKct and of GRK2 mutant. Given this evidence, it was expected that both βARKct and the expression of the mutant form of GRK2 would cause the total reduction of the kinase within the cell. Actually, total levels of GRK2 were not modified in both Chen's and Sato's studies which are therefore in contrast with literature. Given these

discrepancies, the exact role of mitochondrial GRK2 in apoptotic processes still remains to be clarified.

GRK2 in Mitochondria: Detrimental or Cardioprotective?

Actually, contrasting evidence exists on the role of GRK2 in mitochondria. To reconcile these opposing findings, several considerations are to be taken into account.

(a) Membrane displacement of GRK2 from the plasma membrane by means of βARKct overexpression does not mean lack of kinase activity. βARKct reduces GRK2 levels on the plasma membrane but also induces its localization in other cellular compartments, such as mitochondria (Sorriento et al., 2013). Giving this notion, the cardioprotective effect of βARKct in ischemic myocytes is in agreement with kinase accumulation in mitochondria.

(b) The overexpression is a complex maneuver that drastically upsets cell biology. Indeed, GRK2 overexpression increased kinase levels in all cellular compartments, including plasma membranes, whereGRK2induces apoptotic events by affecting GPCR signaling. Indeed, GRK2 induces oxidative stress and apoptosis in cardiac myocytes in the same manner of beta-adrenergic receptor stimulation, and kinase inhibition prevents such events (Theccanat et al., 2016). Thus, GRK2 dependent induction of apoptosis depends on its levels of expression on membranes being limited to its effects on GPCR signaling independently from mitochondria.

(c) Moreover, it should be considered that in response to insults the cell activates protective mechanisms to avoid irreversible damage. Mitochondrial fission, for instance, is activated to better respond to stress conditions by eliminating damaged mitochondria and restoring the normal cell activity. The apoptosis is a programmed cell death that is induced when cell damage is irreversible. Therefore, GRK2 dependent cell death could not be strictly dependent on a deleterious action of the kinase but would rather be the result of an advanced and irreversible mitochondrial damage that GRK2 is not able to stop.

(d) Timing is also a critical point to take into account. Indeed, the protective role of GRK2 has been shown in response to acute insults but the chronic responses in animal models could be different and negatively affect mitochondrial biology due to continuous activation of intracellular signaling.

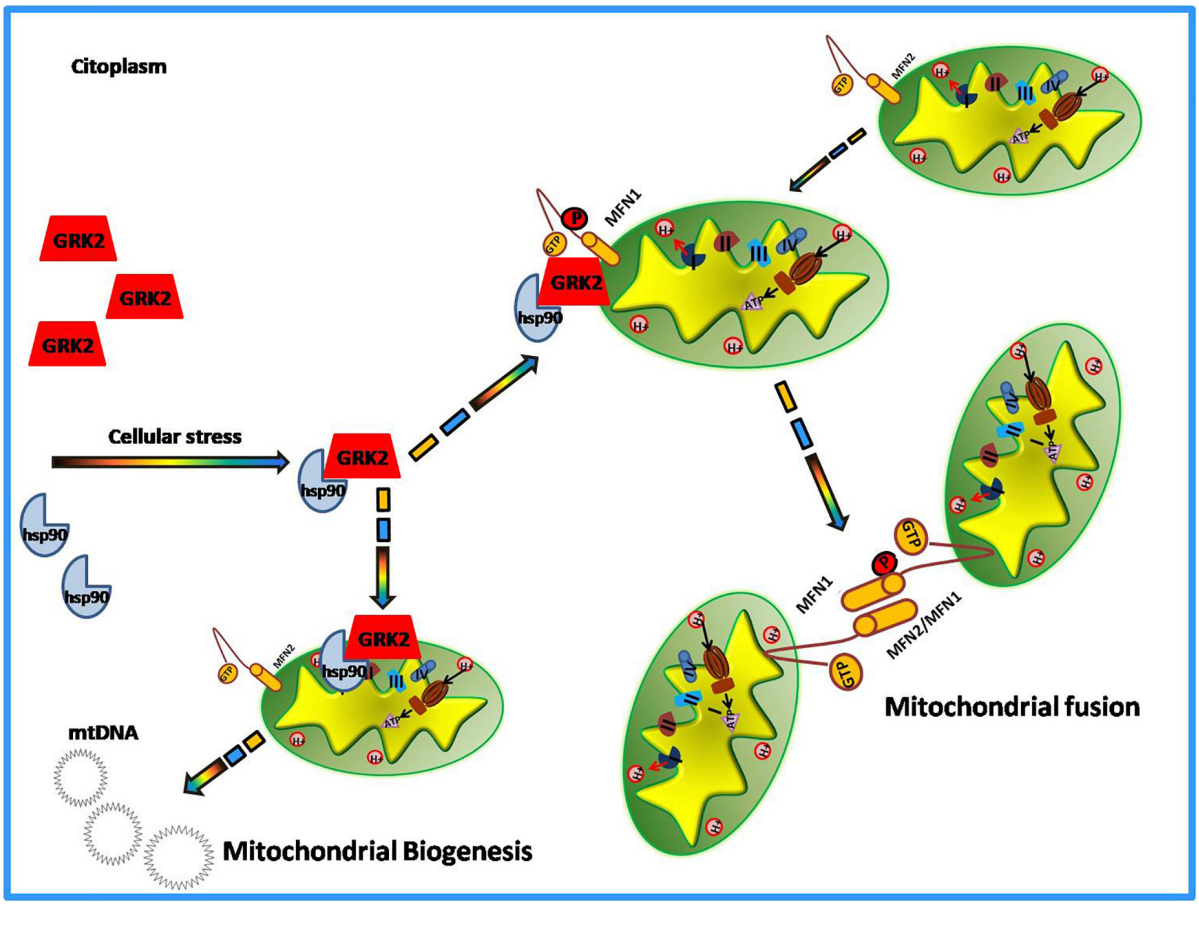

FIGURE 2 | GRK2 activities in mitochondria.

Thus, altogether these findings support the proof of concept that GRK2 is a dynamic and multifunction protein whose actions in mitochondria likely aims to protect the cell from irreversible damage due to external insults (**Figure 2**). This activity is especially significant in those conditions, such as heart failure, characterized by remarkable mitochondrial dysfunction. Thus, the possibility to specifically deliver the kinase to mitochondria (i.e., through means of plasma membrane displacement such as βARK-ct) appears a promising strategy to recover mitochondrial function in damaged cardiac cells.

β-ARRESTINS AND MITOCHONDRIAL ACTIVITY

Among the numerous actions of β-arrestins within the cell, growing evidence suggests their involvement in the regulation of mitochondrial function in the heart. In particular, it has been shown that such proteins interfere with key mitochondrial processes such as cell death, ROS production, and respiration. The involvement of β-arrestins in the regulation of apoptotic processes is very controversial. Indeed, it has been suggested that these proteins can both promote and inhibit cell death probably due to different stimuli and cell type. In Mouse Embryonic Fibroblasts (MEFs), in response to apoptotic stimuli, β-arrestins are cleaved by multiple caspases at Asp380 generating a small fragment of 380 amino acids (Kook et al., 2014). This latter translocates to mitochondria and cooperates with tBID to induce the release of cytochrome C and consequently cell death (Youle and Strasser, 2008; Galluzzi et al., 2012). Conversely, in response to IGF-1 stimulation,β-arrestins mediate the activation of PI3K with subsequent activation of AKT (Gu et al., 2015). The PI3-kinase/AKT signaling pathway blocks caspase activity and consequently apoptosis (Kennedy et al., 1997). Also, in response to oxidative stressβ-arrestins exert an anti-apoptotic effect. Indeed, in HEK-293 cells stimulated with H2O2 β-arrestins bind the C-terminal domain of the Apoptosis Signal-regulating Kinase 1 (ASK1) inducing its ubiquitination and degradation by the proteasome (Zhang et al., 2009). Giving these opposing findings, further data are needed to better clarify the involvement of β-arrestins in apoptotic processes.

It is known that mitochondria represent the major intracellular source of ROS. β-arrestins are able to regulate mitochondrial ROS production (Philip et al., 2015). In cultured human cardiac fibroblasts isolated from failing hearts, there is an upregulation of β-arrestins that is associated with the activation of ERK and subsequent upregulation of Nox4, an NADPH oxidase able to catalyze the production of a superoxide free radical. This increase in oxidative stress promotes collagen synthesis and leads to myocardial fibrosis (Tsutsui et al., 2011). The overexpression of β-arrestins by means of adenoviral-mediated gene transfer

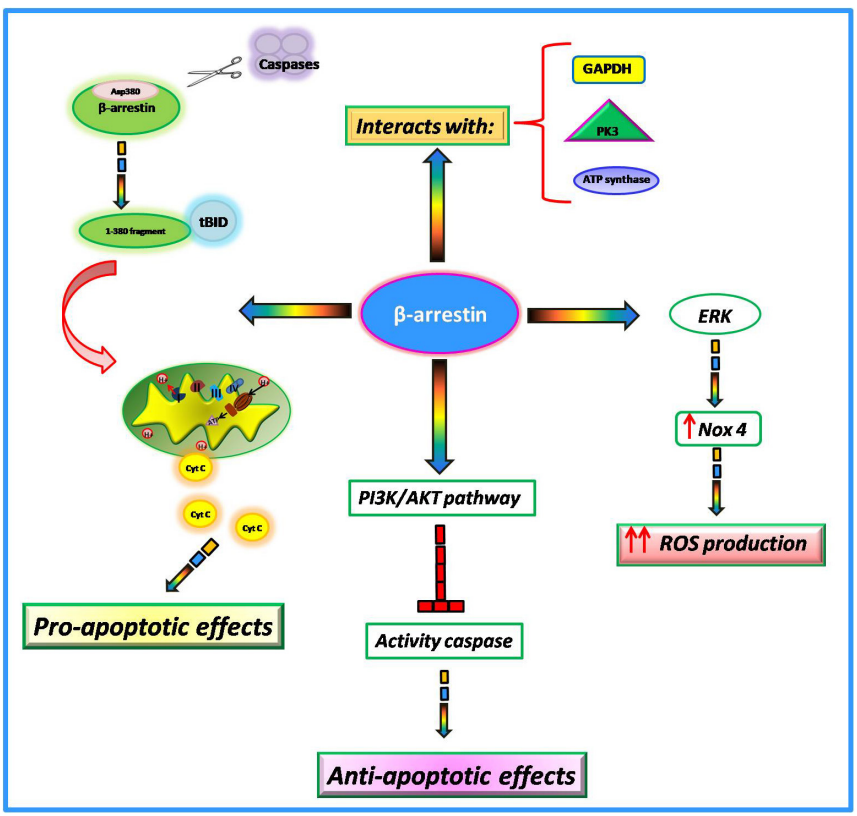

FIGURE 3 | Non-GPCR activities of β-arrestins.

increases mitochondrial superoxide production while the knockdown decreased ROS production and Nox4 expression in failing cardiac fibroblasts (Philip et al., 2015). Thus, targeted inhibition of β-arrestins in cardiac fibroblasts could be an effective strategy to decrease oxidative stress and fibrosis in cardiac tissue. Both β-arrestin 1 and β-arrestin 2 are both expressed in cardiac myocytes but it has been shown that only β-arrestin 1 is involved in βAR induced ROS production (J. Zhang et al., 2017). Indeed, the knockdown of β-arrestin1 inhibited βAR dependent mitochondrial ROS production while the knockdown of β-arrestin 2 exerted no effects. The mitochondrial production of ROS in response to βAR stimulation is activated by two different pathways at different times. Indeed, this study shows that the cAMP/PKA pathway is responsible for faster mitochondrial ROS production, whereas β-arrestin1 signaling is responsible for the slower one (Zhang et al., 2017).

Among β-arrestins interactome, many proteins participate to mitochondrial respiration (Gu et al., 2015), including metabolic enzymes involved in the glycolysis pathway (PK3, GAPDH, and enolase) and oxidative phosphorylation (ATP synthases and SDH2) (Xiao et al., 2007). The functional implications of such interactions are still unknown but likely β-arrestins interactions with these enzymes could be needed to promote energy production.

Compared with GRK2, the involvement of β-arrestins in the regulation of mitochondrial function needs more clarifications. However, the available findings suggest thatβ-arrestins interfere with several intracellular signaling pathways which are involved in the regulation of key mitochondrial processes (**Figure 3**) and could be a promising target for the regulation of mitochondrial function.

CONCLUSION

Several reports support the proof of concept that GRK2 and β-arrestins are able to regulate intracellular signaling in a GPCR independent manner. These activities affect several compartments within the cell, including mitochondria. The involvement of GRK2 in the regulation of mitochondrial function has been recently identified showing its ability to regulate ATP content, ROS production, mitochondrial dynamics, and apoptosis. However, the exact role of the kinase (detrimental or protective) still remains to be elucidated given the opposing results from reports on this issue. Overall, we tried to reconcile these opposing findings pointing to a protective role of GRK2 in mitochondria through its binding to HSP90. Such an effect has important implications in the onset of cardiovascular disease, which are characterized by an impaired mitochondrial function. In this context, β-arrestins are novel identified targets whose activities in mitochondria are not completely clear yet. Few studies are available on β-arrestins dependent regulation of mitochondrial functions thus further investigations are needed. However, the available ones strongly suggest the involvement of β-arrestins in the regulation of mitochondrial ROS production and mitochondrial respiration. The better understanding of the role of these proteins in mitochondria could have important implications providing the basis for new therapeutic approaches to treat mitochondrial dysfunction in cardiovascular diseases.

AUTHOR CONTRIBUTIONS

DS and GI conceived the study. DS, JG, AF, GI, and MI wrote and critically reviewed the manuscript.

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β2-Adrenoceptors and GRK2 as Potential Biomarkers in Patients With Chronic Pulmonary Regurgitation

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Pulmonary regurgitation (PR) is a frequent complication after repair of congenital heart disease. Three different GRK isoforms (GRK2, GRK5, and GRK3) and two β-adrenoceptors (β1-AR and β2-AR) are present in peripheral blood mononuclear cells (PBMC) and their expression changes as a consequence of the hemodynamic and neurohumoral alterations that occur in some cardiovascular diseases. Therefore, they could be useful as biomarkers in PR. A prospective study was conducted to describe the expression (TaqMan Gene Expression Assays) of β-ARs and GRKs in PBMC isolated (Ficoll® gradient) from patients with severe PR before and after pulmonary valve replacement and establish if this expression correlates to clinical status. 23 patients with severe PR were included and compared with 22 healthy volunteers (controls). PR patients before the PVR had a significantly lower expression of β2-AR (513.8 ± 261.2 mRNA copies) vs. controls (812.5 ± 497.2 mRNA copies), so as GRK2 expression (503.4 ± 364.9 copies vs. 858.1 ± 380.3 mRNA copies). The expression of β2-AR and GRK2 significantly decreases in symptomatic and asymptomatic patients, as well as in patients under treatment with beta-blockers and non-treated patients. The expression of β2-AR and GRK2 in PR patients recovers the normal values after pulmonary valve replacement (754,8 ± 77,1 and 897,8 ± 87,4 copies, respectively). Therefore, changes in the expression of β2-AR and GRK2 in PBMC of PR patients, could be considered as potential biomarkers to determine clinical decisions.

Keywords: pulmonary regurgitation, pulmonary valve replacement, right ventricle, congenital heart disease, β2-adrenoceptor, GRK2

INTRODUCTION

The β-adrenergic system plays a key role in the regulation of heart function and it is also known that it is involved in the pathogenesis of heart failure (HF). In this pathology, levels of circulating catecholamines increase and this determines an adaptation in the expression and activity of adrenoceptors (AR) as well as the G-protein coupled receptors kinases (GRK), which phosphorylate AR when they are occupied by agonists.

Three different GRK isoforms (GRK2, GRK5 and, in a minor proportion, GRK3) are present in the heart and also in peripheral blood mononuclear cells (PBMC) (Vinge et al., 2007; Grisanti et al., 2018) and their expression (especially GRK2) changes as a consequence of the hemodynamic alterations that occur in HF or hypertension (Iaccarino et al., 2005; Penela et al., 2006; Vinge et al., 2007; Izzo et al., 2008; Cohn et al., 2009; Santulli et al., 2011, 2013; Piao et al., 2012; Grisanti et al., 2018). Moreover, it is remarkable that expression of β2-ARs and GRK2 follow a similar pattern of changes in different tissues and pathologies (Montó et al., 2015). Initially, changes in the expression of GRKs and β-ARs were determined in myocytes (endomyocardial biopsies, cardiac explant), but it is also possible to study these changes in PBMC (Iaccarino et al., 2005; Agüero et al., 2008; Oliver et al., 2010). Thus, their determination in peripheral blood can become a useful parameter for the follow-up of cardiovascular disease. In fact, in the study of HF, the expression of GRK2 in circulating lymphocytes (Rengo et al., 2016) has been used as a molecular marker.

Pulmonary regurgitation (PR) occurs frequently after surgical repair of tetralogy of Fallot and other congenital heart diseases (CHD), which results in a right ventricle (RV) volume overload and progressive dysfunction (Gregg and Foster, 2007). Chronic severe PR is associated with reduced exercise capacity, increased risk of arrhythmias and sudden cardiac death (Bouzas et al., 2005). Pulmonary valve replacement (PVR) is indicated in symptomatic patients or with moderate-severe RV dilatation/dysfunction (Warnes et al., 2008; Baumgartner et al., 2010). It results in improvement of functional class and reduction of RV volumes, but after surgery there is no evidence of arrhythmias reduction, improvement in exercise capacity, RV systolic function or survival (Ferraz Cavalvanti et al., 2013). Therefore, many authors recommend performing PVR earlier. The risk of the surgery is low (Geva, 2013), but the tissue valves deteriorates and multiple re-interventions are necessary (Oliver et al., 2015) then, the time for PVR must be adequately selected to obtain the maximal clinical benefit with the minimum number of re-interventions. In this context, the existence of a molecular marker, as occurs with GRK2 in HF, could be a useful tool to determine the clinical actuation.

In a previous work we have found that the gene expression pattern of GRK2 and the β2-AR was altered in patients with severe PR in a similar way to patients with advanced HF (Rodríguez-Serrano et al., 2018). In the present work, we propose a molecular approach to improve the pathophysiological knowledge of PR in order to help us to decide the right time for PVR. Our main objective was to describe changes in gene expression of β1 and β2 ARs and GRKs (GRK2, GRK3, and GRK5) in PBMC from patients with severe PR, before and after PVR, and establish if this expression correlates to clinical status.

MATERIALS AND METHODS

Patients with severe PR followed in the adult congenital heart disease unit from December 2011 to July 2015 were selected prospectively. The diagnosis of severe PR was made following echocardiographic criteria (Lancellotti et al., 2013) and was confirmed by magnetic resonance imaging. Patients with left ventricular dysfunction or other severe valve disease were excluded. After signing the informed consent, a blood sample was taken for analysis of the gene expression of β-ARs (β1 and β2) and GRKs (GRK2, GRK3, and GRK5) in PBMC. Clinical data (functional class of the New York Heart Association, rhythm and width of QRS) and RV assessment by cardiac magnetic resonance (ventricular ejection fraction and volumes) were also collected.

Patients included in this study are part of the cohort of PR patients recruited in a previous study (Rodríguez-Serrano et al., 2018) that has been submitted to pulmonary valve replacement (PVR) and clinically followed by one year. PVR was carried out on patients who met criteria according to the guidelines of clinical practice (Warnes et al., 2008; Baumgartner et al., 2010). A blood sample was taken for analysis of gene expression of β-ARs and GRKs in PBMC in this group of patients previous chirurgical intervention and at least one year after surgery. Clinical, electrocardiographic and RV assessment data were collected in the follow-up.

A group of healthy volunteers, comparable in age and sex with the patients, was included in the study to perform a single blood extraction for the analysis of gene expression of β-ARs and GRKs. Patients with inflammatory processes that could alter the expression of these molecules were excluded. The inclusion of control patients was performed in two different periods with a time lapse of 6–12 months between them and the results obtained were not statistically different (results not shown).

The study was made according to the Declaration of Helsinki and approved by the ethics committee of the Hospital La Fe with registry number 2011/0241.

Analysis of Gene Expression of β-Adrenoceptors and GRKs in Peripheral Blood Mononuclear Cells

The biological material to study was a sample of fresh blood (10 ml) obtained by venipuncture and preserved in a tube with anticoagulant (EDTA). Immediately after the extraction, PBMC were isolated using Ficoll gradient (Montó et al., 2015). The sample obtained was stored at −80 °C until be processed.

Total RNA was obtained as previously described (Oliver et al., 2010). After RNA isolation, quality control was carried out by microfluidic electrophoresis using the Experion automated electrophoresis system (BioRad, Madrid, Spain) following the manufacturer's conditions. Complementary DNA synthesis was carried out as previously described (Montó et al., 2015). The mRNAs encoding the human β-ARs (β1 and β2), the 3 GRKs mainly expressed in the myocardium (GRK2, GRK3, and GRK5), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as internal standards were quantified by Taq-Man real-time RT polymerase chain reaction (PCR) with a GeneAmp 7500 Fast System (Applied Biosystems, Carlsbad, CA, United States). We analyzed (in duplicate reactions) a 10-fold dilution of the RT reaction of each sample using the TaqMan Gene Expression Assays (Applied Biosystems). The specific primer probes were: β1-AR, Hs00265096_s1; β2-AR, Hs00240532_s1;

GRK2, Hs00176395_m1; GRK3, Hs00178266_m1; GRK5, Hs00178389_m1, and *GAPDH*, Hs99999905_m1 (Applied Biosystems). Real-time PCR reactions were done in 25 mL of TaqMan Universal PCR Master Mix (Applied Biosystems), including 5 mL of diluted RT reaction and 1.25 mL of 20 x TaqMan Gene Expression Assay Mix (250 nmol/L for the probe and 900 nmol/L for each primer). Complementary DNA was amplified following the manufacturer' s conditions: one initial hold step at 95°C for 10 min, a second step with 40 cycles, 15 s at 95°C (denaturation), and 1 min at 60°C (annealing/extension). The targets and reference (*GAPDH*) were amplified in parallel reactions. The Ct values obtained for each gene were represented in the **Supplementary Figure S1**. We calculated the relative mRNA levels of β-ARs and GRKs referenced to *GAPDH* and converted into the linear form using the term 2^{-dCt} as a value directly proportional to the mRNA copy number (Montó et al., 2015).

Statistical Analysis

The number of patients was adapted during the study, so in the **Online Supplement** the effects size with their corresponding confidence intervals are included. The statistical analysis was performed using Graph Pad software. A normality test, followed by one-way analysis of variance (ANOVA) and Student's *t*-test or Newman Keuls's test were used. Linear regression and Pearson's correlation test were used to establish association between β-adrenoceptors and GRKs with hemodynamic and clinical variables in a post-hoc analysis. A probability value of $p < 0.05$ was considered significant.

RESULTS

Study Population

23 patients with severe PR were included in the study (60.9% males, mean age of 35.7 ± 10.6 years). The most frequent underlying heart disease was Tetralogy of Fallot and pulmonary valve replacement with extension of the RV outflow tract was the most frequent surgical procedure. At recruitment, more than a half of patients were asymptomatic and 39.1% of the patients was under treatment with beta-blockers. The rest of the baseline characteristics of the population are shown in **Table 1** and clinical variables in **Table 2**. According to the criteria of cardiac magnetic resonance, 47.8% had RV dysfunction (RV ejection fraction < 45%) and 73.9 % significant RV dilation (indexed RV end-diastolic volume measured by cardiac magnetic resonance ≥ 150 ml/m^2). The comparisons were made with 22 healthy volunteers comparable in age and sex (62% males, mean age of 36.7 ± 5.2 years).

Analysis of Gene Expression of β-ARs and GRKs in Peripheral Blood Mononuclear Cells From Patients With PR

In a previous work (Rodríguez-Serrano et al., 2018), we have found that patients with severe PR had lower genic expression of β2-AR and GRK2 in PBMCs. In the present study, the group of patients with severe PR also exhibit a significantly lower expression of β2-AR (PR, 513.8 ± 261.2 copies of mRNA) vs. healthy volunteers (control, 812.5 ± 497.2 copies of mRNA). The same occurs if we analyze GRK2 expression (503.3 ± 364.9 copies in PR vs. 858.1 ± 380.3 copies in control) The expression of β1-ARs, GRK3 and GRK5 did not change significantly in patients with severe PR vs. controls (**Figure 1** and **Supplementary Table S1**).

These results were also analyzed considering the NYHA functional class. Only the expression of GRK2 was lower in patients NYHA class ≥ 2 vs. patients in NYHA class 1 (**Supplementary Table S2**). When comparing the levels with the control group, as **Figure 2** shows, the expression of β2-ARs and GRK2 significantly decreases as well as the NYHA class increases.

TABLE 1 | Baseline characteristics of patients with severe pulmonary regurgitation.

Males, n (%)	**14 (60.9)**
Age (years)	35.7 +/− 10.6
Congenital heart disease, n (%)	
Tetralogy of Fallot	18 (78.3)
Valvular pulmonary stenosis	2 (8.7)
Other	3 (13.0)
Corrective intervention on the pulmonary valve, n (%)	21 (91.3)
Isolated miectomy /valvulotomy	5 (21.7)
Extension of the RV outflow tract	16 (69.3)
Functional class, n (%)	
NYHA I	15 (65.2)
NYHA II	8 (34.8)
Treatment with beta-blockers, n (%)	9 (39.1)
Bisoprolol	7 (30.4)
Atenolol	1 (4.3)
Carvedilol	1 (4.3)
Characteristics of ECG	
Sinus rhythm, n (%)	20 (87)
QRS duration (ms)	157.6 ± 26.3

CMR: cardiac magnetic resonance; ECG: electrocardiogram; NYHA: New York Heart Association.

TABLE 2 | Clinical variables of patients with severe PR before and after PVR.

	Before PVR	**After PVR**
RVEDVi (ml/m2)	163.4 ± 24.1	101.7 ± 30.5***
RVESVi (ml/m2)	87.7 ± 19.1	57.8 ± 20.4***
RVEF (%)	44.4 ± 9.6	45.8 ± 9.4
LVEF (%)	59.4 ± 9.3	57.8 ± 10.2
NYHA (%)		
I	65.2	91.3
II	34.8	8.7

LVEF: ejection fraction of left ventricle, RVEDVi: right ventricular end-diastolic volume indexed, RVEF: ejection fraction of right ventricle; RVESVi: right ventricular end-systolic volume indexed.
*** *P < 0.001; Student t-test*

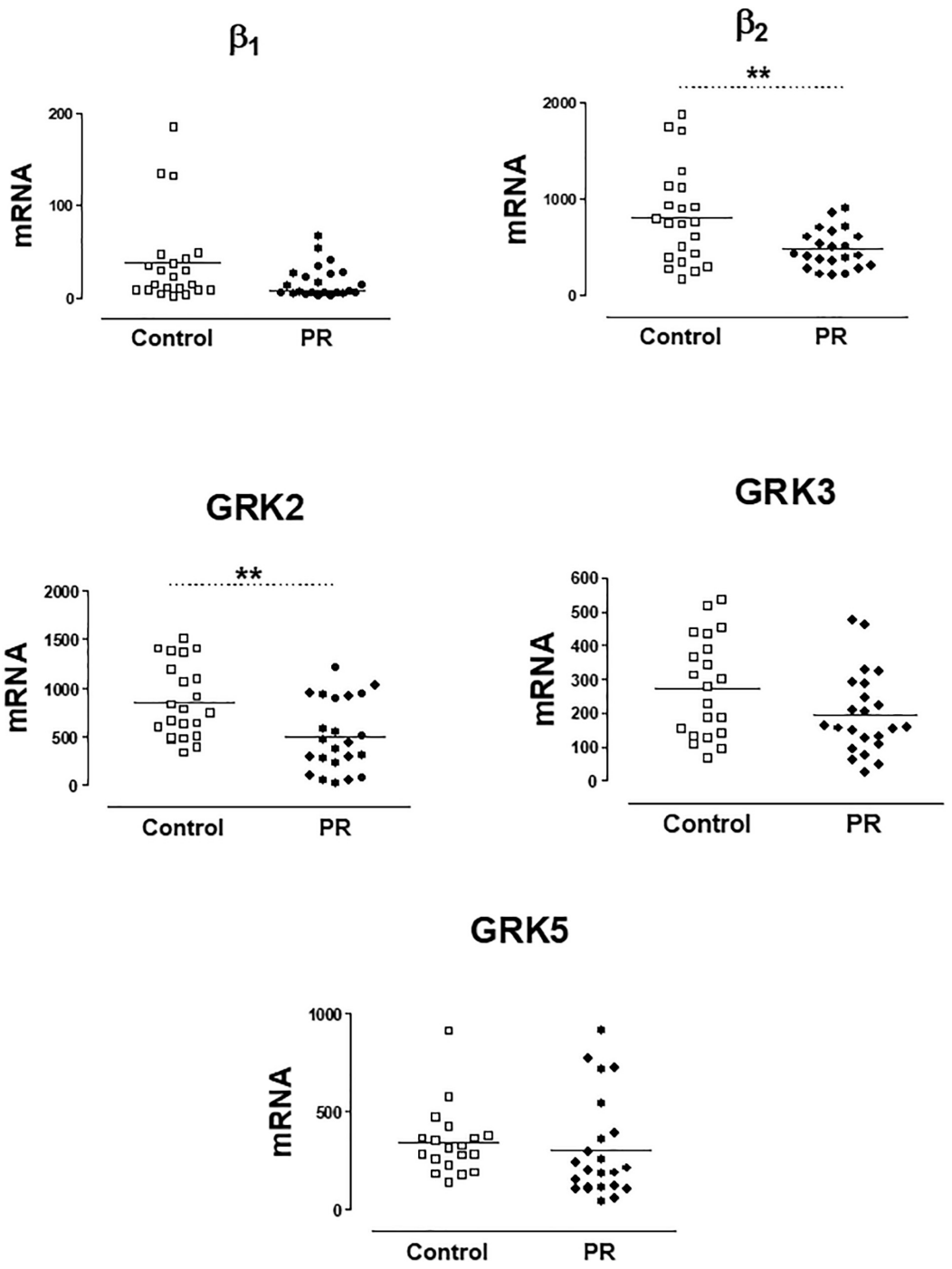

FIGURE 1 | mRNA levels for β1- and β2-adrenoceptors, and G-protein coupled receptor kinases GRK2, GRK3, and GRK5, in peripheral blood mononuclear cells from healthy volunteers (control, $n = 22$) and patients with pulmonary regurgitation (PR, $n = 23$). Data were calculated as 2^{-dCt} vs. GAPDH as reference gene, and the mean was represented by the continuous line. $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's t-test).

No differences were observed in gene expression of β-ARs and GRKs in PR patients in function of gender (results not shown), nor in patients under treatment with beta-blockers vs. non-treated patients (**Table 3**).

No significant correlations were found between the expression of β-ARs or GRKs with the age and the clinical variables of RV assessment. Only the expression of GRK3 was inversely related to the RVEDV (**Figure 3** and **Supplementary Table S3**).

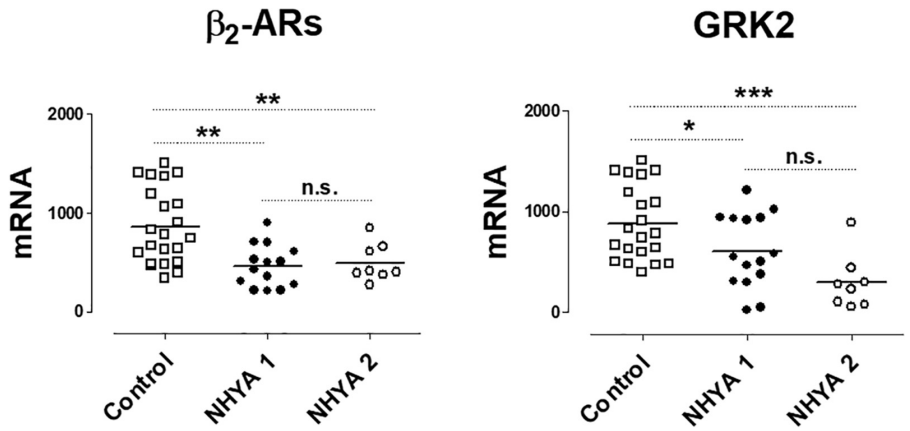

FIGURE 2 | mRNA levels for β2-adrenoceptors (β2) and G-protein coupled receptor kinase 2 (GRK2) in peripheral blood mononuclear cells from healthy volunteers (control, $n = 22$) and symptomatic patients with pulmonary regurgitation, belonging to the NHYA class ≥ 2 (NHYA 2, $n = 8$) and asymptomatic patients, belonging to NHYA class 1 ($n = 15$). Data were calculated as 2^{-dCt} vs. GAPDH as reference gene and the mean was represented by the continuous line. $^*P < 0.05$, $^{**}P < 0.01$, n.s. = no significant (one way ANOVA and Newman Keuls's test).

Changes in Clinical Variables and Genic Expression of β-ARs and GRKs in PR Patients After PVR

The changes observed in the clinical, electrocardiographic and RV assessment after the PVR are shown in **Table 2**. Significant improvement was observed in the NYHA functional class of patients and RV volumes, indicating an adequate response after surgical intervention. All patients receiving beta-blockers before PVR continue with the treatment and two patients begin treatment after the surgery.

mRNA levels of β-ARs and GRKs in PBMC after the PVR are shown in **Figure 4**. The lower expression of GRK2 and β2-AR observed in PBMC from PR patients respect to controls significantly increases after PVR (**Figure 4** and **Supplementary Table S4**). No significant differences were found in the mRNA levels of β1-AR, GRK3 and GRK5 between PR patients before and after PVR (**Figure 4** and **Supplementary Table S4**).

Supplementary Figure S2 show the analysis of results obtained before and after PVR for each patient included in the study. It is interesting that the increase in the expression of GRK2 and β2-ARs observed after PVR, implies the recovery of normal values not statistically different from those observed in the group of healthy subjects (**Table 4**).

Significant Correlation Between mRNA Levels of b2-ARs and GRK2 in PBMC From Healthy Volunteers and Patients With Pulmonary Regurgitation

A linear regression analysis was performed between the mRNA values of β1-AR and β2-AR and the three GRKs isoforms expressed in PBMC obtained from healthy subjects (control group) and patients with pulmonary regurgitation (**Supplementary Table S5**).

A significant correlation was observed between β2-AR and GRK2 expression in healthy volunteers and in patients with PR. The same correlation was observed in this subgroup of patients after the chirurgical intervention. No correlation was observed between β1-ARs and GRK2 in any group studied. **Figure 5** provides data on the relationship of mRNA expression of β1-ARs or β2-ARs and GRK2 in each subgroup of subjects.

Supplementary Table S5 includes a more complete description of the correlations obtained for the other GRKs isoforms but the more consistent observation is related to that described in **Figure 5** and suggest the existence of a common regulatory mechanism for both genes as previously proposed (Montó et al., 2015).

TABLE 3 | mRNA levels for β-adrenoceptors and G-protein coupled receptor kinases GRK2, GRK3 and GRK5 in peripheral blood mononuclear cells from patients with pulmonary regurgitation under treatment ($n = 9$) or not ($n = 14$) with betablockers.

mRNA ($2^{-\Delta Ct}$)	Beta-blockers	No beta-blockers	Effect size (confidence interval)
β1-adrenoceptor	20.23 (22.58)	16.12 (13.99)	−4.11 (−19.89 to 11.66)
β2-adrenoceptor	506.07 (350.40)	518.76 (199.67)	12.69 (−224.82 to 250.19)
GRK2	326.35 (287.40)	617.14 (372.74)	290.79 (−13.74 to 595.32)
GRK3	193.46 (128.13)	196.22 (121.07)	2.76 (−107.25 to 112.76)
GRK5	311.69 (258.1)	334.39 (263.59)	22.70 (−223.32 to 268.72)

Values are expressed as 2^{-dCt} vs. GAPDH as reference gene and represented mean ± SD and the effect size with its corresponding confidence interval.

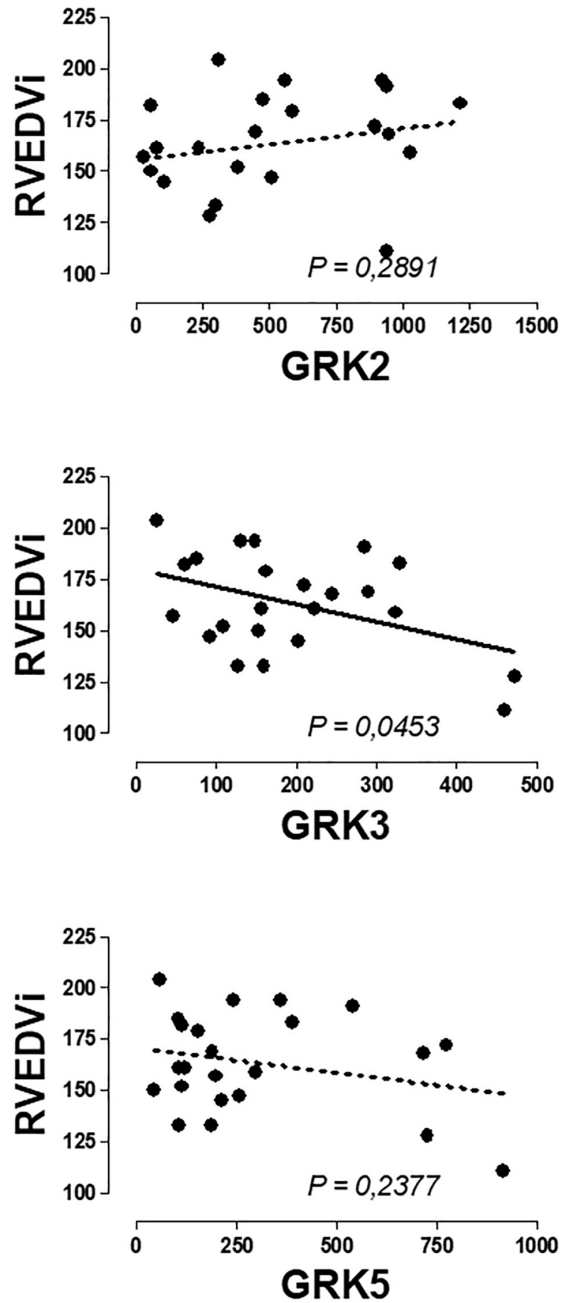

FIGURE 3 | Graphical representation of the linear regression observed between the genic expression of GRK3 in the peripheral blood mononuclear cells of patients with pulmonary regurgitation. Significant linear regression and Pearson's correlation if $P < 0.05$.

DISCUSSION

Pulmonary regurgitation (PR) is a frequent and important consequence of the surgery performed in patients with Tetralogy of Fallot and pulmonary stenosis. Right ventricle is subject to chronic volume overload due to the presence of PR and it leads to its dilatation and dysfunction, associating arrhythmias, exercise intolerance, HF and death. PVR is indicated in symptomatic patients but this indication is still under debate in asymptomatic patients. Therefore, new parameters that identify the evolution of patients are being studied (Geva et al., 2018). At the time, as shown in the guidelines (Warnes et al., 2008; Baumgartner et al., 2010), assessment data of the right ventricle, such as end-diastolic volume, have been important for the PVR indication, but currently other data such as patient age, end-systolic volume and RV function seem more important (Frigiola et al., 2008; Geva et al., 2018). To expand the knowledge of this pathology, and as a useful tool to decide clinical interventions, it could be interesting to obtain a molecular marker that would orientate about the adequate time to PVR.

β1- and β2-ARs are expressed in PBMC, as well as three GRK isoforms: GRK2, GRK3 and GRK5 (Agüero et al., 2009; Oliver et al., 2010; Montó et al., 2015). The low mRNA level of β1-AR implies a poor protein expression and function of this subtype, being the β2-AR the main responsible of the adrenergic modulation of PBMC (Sanders, 2012). Alterations in the expression of β-adrenoceptors and GRKs have been observed in PBMC of patients with cardiovascular diseases such as hypertension or HF (Iaccarino et al., 2005; Penela et al., 2006; Vinge et al., 2007; Agüero et al., 2008, 2012; Oliver et al., 2010; Rengo et al., 2011; Santulli et al., 2011; Montó et al., 2015; Sato et al., 2015), and, in particular, GRK2 has been proposed as a biomarker for the evolution of the HF (Rengo et al., 2016). These alterations vary according to the origin (Montó et al., 2012), stage of the disease (Agüero et al., 2012), or use of betablockers (Leineweber et al., 2005). There is previous evidence that some changes in the heart are reproduced in PBMC (Brodde et al., 1986; Iaccarino et al., 2005; Oyama et al., 2005), and this would allow the use of PBMC as a mirror of cardiac changes. However, there is not always a concordance in the changes produced in heart and PBMC. So, high levels of GRK2 (Penela et al., 2006; Montó et al., 2015) and β2-AR (Montó et al., 2015) in myocardium of patients with dilated cardiomyopathy have been described, but our previous results indicate that the expression of GRK2 and β2-ARs decreases in PBMC of the same patients (Montó et al., 2015). However, the utility as biomarkers of β2-AR and/or GRK2 expression in PBMC could be the same if a direct relationship to pathology was established.

We have shown that β2-AR and GRK2 expression decrease in PBMC from patients with severe PR vs. healthy volunteers, as occurs in HF patients (Rodríguez-Serrano et al., 2018). Therefore, the expression of both genes in PBMC could be an interesting marker of the evolution of the PR patients. For this reason, in the present study, we analyze the mRNA levels of the β-adrenoceptors (β1 and β2), and the three GRKs (GRK2, GRK3, and GRK5) present in PBMC from symptomatic (NHYA $\geq$ 2 class) and asymptomatic patients (NHYA 1 class) with PR, before and after PVR, in order to determine its utility as clinical biomarkers.

Of the five genes studied, a markedly lower expression of GRK2, and β2-ARs in PBMC was found in the group of patients

with PR vs. controls, as we described previously (Rodríguez-Serrano et al., 2018), together to a slight decrease in the GRK3 expression. However, the more interesting contribution of the present work is that the reduced expression of GRK2 and β2-ARs was found not only in symptomatic patients (NHYA $\geq$ 2 class) but also in asymptomatic patients (NHYA 1 class). Then, the decrease in the mRNA levels of β2-AR or GRK2 in PBMC could precede the clinical symptomatology and could alert about a deteriorated situation that other clinical parameters does not evidence. This could mean that, although the parameters used for the surgical indication are RV volume and function values, the points that are marked to indicate surgery may be late and the myocardial damage was already established before changes in these clinical variables.

Previous studies showed that betablockers up-regulate β-AR density in a β-AR subtype-selective manner, whereas they cause down-regulation of GRK2 (Iaccarino et al., 1998; Leineweber et al., 2005). However, this effect depends on the betablocker used, i.e., carvedilol exhibit a peculiar behavior decreasing β-AR density (Iaccarino et al., 1998). In our group of PR patients, a similar expression of β2-AR and GRK2 was observed independently of betablockers treatment, which excludes the possible bias due to the pharmacological treatment.

We performed an analysis of the possible correlation between PBMC genic expression of β-AR and GRKs and clinical variables characteristics of the pathology, but no significant correlation was observed between β2-AR and GRK2 expression and any of the variables studied, confirming that changes in the expression precedes and did not follow clinical symptoms. Therefore, the expression of these genes could be used as biomarker to detect cardiac damage even when this damage was not clinically very evident. An interesting result, not directly related to the main objective of the work, was the fact that a significant inverse correlation was found between GRK3 expressed in PBMCs and the right ventricular end diastolic volume indexed (RVEDVi) of patients with PR, adding more information to previous findings supporting a protective role of GRK3 expressed in human heart or PBMC in the cardiovascular system (Vinge et al., 2007; Oliver et al., 2010; Montó et al., 2012). Focus on the expression of GRK3 in heart and PBMC and its relationship to cardiac damage could be an interesting topic for future research and could contribute to a better understanding of the position of the GRK3 gene in a locus on a chromosome associated with left ventricular mass and contractility (Arnett et al., 2001).

At present, we do not know the mechanisms that leads to changes in the expression of β2-AR and GRK2 in PBMC from PR patients, and a similar decrease was found in PBMC from HF patients, which reverts after cardiac transplantation (Montó et al., 2015), confirming its relationship to the evolution of the pathology. For this reason, the objective of our study was to analyze the expression of β2-AR and GRK2 in PBMC of PR patients after PVR.

We observed significant differences in the expression of GRK2 and β2-AR in PBMC from PR patients before and after the PVR. In fact, PVR restored normal expression levels indicating a reversibility of the pathological situation involved in these changes.

It is interesting to note that a common pattern of changes between β2-AR and GRK2 genes has been previously described by us in different human and murine tissues (Montó et al., 2015) and the same occurs in PBMC from PR patients, where β2-AR and GRK2 expression decreases, whereas not significant changes were found in β1-AR or the other two GRKs studied (GRK3 and GRK5). In fact, genic expression of GRK2 and β2-AR in PBMC follow a common pattern of changes and a significant correlation was found between the mRNA levels of these genes in the different subgroups of patients studied (healthy volonteers and PR patients before and after PVR). This observation confirms the existence of a common regulatory mechanism.

As far as we know, our study is the first to analyze the behavior of β-ARs and GRKs in patients with congenital heart disease or with right ventricle disease in a stable situation. Only, one paper has been published analyzing alterations in the expression of GRK2 and ARs in atrial myocytes of patients with congenital heart disease operated on with cardiopulmonary bypass (Oliveira et al., 2017) but, in this case, the alterations were related to the vulnerability of the myocardium to catecholamine levels in an acute surgical context. These findings, although interesting, are not comparable to the situation of our patients, since post-RVP extraction has been performed in a stable situation, at least one year after surgery.

The decreased expression of β-ARs in PBMC has been related to a chronic inflammatory response (Grisanti et al., 2018). β2-AR expressed in immune cells are required for leukocyte recruitment to the heart following acute myocardial infarction. This response could be targeted to promote reparative processes while preventing chronic inflammatory events that are detrimental to healing (Grisanti et al., 2016).

Present results indicate that in PR patients, there is a lower expression of the β2-ARs and GRK2 expressed in PBMC. As we studied a mixed population of cells (PBMC) it is possible that the decrease in β2-AR and GRK2 in PR patients was general or limited to certain subsets of these cells and/or that altered composition of the PBMC population could be responsible for the low expression found, since the level of expression of β2-ARs is not similar among them (Murray et al., 1993). In any case, this change would be related to the right ventricle damage since it was similar to that found in PBMC from HF patients with damaged left ventricle (Rodríguez-Serrano et al., 2018). Therefore, the PBMC response to cardiac injury in PR patients mimics the response in patients with severe HF despite the PR patients are a population with few symptoms.

Analyzing the changes in the clinical variables after surgery, decrease of right ventricle volume and improvement of the functional class were observed, data similar to other studies (Ferraz Cavalvanti et al., 2013). The fact that gene expression of β2-AR and GRK2 in PBMC was normalized after PVR, as occurred in patients with HF after transplantation (Montó et al., 2015), indicates that a reversal of the response was obtained when cardiac damage disappears.

PR reappears after PVR in the follow-up, so new determinations when the dysfunction of the homograft or bioprosthesis is detected could be useful for planning therapeutic measures. In some series analyzing long-term right ventricle remodeling (Hallbergson et al., 2015), data observed indicates

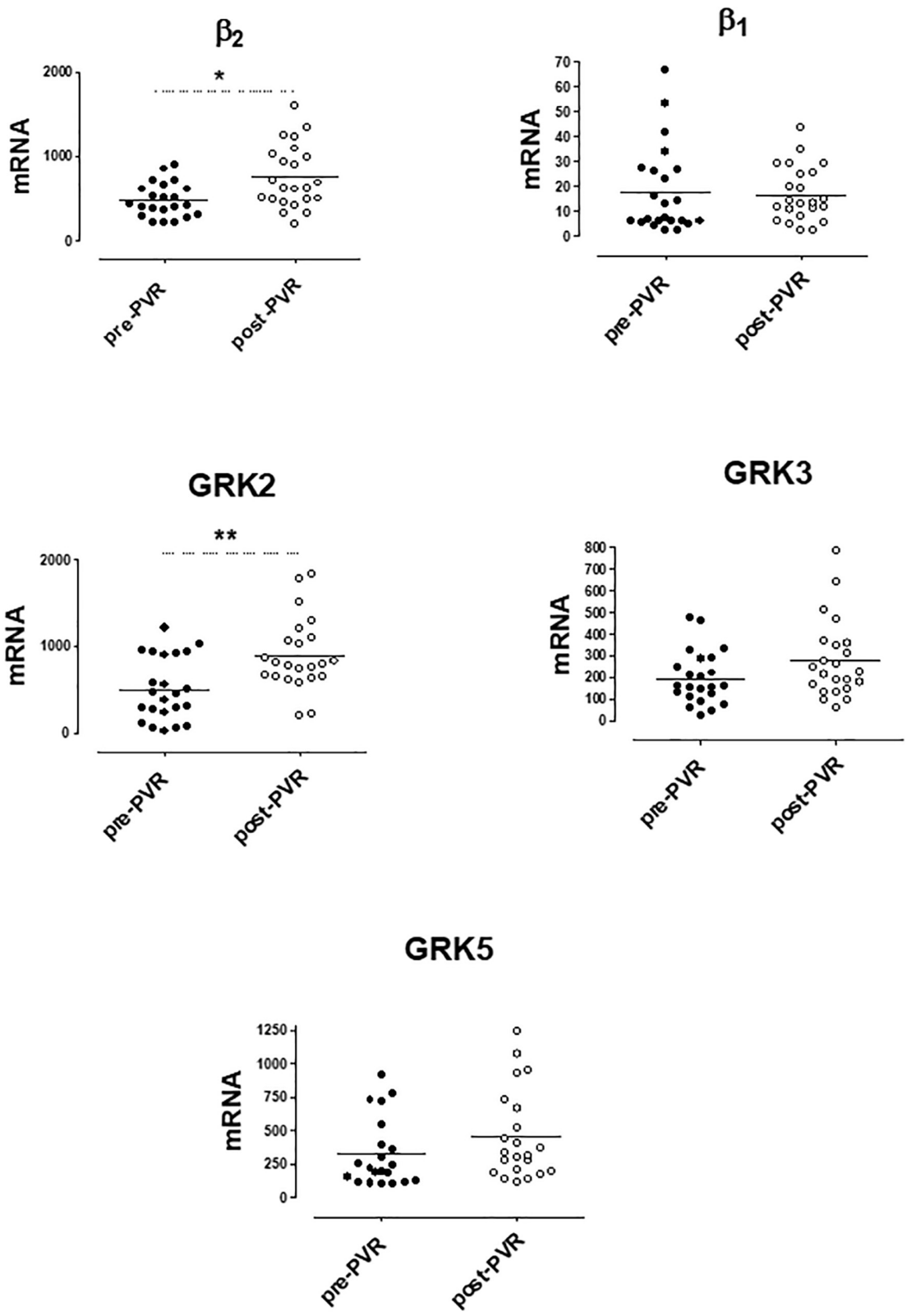

FIGURE 4 | mRNA levels for β1- and β2-adrenoceptors and G-protein coupled receptor kinases (GRK2, GRK3, and GRK5) in peripheral blood mononuclear cells from patients with pulmonary regurgitation ($n = 23$) before (pre PVR) and after (post PVR) pulmonary valve replacement. Data were calculated as 2^{-dCt} vs. GAPDH as reference gene and the mean was represented by the continuous line. $^{*}P < 0.05$; $^{**}P < 0.01$ (Paired Student's *t*-test).

TABLE 4 | Comparison of the mRNA levels of β-adrenoceptors and GRKs in PBMC from healthy volunteers (control) and patients with pulmonary regurgitation after pulmonary valve replacement (PVR)

mRNA	Control	PVR	Effect size (confidence interval)
β1-adrenoceptor	30.9 ± 37.0	16.8 ± 11.1	14.07 (−2.2 to 30.4)
β2-adrenoceptor	812.5 ± 497.2	754.8 ± 372.7	57.8 (−205.7 to 321.3)
GRK2	858.1 ± 380.3	897.8 ± 419.1	39.6 (−280.7 to 201.4)
GRK3	292.4 ± 164.2	278.8 ± 181.0	13.63 (−90.5 to 117.7)
GRK5	342.0 ± 175.5	454.6 ± 333.2	−112.7 (−285.0 to 59.7)

Values are expressed as 2^{-dCt} vs. GAPDH as reference gene and represented mean ± SD and effect size with its corresponding confidence interval

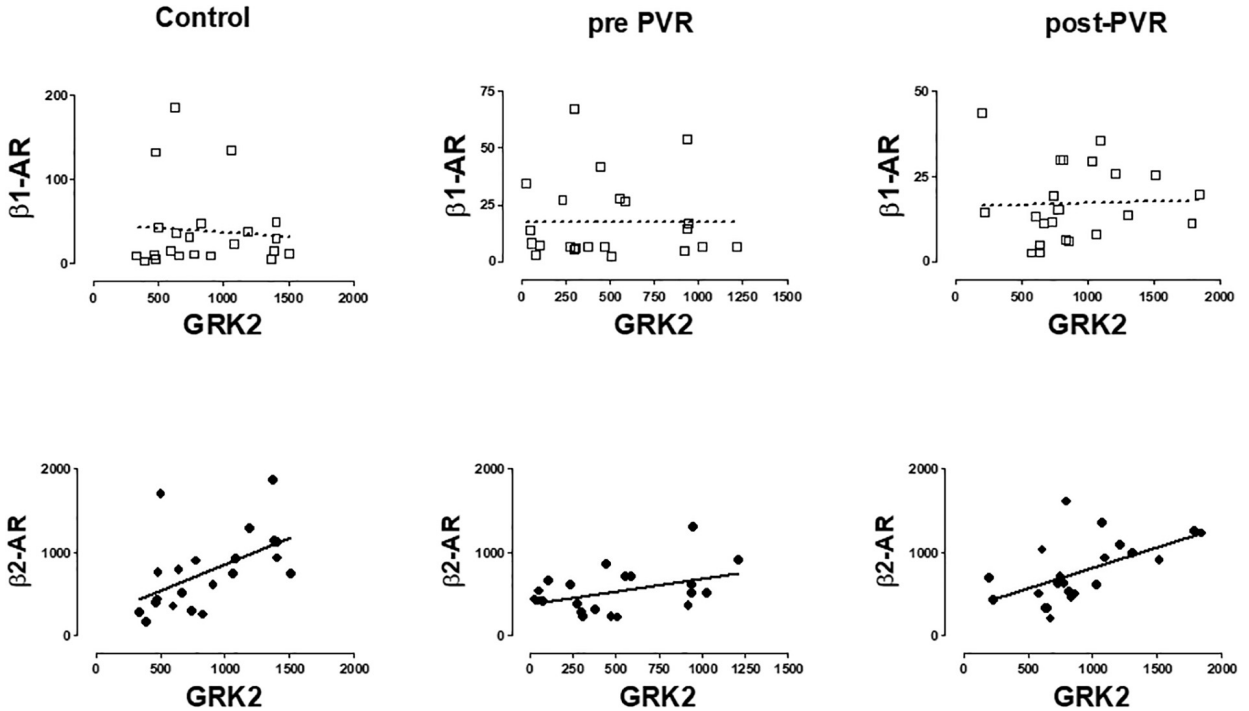

FIGURE 5 | Graphical representation of the linear regression observed between the mRNA levels of β-AR subtypes (β1, β2) and GRK2 in PBMC from healthy volunteers (control) and patients with pulmonary regurgitation before (pre-PVR) and one year after (post-PVR) pulmonary valve replacement. Significant linear regression and Pearson's correlation ($P < 0.05$) is represented as a continuous line and no significant regression ($P > 0.05$) as a discontinuous line.

that in the first two years after PVR there was a decrease in right ventricle volumes, but at 10 years after the chirurgical intervention, the volume of the right ventricle increased again to the values previous to the PVR, with a lower ejection fraction of the right ventricle, although there were no significant hemodynamic valve changes. This may indicate that ventricular remodeling, that has been caused by a chronic overload, makes the RV vulnerable and this can deteriorate again in the following.

A future multicentric study with prolonged follow-up after PVR intervention, including periodic measurements of GRK2 and/or β2-AR levels in PBMC, would be interesting to obtain prognostic data about the expression of GRK2 on PBMC as a biological marker to early detect myocardial damage in this population. At present, there are not clinical evidence that PVR improves prognostic in PR patients (Harrild et al., 2009; Ferraz Cavalvanti et al., 2013; Bokma et al., 2018). Furthermore, the protein expression has not been determined in the present work, due to the limited amount of samples. This limitation could be addressed in the future work by using flow cytometry to detect expression in the different PBMC populations.

LIMITATIONS

The sample size of the study is small and so the statistical power limited, however, the sample is similar to that presented in previous publications on patients with PR (Ferraz Cavalvanti et al., 2013) in unicentric studies. Although the prevalence of patients with congenital heart disease is increasing (Baumgartner et al., 2010), it is still a pathology of low incidence compared to other heart diseases and therefore, there is a difficulty of carrying out more patient recruitment.

An important limitation of the article is that determination of protein levels of GRK2 and β2-AR has not been made.

Post-transcriptional cellular events can modulate the translation of proteins, so it would have been interesting to see that changes in gene expression correlate with changes in protein levels, although available antibodies for β2-adrenoceptors are not enough reliable (Michel et al., 2009).

Gene expression of GRKs and β-adrenoceptors was analyzed only once after PVR. Long-term evolution of these molecular markers is unknown and they could change over time.

CONCLUSION

Symptomatic and asymptomatic patients with PR exhibit decreased mRNA levels of β2-AR and GRK2 in PBMC that does not depend on the pharmacological treatment. This decrease is similar to that found in HF patients and is restored after PVR as occurs in HF patients after cardiac transplantation. This is the first study that evaluates these changes in patients with PR before and after PVR, and provides the first proof of concept about the use of these genes as potential biomarkers. Present findings support future studies with these molecular markers in order to analyze their prognostic value and usefulness to determine clinical interventions in these patients.

AUTHOR CONTRIBUTIONS

MR-S, JR, FB, LM-D, and PD contributed conception and design of the study. JR, AO, OC, and LM-D obtained clinical data. MR-S, FB, JA, and FM performed experiments. MR-S, FB, FM, JR, JA, AO, LM-D, and PD organized the database and analyzed data. MR-S, JA, and PD performed the statistical analysis. MR-S wrote the first draft of the manuscript. FB, FM, JR, and PD wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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MINI REVIEW
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New Routes in GPCR/β-Arrestin-Driven Signaling in Cancer Progression and Metastasis

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Tumor cells acquire invasive and metastatic behavior by sensing changes in the localization and activation of signaling pathways, which in turn determine changes in actin cytoskeleton. The core-scaffold machinery associated to β-arrestin (β-arr) is a key mechanism of G-protein coupled receptors (GPCR) to achieve spatiotemporal specificity of different signaling complexes driving cancer progression. Within different cellular contexts, the scaffold proteins β-arr1 or β-arr2 may now be considered organizers of protein interaction networks involved in tumor development and metastatic dissemination. Studies have uncovered the importance of the β-arr engagement with a growing number of receptors, signaling molecules, cytoskeleton regulators, epigenetic modifiers, and transcription factors in GPCR-driven tumor promoting pathways. In many of these molecular complexes, β-arrs might provide a physical link to active dynamic cytoskeleton, permitting cancer cells to adapt and modify the tumor microenvironment to promote the metastatic spread. Given the complexity and the multidirectional β-arr-driven signaling in cancer cells, therapeutic targeting of specific GPCR/β-arr molecular mechanisms is an important avenue to explore when considering future new therapeutic options. The focus of this review is to integrate the most recent developments and exciting findings of how highly connected components of β-arr-guided molecular connections to other pathways allow precise control over multiple signaling pathways in tumor progression, revealing ways of therapeutically targeting the convergent signals in patients.

Keywords: cancer, β-arrestin, G protein-coupled receptors, cytoskeleton, motility

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INTRODUCTION

G protein-coupled receptors (GPCRs) constitute the largest family among the membrane proteins, playing an important role not only in mediating physiological function but also controlling the recruitment and activation of intracellular molecules associated with human diseases, including cancer (Lagerstrom and Schioth, 2008; Hauser et al., 2017; Insel et al., 2018). Agonist-activated GPCRs couple to heterotrimeric G proteins, thus facilitating exchange of GDP by GTP in the Gα subunits followed by dissociation from the βγ dimers and transiently interacting with specific effectors to trigger canonical signal transduction cascades. The phosphorylation of GPCRs by G protein-coupled receptor kinases (GRKs), a subfamily of AGC (protein kinase A/G/C-like) kinases originally identified as inhibitors of GPCR signaling, promotes the recruitment to the phosphorylated receptor of the cytosolic proteins β-arrestins (β-arrs), leading to GPCR desensitization (Gurevich et al., 2012).

In the classical view, GPCR signaling is mediated by G proteins, while β-arr recruitment is associated with signaling desensitization of GPCR. In this old view, the competition between G proteins

and β-arrs, β-arr1 or β-arr2, for an activated GPCR determines the signal termination, hampering further G protein signaling (Luttrell and Lefkowitz, 2002; Sente et al., 2018). Also, β-arrs promote clathrin-dependent-endocytosis of activated GPCR (Luttrell and Lefkowitz, 2002; Sente et al., 2018). One of the most important breakthroughs in GPCR field during the past decades was that the signal transduction of GPCRs is also strictly linked to β-arr. Beyond their known roles in GPCR desensitization/internalization, β-arrs have been implicated in the control of multiple outcomes, acting as multifunctional scaffold proteins and signaling transducers, crucial for intracellular signal propagation and amplification, and governing different cellular effects (Miller and Lefkowitz, 2001; DeFea, 2008; Shukla et al., 2011; Peterson and Luttrell, 2017). Almost two decades after the first evidence, GPCR field now encompasses an extensive knowledge that β-arrs integrate signals arising from GPCR with intrinsic cellular pathways in human disease, initiating waves of intracellular signaling in a G protein-independent manner and allowing the discovery of new therapies targeting selectively β-arr-mediated circuits, known as biased arrestin-biased agonism (Smith et al., 2018). Moreover, since β-arr-biased signaling requires phosphorylation of GPCRs by GRKs to promote high-affinity binding of β-arr to GPCRs and GRK subtypes might have preferential phosphorylation and trigger unique conformational changes in GPCRs, studies of β-arr-biased signaling might consider also the involvement of GRKs in cancer-related signaling pathways (Heitzler et al., 2012). In this regard, different isoforms of GRKs are able to modulate the response to many GPCRs involved in tumoral signaling *via* its direct interaction with other components of transduction cascades, as well outlined in a recent review (Nogués et al., 2018). Therefore, GRKs would also be considered critical to control the fate of β-arr-dependent signaling of GPCRs and as potential therapeutic targets in cancer.

Recent pharmacological studies on the paradigm of biased agonists, where a particular biased ligand can generate a GPCR conformation able to lead to a distinct functional outcome, usually either G-protein or β-arr-dependent signaling but not both, suggest that current GPCR-based therapeutics could be improved by increasing anticancer efficacy (Smith et al., 2018). Moreover, computational and atomic level dynamic simulation approaches provided new details linking phosphorylation of GPCR, β-arr interactions, and β-arr-dependent signaling, supporting the "barcode hypothesis," in which distinct patterns of GPCR phosphorylation trigger specific conformational states of β-arr with specific functional outcomes (Srivastava et al., 2015). In addition, remarkable advances in the GPCR structural biology field deeply demonstrated that specific ligands, by stabilizing particular sets of conformations and permitting the interaction with specific effectors, might achieve specific efficacies for selected signaling pathway (Rosenbaum et al., 2009). Recently, this conceptual framework has been refined, whereby the activated GPCR might lead the formation of a "supercomplex," where GPCR and β-arr1 form a unique signaling module with G-protein (Marshall, 2016; Thomsen et al., 2016). These findings support the hypothesis of a new way to signal, by concomitant binding of G proteins and β-arr to activated receptors, further providing an additional paradigm in GPCR-driven signaling transduction.

β-ARRESTINS AS SCAFFOLD PROTEINS IN GPCR SIGNALING

In cancer cells and in a cell context- and cancer type-dependent manner, the pools of β-arr-dependent multiprotein complexes can be found localized to different intracellular compartments, as bound to the cytoskeleton, as endocytic adapters acting on specific signalosomes in endosomes and interacting with signaling proteins involved in gene transcription, protein ubiquitination, and cytoskeletal remodeling, among others (Ma and Pei, 2007; Sobolesky and Moussa, 2013; McGovern and DeFea, 2014; Black et al., 2016; Jean-Charles et al., 2016; Rosanò and Bagnato, 2016; Chaturvedi et al., 2018; Eichel and von Zastrow, 2018; Song et al., 2018). β-arr-dependent multiprotein complexes, transducing the GPCR signals, regulate the functionality of different tyrosine kinase receptor family members and directly control cytosolic, cytoskeletal remodeling or nuclear signaling components of pathways relevant for tumor growth, invasiveness, and metastatic progression (**Figure 1**). Through these functions, both β-arrs foster a plethora of signaling pathways, including members of the mitogen-activated protein kinase (MAPK), AKT, PI3K, Wnt, Hedgehog, E3 ubiquitin ligases, PTEN, nuclear factor-kB, and regulators of small GTPase activity. To expand the intracellular communication, agonists of GPCRs can activate tyrosine kinase receptors (RTK), through a signal cross talk. This can occur *via* a mechanism by a GPCR-mediated activation of proteases operating the ectodomain shedding of a membrane bound pro-ligand, such as heparin-binding epidermal growth factor (Hb-EGF), or by the intercellular activity of GPCR-activated tyrosine kinase, completely independent of ligand binding (Rosanò and Bagnato, 2016; Crudden et al., 2018). Moreover, accumulating evidence recognizes that the transactivation of RTKs by GPCRs is not unidirectional, as the cross talk between RTKs and GPCRs is reciprocal, GPCRs can be activated by RTKs, and β-arr can be used by RTKs, as in the case of insulin-like growth factor type 1 receptor (Girnita et al., 2005, 2007; Zheng et al., 2012; Crudden et al., 2018) or platelet-derived growth factor receptors (Pyne and Pyne, 2017). In both mechanisms, it is well known that some GPCRs use β-arr to execute and transduce this cross talk between GPCRs and RTKs, governing multiple cellular processes in cancer invasion and metastasis. Proteomic studies in cancer cells demonstrated a very impressive diversity of signaling cascade molecules, which can be engaged by β-arrs for a positive or negative signaling regulation (Xiao et al., 2007; Parisis et al., 2013; Xiao and Sun, 2018), underscoring the importance of GPCR-driven β-arrs in shaping and fine-tuning signaling in cancer progression.

β-arrs are expressed in human tumors and mediate multiprotein signaling complexes, in which β-arr acts as membrane, cytosolic, or nuclear scaffold and signal transducer, culminating in multifaceted signaling processes, such as cell growth and proliferation, drug resistance, cell migration, invasion, and metastasis (Ma and Pei, 2007; Sobolesky and Moussa, 2013; Black et al., 2016; Jean-Charles et al., 2016; Rosanò and Bagnato, 2016; Chaturvedi et al., 2018; Song et al., 2018) (**Table 1**). In this review, we summarize new specific routes

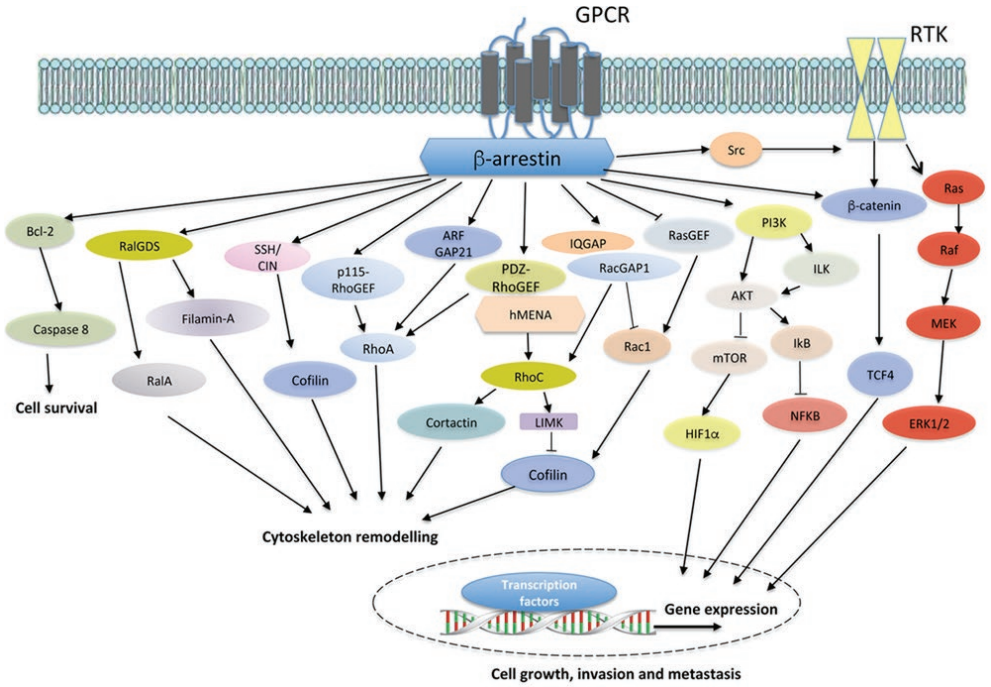

FIGURE 1 | Model of GPCR/β-arr-dependent signal pathways controlling cell survival, cytoskeleton remodeling, and gene expression, leading to enhanced cell growth, invasion and metastasis. In different cancer cells, the binding of ligands to cognate GPCRs leads to the recruitment of β-arrestin (β-arr), which might activate diverse signal-transduction pathways, including Bcl2 and downstream caspase 8. The crosstalk with receptor tyrosine kinases (RTKs), through the recruitment and activation of Src, results in downstream pathway activation, such as members of the Ras/Raf/MEK/ERK family and β-catenin/TCF4. Moreover, GPCR stimulation activates PI3K, leading to AKT/integrin-linked kinase (ILK) signaling and mTOR inhibition. Beyond the cytosolic functions, β-arr might regulate hypoxia-inducible factor 1α (HIF1α) at the levels of transcription, leading to nuclear entry and binding to hypoxia-response elements and the gene transcription. Similarly, β-arr might activate nuclear factor-κB (NF-κB) signaling *via* inhibition of NF-κB inhibitor (IκB), resulting in the dissociation and subsequent nuclear localization of active NF-κB. At the same time, the interaction of β-arr with actin regulators, such as Filamin-A and LIMK, and ser/thr phosphatases, such as SSH and CIN, leads to enhanced cofilin activity in actin cytoskeleton effects. In addition, GPCR activation might promote the interaction between β-arr and either RHO-guanine nucleotide exchange factors (RHO-GEFs), such as p115RhoGEF or PDZ-RhoGEF, or Rho GTPase-activating proteins (RhoGAPs), such as ARF-GAP21, to activate RhoA GTPase and regulate actin remodeling. β-arr can also bind RalGDS to activate RalA GTPase in cytoskeletal reorganization. Moreover, the interaction of β-arr1 with PDZ-RhoGEF and members of ENA/VASP family, hMENA, might lead to RhoC GTPase activation, causing LIMK-dependent cofilin inhibition and cortactin activation, enhancing invasive behavior. At the same time, β-arr might bind IQGAP1 and RacGAP1, leading to the suppression of Rac1 activity and favoring activation of RhoC and invadopodia functions. The inhibition of β-arr-dependent RASGFR2 activates Rac1 promoting actin polymerization through cofilin activity. β-arr acts as hub regulating several cellular processes related to cancer progression *via* its interaction with different components of transduction cascades.

TABLE 1 | β-arrestins in cancer.

Tumor	β-arr	Role in tumor progression and metastasis	References
Ovarian cancer	β-arr1/β-arr2	Chemoresistance, angiogenesis, invadopodia, invasion, EMT, metastasis	Rosanò et al., 2009, 2012, 2014; Cianfrocca et al., 2010, 2014, 2016; Semprucci et al., 2016; Chellini et al., 2018; Di Modugno et al., 2018
Lung cancer	β-arr1/β-arr2	EMT, invasion, chemoresistance	Wang et al., 2018; Tsai et al., 2019
Prostate cancer	β-arr1/β-arr2	Cell growth, migration, invasion, EMT, angiogenesis, metastasis	Ma et al., 2014; Zecchini et al., 2014; Purayil et al., 2015; Duan et al., 2016; Kong et al., 2018
Acute lymphoblastic leukemia	β-arr1	Cell propagation and senescence	Qin et al., 2014; Liu et al., 2017; Ferrandino et al., 2018
Chronic myeloid leukaemia	β-arr1/β-arr2	Tumor cells initiation and growth, stemness	Fereshteh et al., 2012; Qin et al., 2014
Colorectal cancer	β-arr1/β-arr2	Cell proliferation, apoptosis, chemoresistance, migration, invasion and metastasis	Buchanan et al., 2006; Jin et al., 2013; Cianfrocca et al., 2017; Ren et al., 2018
Gastric cancer	β-arr1	Cell proliferation	Alvarez et al., 2009
Osteosarcoma/ Ewing's sarcoma	β-arr1	Cell sensitivity, proliferation and invasion	Zheng et al., 2012; Zhang et al., 2014
Breast cancer	β-arr1/β-arr2	Cell proliferation, apoptosis, chemotaxis, invasion, invadopodia, metastasis, angiogenesis, multidrug resistance	Sun et al., 2002; Ge et al., 2004; Li et al., 2009; Zhao et al., 2009; Zajac et al., 2011; Shenoy et al., 2012; Alemayehu et al., 2013; Jing et al., 2015; Goertzen et al., 2016
Medulloblastoma	β-arr1/β-arr2	Cancer stem cells self-renewal	Miele et al., 2017; Infante et al., 2018
Renal cancer	β-arr2	Cell growth, metastasis	Masannat et al., 2018
Melanoma	β-arr1	Cell migration, vasculogenic mimicry	Spinella et al., 2013
Pancreatic cancer	β-arr2	Cell proliferation and invasion	Heinrich et al., 2012

of β-arr-mediated signaling of GPCR in cancer, focusing on invasive behavior, sustained by complex machinery that includes physical interaction with adaptor proteins.

β-ARR1 AS SCAFFOLD FOR CYTOSKELETON REMODELING IN TUMOR CELL MOTILITY

Cell motility is a complex process in which cells change shape following activation of signaling pathways that control cytoskeleton dynamics and the turnover of cell-matrix and cell-cell junctions (Friedl and Alexander, 2011; Lambert et al., 2017). The primary driving force guiding cell motility is linked to actin assembly/disassembly within cells, in which different proteins might regulate the major steps in actin remodeling, as activation of proteins breaking the existing filaments into smaller fragments to create free barbed ends and protein nucleators facilitating association of actin monomers into filaments and membrane protrusions. Some of these processes depend on environmental cues, including ligand-dependent activation of GPCR, and their spatial/temporal regulation is mediated by proteins interacting directly or indirectly with the actin and microtubule cytoskeleton elements, in proximity with signaling proteins. During tumor cell migration, chemotaxis, and metastasis, β-arrs mediate GPCR-driven effects on actin cytoskeleton remodeling by orchestrating activation and localization of selected proteins at the leading edge and generating the necessary forces for movement (Zoudilova et al., 2007, 2010; Min and Defea, 2011; Ma et al., 2014). Although the first evidence showed that β-arr-MAPK complexes control actin cytoskeletal reorganization at the leading edge during cell migration (Sun et al., 2002; Ge et al., 2004; Décaillot et al., 2011), many other studies highlighted that β-arrs function as master signalosome scaffold for specific cytoskeleton-related signaling molecules, including c-Src, filamin, cofilin, and small monomeric GTPases, to connect GPCRs to the cytoskeleton and cell shape changes (Bhattacharya et al., 2002; Barnes et al., 2005; Hunton et al., 2005; Buchanan et al., 2006; Scott et al., 2006; Zoudilova et al., 2007, 2010; Li et al., 2009; Rosanò et al., 2009; Godin et al., 2010; Min and Defea, 2011; Ma et al., 2012; Semprucci et al., 2016; Shishkin et al., 2016; Tocci et al., 2016). Cofilin is considered as one of the primary actin filament severing proteins, operating a rapidly disassembling of existing filaments and promoting generation of actin filament extension and the formation of the leading edge during chemotaxis (Shishkin et al., 2016). The function of β-arr, by interacting with cofilin and phosphatases/proteases or by mediating the activity of small GTPases, is a prerequisite for controlling phosphorylation/inactivation-dependent cofilin activity (Zoudilova et al., 2007, 2010; Min and Defea, 2011). Growing evidence demonstrated that β-arr regulates the activity of RhoA, in the family of Rho GTPases, by interacting with guanine nucleotide exchange factors (GEFs), such as p115RhoGEF and PDZ-RhoGEF, or GTPase activating protein (GAPs), such as ARFGAP21, in regulating stress fiber assembly/disassembly, or with Ral-GDS to regulate RalA for controlling membrane ruffling and cell migration, as observed for activation of cognate receptors for angiotensin AT1A (ATII1A), beta-2 adrenergic (β2AR), formyl-Met-Leu-Phe, lysophosphatidic acid (LPA), or endothelin-1 (ET-1) (Bhattacharya et al., 2002; Barnes et al., 2005; Hunton et al., 2005; Li et al., 2009; Godin et al., 2010; Ma et al., 2012; Semprucci et al., 2016; Tocci et al., 2016). Other studies showed that GPCR-dependent cytoskeletal rearrangement and membrane protrusion formation might depend on the interaction of β-arr with the actin-binding protein filamin A (Scott et al., 2006).

The dissemination of cancer cells from primary tumors and their seeding in the metastatic niche often involves the local movement of tumor cells and their penetration into surrounding tissue, which require dramatic reorganization of the cell cytoskeleton and remodeling of extracellular matrix (ECM) (Friedl and Alexander, 2011; Eddy et al., 2017; Lambert et al., 2017; Mrkonjic et al., 2017; Paterson and Courtneidge, 2018). Therefore, invading cells adopt mesenchymal, elongated morphology with focalized proteolytic activities toward ECM, by forming invadopodia (Eddy et al., 2017; Mrkonjic et al., 2017; Paterson and Courtneidge, 2018). In this context, it has been demonstrated that β-arr-related molecular complexes might govern invasive and metastatic behavior by promoting invadopodia formation. The activation of GPCRs, such as ET-1R, initiates downstream signaling cascade and formation of protein complexes mediating actin rearrangement leading to invadopodia formation and ECM degradation, where β-arr1 regulates the spatial distribution of actin cytoskeleton and actin regulators (Bagnato and Rosanò, 2016; Semprucci et al., 2016). This cascade, starting with the interaction of β-arr1 with PDZ-RhoGEF, controlling the spatial distribution of RhoC GTPase and cofilin pathway, represents a critical route by which the tumor cells form active invadopodia, and acquires maximally proinvasive capabilities (Semprucci et al., 2016). Following studies demonstrated that β-arr1-associated molecular complexes during invasive protrusions involve members of the ENA/VASP family, known to regulate the actin-based motility of various cell types, and in particular, hMENA and the invasive isoform hMENAΔv6 (Krause et al., 2003; Gertler and Condeelis, 2011; Di Modugno et al., 2012, 2018). Inputs derived from ET-1R promote the formation of a signaling platform containing β-arr1-hMENA/hMENAΔv6/PDZ-RhoGEF converging on RhoC pathway, favoring pericellular matrix degradation, and conferring also a fitness advantage to tumor cells to breach the endothelial barrier and start the transendothelial migration process (Di Modugno et al., 2018). More recently, a deep understanding of how protein complexes are assembled into a functional unit to enhance specific signaling pathways revealed a new mechanistic link between β-arr1 and the integrin-related protein IQ-domain GTPase-activating protein 1 (IQGAP1), downstream of ET-1R signaling, in shaping cytoskeleton remodeling and invadopodia-dependent ECM degradation (Chellini et al., 2018). Specifically, IQGAP1/β-arr1 acts as small GTPase scaffolding platform, as RacGAP1, to promote Rac1 inhibition and concomitant RhoA,C activation, suggesting that ET-1R-guided β-arr1 interactions determine the convergence and activation/inhibition of specific signals for invadopodia, such as Rho GTPases, where IQGAP1 helps to define the discrete locations and/or time (Chellini et al., 2018). In line with these

findings, the activation of the GPCR kisspeptin receptor promotes invadopodia formation in human breast cancer cells *via* β-arr2/ERK (Goertzen et al., 2016). Concordantly, new findings demonstrated a functional link between the tumor suppressor PTEN, scaffolding function of β-arr1, ARHGAP21 and cytoskeletal rearrangements, in driving evolution of 3D morphology phenotypes mimicking colorectal cancer in early step of metastatization (Jagan et al., 2013; Javadi et al., 2017). These new findings disclose so far an unexpected role of β-arr capable to rewire the GPCR signaling networks and activate specific machinery for changing shape, generating invasive protrusions, and remodeling ECM in invasive and metastatic cancer cells, and update the signaling paradigm that targeting GPCR/β-arr1 pathways can represent a possible route of therapeutic intervention (Rosanò et al., 2013; Bagnato and Rosanò, 2016; Goertzen et al., 2016; Semprucci et al., 2016; Chellini et al., 2018; Di Modugno et al., 2018).

NUCLEAR FUNCTION OF β-ARR1 IN CANCER

Among the non-canonical functions of β-arr, many studies demonstrated that nuclear β-arr1 might generate coordinated transcriptional responses to environmental changes, uncovering additional functions of β-arr1 in tumor progression (Kang et al., 2005; Shi et al., 2007; Hoeppner et al., 2012; Yang et al., 2015). To dissect a genomic landscape of β-arr1 in cancer and find direct transcriptional targets, an integrated whole-genome ChIP-Seq analysis and gene expression profiling have been performed in prostate cancer cells exposed to pseudohypoxia, a condition that mimics hypoxia that is frequently encountered within solid tumors. The results of this study revealed a partial overlap between β-arr1 and p300 acetyltransferase-binding sites in the same gene-proximal regions and the presence of non-overlapping sites, suggesting a double-hedged sword of β-arr1 in modulating gene, dependently or independently of p300 (Zecchini et al., 2014). A functional analysis of β-arr1 transcriptome also revealed an enrichment of genes involved in cellular metabolism and the cell cycle, with an overlap with the hypoxia-induced factor-1α (HIF-1α) transcriptome, including known HIF1α target genes involved in angiogenesis and aerobic glycolysis (Shenoy et al., 2012; Zecchini et al., 2014). Concordantly, in ovarian cancer, the activation of ET-1R, by mimicking hypoxia, promotes the interaction between β-arr1/p300 and HIF-1α, enhancing the transcription of genes, such as ET-1 and VEGF, required for tumor cell invasion and proangiogenic effects, operating a self-amplifying HIF-1α-mediated transcription of genes that sustain metastatic process (Cianfrocca et al., 2016). The findings further supporting the nuclear role of β-arr1/p300 in maintaining a more aggressive phenotype demonstrated the interplay with Wnt/β-catenin signaling (Chen et al., 2001; Bryja et al., 2007; Bonnans et al., 2012; Rosanò et al., 2013, 2014; Duan et al., 2016). Downstream of ET-1R activation, β-arr1/p300/β-catenin pathway, also represents a novel bypass mechanism through which this receptor is linked to chemoresistance, cancer stem cells like phenotype, and metastatic behavior (Rosanò et al., 2014). In both androgen-dependent and castration-resistant prostate cancer cells, β-arr1 enhances the binding of androgen receptor (AR) to androgen response elements, favoring cell proliferation, growth, and invasion, as well as *in vivo* tumor formation, local invasion, and distant metastasis (Purayil et al., 2015). Moreover, nuclear β-arr1 suppresses RasGRF2 gene expression through promoter hypermethylation, with consequent controlling of Rac1/cofilin pathways (Ma et al., 2014). An important interplay between nuclear β-arr1 and E2F transcription factor has been demonstrated in non-small cell lung cancers, contributing to the growth and progression of this tumor (Dasgupta et al., 2011; Perumal et al., 2014; Pillai et al., 2015). In myeloid leukemia where β-arr mediates the initiation and maintenance of tumor cells (Fereshteh et al., 2012; Kotula et al., 2014), the interaction of β-arr1 with the DNA-binding Enhancer of Zeste Homologue 2 (EZH2) protein mediates BCR/ABL histone acetylation during tumor progression (Qin et al., 2014). In addition, it has been proved that the self-renewal ability of the leukemia initiating cell-enriched subpopulation is linked to the ability of β-arr1 to promote the activity of the DNA methyltransferase 1 on PTEN promoter region, thus reducing the expression of PTEN (Shu et al., 2015). At the same time, nuclear β-arr1 represses the senescence of leukemic cells by interaction with hTERT, thus enhancing telomerase activity and telomere length (Liu et al., 2017), providing novel insights into the β-arr1-mediated regulation of leukemic cells. In Sonic Hedgehog medulloblastoma, where aberrant Sonic Hedgehog/Gli (Hh/Gli) signaling pathway is a critical regulator of tumor initiation and progression, β-arr1 promotes p300-mediated acetylation of Gli1 inhibiting its function, acting as negative regulators of self-renewal (Miele et al., 2017). All these findings, and in particular the specific contributions of β-arr1 for acetylation/methylation mechanisms or interactions with transcriptional factors or regulators, establish a new paradigm in multimodality of β-arr1 in controlling gene expression in cancer. However, their integration will have to be complemented with other studies in specific tumors and cell types, occurring during tumor development and metastasis. Future research will need to address whether similar mechanisms might occur for other GPCRs and open new ways to understand new nuclear interactions of β-arr1 in cancer and to obtain the effective knowledge of how β-arr1 is complicit in the epigenetic control of cancer progression.

ROLE OF β-ARR2 IN CANCER PROGRESSION

Although β-arr1 and β-arr2 show high degree of sequence and structural similarity and functional overlap (Srivastava et al., 2015), emerging evidences establish an involvement of β-arr2 in cancer growth and progression, with contradictory results. Previous studies demonstrated that β-arr2 depletion promoted tumor growth and angiogenesis in a murine model of lung cancer (Raghuwanshi et al., 2008) and that low expression of β-arr2 is significantly associated with aggressive pathologic features and is predictive of poor patient prognosis, as observed in lung and hepatocellular carcinoma (Sun et al.,

2016; Cong et al., 2017). In prostate cancer, β-arr2 inhibits cell viability and proliferation by downregulation of FOXO1 and represses AR signaling, and AR expression/activity negatively correlates with β-arr2 expression (Lakshmikanthan et al., 2009; Duan et al., 2015). By contrast, other results are consistent with the idea that β-arr2 action provides a supportive role in the development of human tumors, and β-arr2 is overexpressed in different human tumors, including breast and renal cell carcinoma, correlating with advanced stage and decreased patient survival, and mediates different tumor-promoting effects, such as cell migration and invasion (Sun et al., 2002; Ge et al., 2004; Alemayehu et al., 2013; Masannat et al., 2018). In both myeloid leukemia and ovarian cancer cells, the cross-signaling between β-arr2 and Wnt controls cell proliferation and metastasis through the interaction with c-Src followed by EGFR transactivation (Luttrell et al., 1999; Rosanò et al., 2009). The interaction of β-arr2 with c-Src is also implicated in regulating cell cycle progression and metastatic tumor growth in mice and further expanding the role of β-arr2 (Zhang et al., 2011). Very recently, a new role of β-arr2 has been linked to Hh signaling and medulloblastoma tumorigenesis, controlling SuFu-Gli3 complex, as a major control node in Hh signaling. In particular, it has been demonstrated that in the absence of Hh signaling the interaction of β-arr2 with the E3 ligase Itch and Suppressor of Fused (SuFu), a tumour suppressor gene, promotes the processing of Gli3 transcription factor into a cleaved repressor form, GLI3R, unveiling a new role of β-arr2 in controlling the immunosuppressive function of SuFu and maintaining the signaling off in the absence of ligand (Infante et al., 2018). These results further point out that function specialization of β-arr isoforms might exist in cancer, implying that different roles of β-arr2 function may be cell context- and cancer type-dependent, and that many other studies are needed to fully understand the mechanisms underlying the role of β-arr2 in cancer.

FUTURE EXPLORATION OF TARGETING CONVERGENT GPCR/β-ARR-DEPENDENT MECHANISMS IN CANCER

The role of β-arr-mediated network in executing the functional consequences of GPCR signaling in tumor cells is providing new insight into the mechanisms that underlie cell migration and the acquisition of invasive traits. Besides the complexity of GRK-mediated signaling that could allow compensatory networks to strengthen cancer progression suggesting the feasibility of therapeutic strategies using GRK inhibitors (Nogués et al., 2017, 2018), the central theme that has emerged from this review is the critical role of GPCR/β-arr-driven signaling in rewiring the complex signaling network sustaining cancer progression. This indicates that targeting GPCR/β-arr pathways might represent a new route of therapeutic intervention by developing precision medicines with tailored efficacy profiles for cancer-specific context. The existence of biased ligands that can transduce intracellular signaling from a GPCR by favoring either the G-protein or the β-arr-mediated signaling pathways strongly leads to the development of signal-biased drugs (Smith et al., 2018; Xiao and Sun, 2018). In this context, findings obtained by specifically targeting ET-1R/β-arr1-driven pathways by using small molecule ET-1R antagonist demonstrated a potential therapeutic approach for controlling metastatic progression (Rosanò et al., 2014; Cianfrocca et al., 2016, 2017; Semprucci et al., 2016; Chellini et al., 2018; Di Modugno et al., 2018). In addition to directly targeting GPCRs, emerging data suggest the possibility to selective disruption of β-arr interactions by inhibiting the linked functional responses, as an RNA aptamer targeting βarr2 and a synthetic intrabody fragment recognizing βarr, or by specifically interfering with GPCR/β-arr interaction, revealing new ways of therapeutically targeting β-arr-driven convergent signals in patients to hamper metastatic dissemination (Chaturvedi et al., 2018).

CONCLUSIONS

Recent technological advancements in structural and cell biology have provided crucial insights into the molecular mechanisms of GPCR signaling mediated by both β-arrs and G-proteins, shedding additional light on the dynamic assembly and disassembly of GPCR signaling complexes (Alvarez-Curto et al., 2016; O'Hayre et al., 2017; Eichel et al., 2018; Grundmann et al., 2018; Gurevich and Gurevich, 2018; Gutkind and Kostenis, 2018; Latorraca et al., 2018; Luttrell et al., 2018). In these new advancements, studies limited to ERK signaling by using genome editing to modulate G protein or β-arr expression and/or function suggest that β-arrs, rather than being active GPCR transducers, are critical initiator of G-protein-mediating signaling cascade, acting as "rheostat" (Gutkind and Kostenis, 2018), uncovering new role of β-arr as dictating factors of G-protein-dependent signaling activation.

Although aspects of the GPCR/β-arr signaling network had been established previously, the novelty of the recent studies highlighted in this review is the ability of β-arr to orchestrate a complex signaling network that specifically controls in a fine-tuning manner, the time, intensity, and space of GPCR-mediated signaling flow to regulate distinct steps of tumor invasion, extravasation, and metastatic spread. Looking forward, a better understanding of how the different types of GPCRs contribute to β-arr-driven signaling activation in tumor cells and by tumor microenvironment and their interaction with other surface receptors is needed. These studies should consider the role of mechanical forces imposed by the ECM and tissue microenvironment in GPCR/β-arr-mediated actin cytoskeleton remodeling and should attempt to delineate the specific contributions of different effectors (such as Rho GTPase family members) to promote cytoskeleton effects related to cell motility and invasiveness in the context of GPCR/β-arr signaling (**Figure 1**). However, remains to be learned both about how β-arr-1 mediates gene expression changes to execute the GPCR-induced pro-metastatic effects in tumor cells and about how β-arr-1/-2 and G-protein-mediated effects may differ in

this regard. The possibility that cytoplasmic and nuclear β-arrs are regulated by several cues and contribute to unexplored relevant aspects of tumor progression should also be considered. The impact of this work is likely to be substantial, given the intense interest in targeting GPCR/β-arr signaling as a therapeutic approach to inhibiting metastatic progression in cancer patients and highlighting the need for translation of preclinical insights into clinical applications. Considering the role of GPCR/β-arr-driven signaling in cancer progression and many efforts in using GPCR antagonists to dampen specifically β-arr-dependent signaling, other studies are needed not only to rewire the complexities of β-arr signaling networks and the functional effects in cancer but also to strongly improve therapeutic targeting of GPCR in cancer. In this regard, additional studies using adequate patient-derived models to further analyze the blockade of potential β-arr-dependent signaling machinery are needed to instruct treatment options in the clinic.

AUTHOR CONTRIBUTIONS

Both authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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GPCR Signaling Regulation: The Role of GRKs and Arrestins

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Every animal species expresses hundreds of different G protein-coupled receptors (GPCRs) that respond to a wide variety of external stimuli. GPCRs-driven signaling pathways are involved in pretty much every physiological function and in many pathologies. Therefore, GPCRs are targeted by about a third of clinically used drugs. The signaling of most GPCRs via G proteins is terminated by the phosphorylation of active receptor by specific kinases (GPCR kinases, or GRKs) and subsequent binding of arrestin proteins, that selectively recognize active phosphorylated receptors. In addition, GRKs and arrestins play a role in multiple signaling pathways in the cell, both GPCR-initiated and receptor-independent. Here we focus on the mechanisms of GRK- and arrestin-mediated regulation of GPCR signaling, which includes homologous desensitization and redirection of signaling to additional pathways by bound arrestins.

Keywords: GPCR, GRK, arrestin, signaling, protein engineering

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GPCR SIGNALING VIA G PROTEINS

G protein-coupled receptors are the largest family of signaling proteins. Structurally, the cores of all GPCRs are very similar: extracellular N-terminus, seven membrane-spanning α-helices (TM), and intracellular C-terminus, with variable extracellular and intracellular elements (Bockaert and Pin, 1999; Fredriksson et al., 2003). GPCRs mediate cellular response to various stimuli, from light and odorants to hormones, neurotransmitters, and even extracellular protease activity and calcium (Bockaert and Pin, 1999). GPCRs are usually localized on the plasma membrane, serving as "eyes and ears" of the cell. Accordingly, GPCRs have ligand-binding elements exposed on the extracellular side. Most receptors belong to class A that have a ligand-binding pocket between the helices, which could be either close to the extracellular surface or buried almost to half the depth of the membrane (Fredriksson et al., 2003). Even covalently attached 11-cis-retinal in the light receptor rhodopsin sits in a pocket similar to those of the receptors that bind dissociable small molecule ligands. Class B GPCRs interact with peptides. These receptors have large N-termini, which contain the high-affinity part of the ligand-binding site, with the pocket between helices constituting the lower affinity part. Class C GPCRs are dimers, consisting of two 7TM units, with ligand-binding pocket localized on a separate extracellular Venus flytrap domain homologous to bacterial proteins involved in transporting amino acids and ions (Fredriksson et al., 2003; Pin and Bettler, 2016). In class C GPCRs many allosteric modulators bind to the pocket between helices in the 7TM part, so that after the deletion of the extracellular elements the remaining heptahelical domain functions pretty much like a class A receptor binding allosteric regulators as ligands (Goudet et al., 2004). GPCR orthosteric (i.e., binding to the site natural ligand occupies) ligands fall into three categories: activating (agonists), inactivating (inverse agonists that suppress constitutive activity), and neutral (antagonists, that occupy the site but do nothing else). Most natural ligands are agonists,

but some are antagonists (e.g., agouti and agouti-related peptide are antagonists of melanocortin receptors). Covalently attached to rhodopsin 11-cis-retinal is an inverse agonist, which ensures virtually zero constitutive activity of rhodopsin in the dark, whereas a photon of light converts it into all-*trans*-retinal, which is an agonist that also remains covalently attached.

G protein-coupled receptor activation is accompanied by the outward movement of transmembrane helices V and VI, which creates a cavity on the cytoplasmic side of the heptahelical domain (Farrens et al., 1996). Activation-induced movement appears to be smaller in case of Gi-coupled GPCRs, as compared to Gs-coupled (Rasmussen et al., 2007, 2011a,b; Koehl et al., 2018; Van Eps et al., 2018). Recent structural studies showed that this cavity serves as a docking site for the heterotrimeric G proteins of all subtypes (Scheerer et al., 2008; Rasmussen et al., 2011b; Szczepek et al., 2014; Carpenter et al., 2016; Liang et al., 2017; Zhang et al., 2017; Koehl et al., 2018; Van Eps et al., 2018). Agonist-activated GPCRs act as guanyl nucleotide exchange factors for heterotrimeric G proteins. In the receptor-bound G protein its nucleotide-binding pocket opens (Oldham and Hamm, 2008; Mahoney and Sunahara, 2016), which results in the loss of GDP occupying this site in the inactive form, and binding of GTP, which is much more abundant in the cytoplasm (Traut, 1994). GTP-liganded G protein α-subunit then dissociates from the receptor and βγ-subunit, whereupon both G protein subunits bind their respective effectors. Freed active receptor can bind and activate another G protein molecule, which provides signal amplification at this level. The effectors of G proteins are either ion channels or enzymes, so that the activation of a single effector molecule induces the movement of numerous ions across plasma membrane or the conversion of many substrate molecules into product, providing further signal amplification. Among GPCR-driven systems signal amplification is the greatest in rod photoreceptors, which gives these cells single photon sensitivity (Baylor et al., 1979). However, everything the cell does costs energy. So, as soon as the cell gets the message, it makes biological sense to stop signaling. In case of GPCRs, rapid signal turnoff is accomplished by a conserved two-step mechanism: receptor phosphorylation by GRKs followed by arrestin binding (Carman and Benovic, 1998).

PHOSPHORYLATION OF ACTIVE RECEPTORS BY GRKs

Activation-induced rhodopsin phosphorylation was discovered in the early 1970s (Kühn and Dreyer, 1972; Kühn, 1974), before it became clear that there is a GPCR family to which rhodopsin belongs. Subsequent studies revealed that rhodopsin kinase binds rhodopsin-containing membranes only upon its activation by illumination (Kuhn, 1978). Later another receptor kinase, originally named β-adrenergic receptor kinase, was discovered, which specifically phosphorylated activated β2-adrenergic receptor (β2AR) (Benovic et al., 1986b). That kinase was also shown to phosphorylate rhodopsin in activation-dependent manner (Benovic et al., 1986a). The cloning and sequencing of this kinase (modern systematic name GRK2, whereas rhodopsin kinase is now called GRK1 Gurevich et al., 2012) suggested that there is a family of GRKs likely targeting different GPCRs (Benovic et al., 1989).

The key question that needed to be answered was why these kinases specifically phosphorylate active receptors, whereas other protein kinases known at the time simply recognized specific sequences within targeted proteins. This question was answered in the visual system, where by proteolysis one could eliminate the rhodopsin C-terminus with all phosphorylation sites, while leaving the rest of the rhodopsin molecule as a functional light receptor. Rhodopsin kinase was shown to be activated by physical interaction with light-activated rhodopsin, whereupon it could phosphorylate anything accessible, including exogenous peptides (Palczewski et al., 1991a). Similar activation mechanism was described for GRK2 (Chen et al., 1993). Apparently, when GRK binds a full-length GPCR, the receptor activates it. Parts of that same receptor happen to be in the vicinity and are therefore phosphorylated by the kinase. However, when the receptor is crowded, like rhodopsin in rod disk membranes, where rhodopsin molecules cover about half of the area, inactive receptors can come close by diffusion and get phosphorylated by the kinase activated by the active receptor (Binder et al., 1990; Binder et al., 1996). This high-gain phosphorylation, which is best observed when a very small fraction of rhodopsin molecules is activated, essentially confirms the mechanism of GRK activation. This mechanism, the phosphorylation of inactive pigment molecules by GRK1 activated by bleached rhodopsin, was further supported by detected phosphorylation of an inactive cone pigment co-expressed in rods with rhodopsin (Shi et al., 2005).

GRKs are soluble proteins, so they need specific mechanisms to bring them to the vicinity of membrane-embedded GPCRs. Visual GRK-1 and -7 are prenylated at their C-termini, which ensures their constitutive membrane localization. The pleckstrin homology (PH) domain of GRK2/3 binds Gβγ (Koch et al., 1993; Touhara et al., 1994). GRK2 was even crystallized in complex with Gβγ bound to its PH domain (Lodowski et al., 2003). The Gβγ binding recruits GRK2/3 to the membrane, where the receptors reside. Thus, Gβγ released after G protein activation by a GPCR helps to recruit GRK2/3 to the receptor to shut off signaling (Haga and Haga, 1992; Pitcher et al., 1992, 1995; Li et al., 2003). It has recently been reported that the dopamine D2 receptor can recruit GRK2 even without G protein activation (Pack et al., 2018), although this observation is puzzling considering that no agonist-dependent phosphorylation of the receptor was observed. Interestingly, the expression of the plekstrin homology domain of GRK2 separately from the rest of the molecule suppresses Gβ-mediated signaling in the cell (Inglese et al., 1994). The GRK4/5/6 subfamily lacks the PH domain as well as the C-terminal prenylation (Gurevich et al., 2012). Instead, the GRKs of this subfamily associate with the plasma membrane via palmitoylation of their C-terminal cysteines and/or via an amphipathic helix interacting with the membrane phospholipids (Gurevich et al., 2012 and references therein).

Interestingly, out of three branches of the GRK family, The GRK1/7, GRK2/3, and GRK/4/5/6, only the first two appear to be activated exclusively by the binding to active GPCRs. GRK4

constitutively phosphorylates the dopamine D1 receptor (Rankin et al., 2006). GRK5 was shown to be activated by phospholipids *in vitro* (Kunapuli et al., 1994) and GRK5 and closely related GRK6 phosphorylate even inactive GPCRs both *in vitro* and in live cells (Tran et al., 2004; Baameur et al., 2010; Li et al., 2015). However, the structural data on the GRK-GPCR complexes suggest that GRK1 (He et al., 2017), as well as GRK5 (He et al., 2017; Komolov et al., 2017) engage the same inter-helical cavity in active GPCRs that is part of the docking site of G proteins and arrestins. In agreement with this, engineered phosphorylation-independent arrestin-2 was shown to compete with GRK2 for the β2AR (Pan et al., 2003), indicating that the binding sites on GPCRs used by GRKs and arrestins bind overlap and include the cavity on the cytoplasmic side of GPCRs that opens upon receptor activation (Farrens et al., 1996).

Although phosphorylation of rhodopsin (Arshavsky et al., 1985) and β2AR (Sibley et al., 1986; Benovic et al., 1989) reduced signaling via G proteins, it did not stop it. So, another set of players was suspected. These players turned out to be arrestins (**Figure 1**).

ARRESTINS BLOCK G PROTEIN COUPLING

Preferential binding of arrestins to their cognate receptors when they are active and phosphorylated at the same time was demonstrated directly in case of visual arrestin-1 (Wilden et al., 1986) and non-visual arrestin-2 (Krasel et al., 2005). The role of arrestin-1 (called 48 kDa protein at the time of discovery) in preventing the coupling of phosphorylated rhodopsin to its cognate G protein, transducin, was established in mid-1980s (Wilden et al., 1986). Later is shown independently by two labs that visual arrestin-1 does that by successfully competing with transducin for the light-activated phosphorylated rhodopsin (Wilden, 1995; Krupnick et al., 1997a). The need of an arrestin-like protein in the homologous desensitization of β2AR was shown using purified receptor and GRK2 of different levels of purity. It turned out that while highly purified GRK2 phosphorylated the receptor better than partially purified preparation, it failed to significantly suppress its coupling to the cognate G protein, Gs (Benovic et al., 1987). The addition of purified visual arrestin (arrestin-1 in current systematic nomenclature) significantly enhanced the desensitizing effect of receptor phosphorylation by GRK2, which suggested that non-visual homolog of arrestin-1 might be required for homologous desensitization of the non-rhodopsin GPCRs (Benovic et al., 1987). Soon thereafter the first non-visual arrestin was cloned (Lohse et al., 1990). It was termed β-arrestin because it clearly preferred β2AR over rhodopsin (Lohse et al., 1990, 1992). The second non-visual arrestin was cloned soon after the first, and called β-arrestin2, whereas the first one was retroactively renamed β-arrestin1 (Attramadal et al., 1992). The second non-visual subtype was also cloned from human thyroid and named hTHY-ARRX (Rapoport et al., 1992). When it was cloned for the third time, a systematic arrestin nomenclature, with the number indicating the order of cloning, was proposed, which made this member of the family arrestin-3 (Sterne-Marr et al., 1993). Interestingly, only one additional arrestin, cone photoreceptor-specific arrestin-4, was found in mammals (Murakami et al., 1993; Craft et al., 1994). Thus, hundreds of GPCR subtypes expressed by most mammals (from ∼500 in dolphins and ∼800–1,200 in primates including humans to >3,400 in elephants; sevens.cbrc.jp), are served by only four arrestin proteins, two of which (arrestin-1 and -4) are specialized visual, they are expressed in photoreceptor cells in the retina and bind photopigments, leaving the two non-visual subtypes for the rest of GPCRs. The role of arrestins in terminating G protein-mediated GPCR signaling is well established (Carman and Benovic, 1998). Recent structural data revealed the molecular basis of the competition between G proteins and arrestins: both engage the same inter-helical cavity on the cytoplasmic side of the receptor (Rasmussen et al., 2011b; Kang et al., 2015; Carpenter et al., 2016; Liang et al., 2017; Zhang et al., 2017; Zhou et al., 2017), so that the binding of one precludes the binding of another. Bound G proteins readily dissociate from the receptor in the presence of GTP, whereas arrestins do not. Thus, in case of active phosphorylated GPCRs, which arrestins bind with high affinity (Gurevich and Gurevich, 2004), arrestins easily win in the competition with G proteins. However, in addition to the inter-helical cavity, which is a shared docking site of G protein and arrestins, the latter tightly bind receptor-attached phosphates that fit into positive patches on the arrestin surface (Zhou et al., 2017). This dual-site binding, predicted in 1993 based on arrestin mutagenesis (Gurevich and Benovic, 1993), creates a possibility

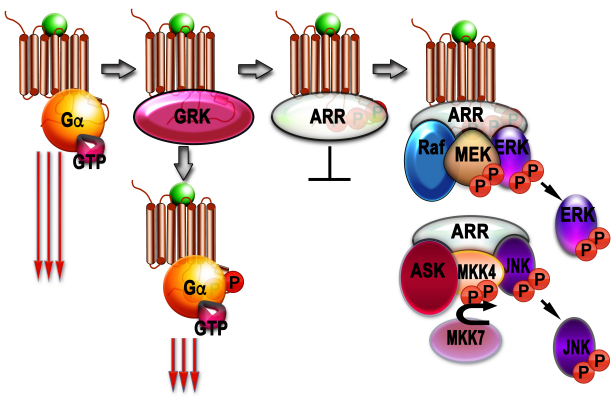

FIGURE 1 | Signaling by G protein-coupled receptors (GPCRs) and arrestins. Agonist-activated GPCRs (agonist is shown as a green ball) bind heterotrimeric G proteins, serving as GEFs: they facilitate the release of GDP bound to the α-subunit of inactive heterotrimer, which subsequently bind GTP. Then Gα subunit dissociates from the GPCR and Gβγ dimer, and both GTP-liganded α-subunit and released Gβγ activate or inhibit various signaling pathways (this signaling is shown as three long arrows). GRKs also bind agonist-activated GPCrs and phosphorylate them. This reduces G protein coupling of active GPCR (three shorter arrows), but complete blockade of G protein-mediated signaling requires arrestin binding to the active phosphorylated GPCR, where arrestins outcompete G proteins. The arrestin-receptor complex acts as a scaffold facilitating different branches of signaling (Raf-MEK-ERK cascade is shown as an example). Free arrestins in the cytoplasm also act as scaffolds, facilitating signaling independently of GPCRs (ASK-MKK4/7-JNK cascade shown as an example).

that arrestin might engage the receptor via only one site. Indeed, it was shown that, at least in case of mutant GPCRs and/or arrestins, the latter can engage solely the phosphorylated receptor C-terminus, leaving the inter-helical cavity accessible for a G protein (Kumari et al., 2016, 2017; Thomsen et al., 2016; Cahill et al., 2017). In this situation "super-complexes" that include a single GPCR simultaneously interacting with G protein and arrestin were observed (Thomsen et al., 2016). Recent study of different subtypes of neuropeptide Y receptors suggests that this mechanism might operate in case of at least some wild type GPCRs (Wanka et al., 2018). However, it appears that simultaneous arrestin interaction with both inter-helical cavity and phosphorylated parts of the receptor, which precludes G protein binding, is the rule, rather than an exception. This mode of arrestin binding is the basis of homologous GPCR desensitization, ensuring direct competition of arrestins with G proteins (Wilden, 1995; Krupnick et al., 1997a).

Both non-visual arrestin subtypes effectively bind clathrin (Goodman et al., 1996) and its adaptor AP2 (Laporte et al., 1999) via specific sites in their C-termini (Kim and Benovic, 2002), which are made more accessible by the release of the C-terminus upon GPCR binding (Zhuo et al., 2014), similar to the rhodopsin binding-induced release of the C-terminus of visual arrestin-1 (Palczewski et al., 1991b; Gurevich et al., 1994; Vishnivetskiy et al., 2002, 2010; Hanson et al., 2006). Thus, non-visual arrestins not only block receptor coupling to the G proteins, but also facilitate GPCR internalization via coated pits (reviewed in Gurevich and Gurevich, 2003), further reducing cell responsiveness. Interestingly, visual arrestin-1 does not have a clathrin-binding site (Goodman et al., 1996), although it has a relatively low affinity AP2 binding site (Moaven et al., 2013). It is likely a relic, as all arrestins apparently arose from an ancestral universal form, similar to a single arrestin in ascidian *Ciona officinalis*, which serves as visual in the eyes of its tadpole and as non-visual in the sessile blind adult (Gurevich and Gurevich, 2006), where it likely promotes GPCR internalization (Nakagawa et al., 2002).

ARRESTIN-MEDIATED SIGNALING

In addition to stopping ("arresting," hence the name) GPCR signaling via G proteins, in the last two decades arrestins have been proposed to serve as signal transducers in their own right [reviewed in Gurevich and Gurevich (2006); Hanson et al. (2006); Peterson and Luttrell (2017); **Figure 1**]. The first signaling function of arrestins was described in 1999: receptor-bound arrestins were found to promote Src-dependent activation of pro-proliferative MAP kinases ERK1/2 (Luttrell et al., 1999). Soon arrestin-3 (but not closely related arrestin-2) was found to scaffold ASK1-MKK4/7-JNK3 cascade, also in receptor-dependent manner (McDonald et al., 2000). Then both non-visual arrestins upon GPCR binding were shown to scaffold yet another three-tiered MAP kinase cascade, c-Raf1-MEK1-ERK1/2 (Luttrell et al., 2001). This finding revealed a previously unappreciated mechanism of GPCR-dependent facilitation of ERK1/2 activation. The number of non-receptor binding partners of non-visual arrestins kept increasing, culminating in a comprehensive proteomics study that described more than a hundred proteins that bind each of the non-visual subtypes, many of which are bona fide signaling proteins (Xiao et al., 2007). The results of protein knockdown using siRNAs even suggested that arrestin-mediated signaling to ERK1/2 is G protein-independent (Shenoy et al., 2006). However, numerous pathways lead to the activation of ERKs (Luttrell, 2003), and many of them, such as receptor tyrosine kinase-dependent (Marshall, 1995), are not even GPCR-driven. Recent findings indicate that in total absence of G protein activity ("zero functional G cells") due to genetic knockout of members of Gs, Gq, and G12/13 families and inactivation of Gi family members by pertussis toxin, arrestin-mediated signaling in response to GPCR activation cannot be detected (Alvarez-Curto et al., 2016; Grundmann et al., 2018). In contrast, in arrestin-2/3 knockout cells ERK1/2 phosphorylation in response to the activation of several GPCRs, which was often considered a hallmark of arrestin-mediated signaling, is similar to that in parental cells with full complement of non-visual arrestins (O'Hayre et al., 2017). These data are incompatible with the idea that arrestin-mediated signaling is G protein-independent, but do not contradict the notion that arrestin-mediated signaling actually exists, as was shown yet again by a recent study that used three independently generated arrestin-2/3 knockout cell lines (Luttrell et al., 2018). In fact, the role of arrestins in enhancing ERK1/2 activation in the presence of G proteins in case of some GPCRs was documented in the study designed to demonstrate G protein dependence of GPCR signaling (Grundmann et al., 2018).

The major aspect overlooked in virtually all studies of arrestin-mediated signaling is signal initiation (Gurevich and Gurevich, 2018). MAPK kinase activation cascades are highly conserved three-tier signaling modules consisting in general terms of upstream MAP3Ks, intermediate MAP2Ks, and downstream MAPKs. MAP3Ks and MAP2Ks activate their downstream target kinases by phosphorylating their activation loops (Tian and Harding, 2014). MAPKs ultimately phosphorylate various nuclear and cytoplasmic proteins to elicit cellular response. Scaffold proteins, such as arrestins, bring the kinases close to each other, thereby facilitating signal transduction. However, the signaling only occurs when the upstream-most MAP3Ks are activated, and arrestins were never implicated in this event. It is entirely possible that before arrestin-mediated scaffolding has a chance to facilitate signaling, MAP3Ks must be activated by arrestin-independent mechanisms, which can be G protein-dependent in case of GPCRs, or G protein- and GPCR-independent in case of growth factor receptors (Garrington and Johnson, 1999) and integrins (Stupack and Cheresh, 2002). This aspect of arrestin-mediated scaffolding needs to be studied experimentally.

GPCR-INDEPENDENT SIGNALING FUNCTIONS OF GRKs

GRKs have been reported to phosphorylate and thus regulate via phosphorylation numerous non-GPCR substrates, including receptor tyrosine kinases, single transmembrane domain

serine/threonine kinases, death receptors, toll-like receptors, transcription factors, various adapter proteins, cytosolic, nuclear and cytoskeletal proteins (for review see Gurevich et al., 2012). Although in most cases the functional role of regulation via GRK-dependent phosphorylation remains poorly understood, the mere number of targets suggests that, in addition to playing the key role in controlling the GPCR signaling, GRKs might play important roles in cell growth, attachment and motility, cell death, proliferation and survival, immunity, cancer, as well as other pathological conditions. One example of such regulation is GRK5-dependent phosphorylation of class II histone deacetylase 5 (HDAC5), which plays a role in pathological cardiac hypertrophy (Martini et al., 2008; Traynham et al., 2016). Another interesting substrate of GRK-dependent phosphorylation is the synuclein family comprising α-, β-, and γ-synucleins, small proteins with poorly defined functions. GRK2 preferentially phosphorylates α- and β-synucleins, whereas α-synuclein is the best substrate of GRK5 (Pronin et al., 2000). The great importance of α-synuclein, which is enriched in the presynaptic terminals in the nervous system, stems from its role in sporadic Parkinson's disease as the main component of Lewy bodies, a hallmark feature of the disorder, as well as from its genetic association with a familial form of the disease (Polymeropoulos et al., 1997; Benskey et al., 2016).

In addition to regulating multiple non-GPCR signaling pathways via phosphorylation, GRKs can control signaling in phosphorylation-independent manner via direct protein-protein interaction. The best studied mode of such regulation is via the function of the GRK RGS homology (RH) domain. The RH domain of GRKs 2/3 acts in a manner similar to other RGS proteins by binding active Gαq/11 (Siderovski et al., 1996). In contrast to canonical RGS proteins, RH domains of GRKs possess only weak ability to activate intrinsic GTPase of G proteins, but instead reduce the Gq/11-mediated signaling mostly by sequestering active Gαq/11 or directly blocking the receptor (Carman et al., 1999; Dhami et al., 2002; Ribeiro et al., 2009). Although all GRK isoforms are equipped with the RH domain, only the RH domain of GRKs 2/3 appears to be functional, whereas those of other GRKs seem to be unable to interact with any G protein, for they are missing the key binding residues (Carman et al., 1999; Picascia et al., 2004; Sterne-Marr et al., 2004; Lodowski et al., 2006).

GRKs 2/3 possess the PH domain in their C-terminus capable of binding Gβγ (Touhara et al., 1994). In addition to mediating the recruitment of the kinases to the plasma membrane upon receptor activation, it also regulates the Gβγ-dependent signaling in the same manner as RH domain regulates the Gq/11-mediated signaling: by sequestering Gβγ and inhibiting Gβγ-mediated signaling processes. This mechanism has been described for G protein-coupled inwardly rectifying potassium channels (GIRK) activated by adenosine A1 and μ-opioid receptors (Raveh et al., 2010) and for κ-opioid receptors (Abraham et al., 2018). GRKs are able to perform other protein binding and/or scaffolding functions (for details see Gurevich et al., 2012). For example, GRK5 biding to calmodulin assists in the nuclear translocation associated with the cardiac hypertrophy (Gold et al., 2013). Furthermore, in addition to phosphorylating HDAC5 in the nucleus, GRK5 contributes to pathological cardiac hypertrophy by activating nuclear factor of activated T cells (NFAT) transcription factor in phosphorylation-independent manner via direct binding (Hullmann et al., 2014). Thus, GRK functions requiring the kinase activity and phosphorylation-independent actions go hand in hand in physiological and pathological processes.

Some of these functions are mediated by GRK interacting proteins (GITs), which are themselves large multidomain scaffolding proteins interacting with multiple partners and playing important role in numerous cellular processes (Premont et al., 1998; Hoefen and Berk, 2006). The involvement of GRKs via their phosphorylation-independent scaffolding function in multiple signaling events in cells led to a suggestion that GRKs might, in concert with arrestins, serve as a critical node within the complex signaling network and a play a role in multiple conditions and/or pathologies, such as aging, cardiovascular and neurodegenerative disorders (Hendrickx et al., 2018).

GPCR-INDEPENDENT SIGNALING FUNCTIONS OF ARRESTINS

Arrestins also serve as signaling molecules independently of GPCRs (**Figure 1**). Arrestin-3-dependent facilitation of JNK3 activation was first shown to be independent of GPCRs as early as 2001 (Miller et al., 2001). This was later confirmed by the use of arrestin mutants that do not bind receptors (Song et al., 2009a; Breitman et al., 2012) and by reconstitution of MKK4/7-JNK3 modules with arrestin-3 using purified proteins *in vitro* in the absence of GPCRs (Zhan et al., 2011; Kook et al., 2014b). A short arrestin-3-derived peptide lacking most known receptor-binding elements was found to facilitate JNK3 activation *in vitro* and in living cells (Zhan et al., 2016). Interestingly, the measurements of the affinities of active and inactive kinases of the ASK1-MKK4/7-JNK3 cascade suggest that the activation of MKK4, MKK7, and JNK3 by phosphorylation reduces their binding to arrestin-3 (Perry et al., 2019). This reduction in binding affinity is the most striking in case of JNK3, suggesting that activated (doubly phosphorylated) JNK3 likely dissociates, freeing the place for another molecule of inactive JNK3 to bind (Perry et al., 2019). This "conveyor belt" mechanism allows the complex of arrestin-3 with upstream kinases to sequentially activate several JNK3 molecules, thereby amplifying the signal (Perry et al., 2019). It would be interesting to test whether other MAP kinase scaffolds also employ similar amplification mechanism. The critical role of caspase-cleaved arrestin-2 in programmed cell death also did not appear to depend on receptor binding (Kook et al., 2014a). Caspase cleavage of the other non-visual subtype, arrestin-3, which generates anti-apoptotic arrestin-3-(1-366) fragment (Kook et al., 2019), also does not require GPCR activation. The function of both non-visual arrestins in focal adhesion disassembly (Cleghorn et al., 2015) or in the activation of small G proteins that regulate cytoskeleton (Cleghorn et al., 2018) also did not require arrestin interactions with the receptor.

Thus, widespread belief that arrestin-mediated signaling is always GPCR-driven appears to be wrong. In fact, there are

known non-receptor partners that free arrestins, including arrestin mutants that do not interact with GPCRs, bind with higher affinity than receptor-bound (presumably "active") arrestins. These include E3 ubiquitin ligases Mdm2 (Song et al., 2006) and parkin (Ahmed et al., 2011), whereas all functional forms of arrestin-3 appear to bind JNK3 comparably (Song et al., 2006). So, the question what is the "active" conformation of arrestins and whether there are several "active" conformations facilitating different branches of signaling, needs to be answered experimentally.

POTENTIAL ROUTES OF INTERVENTION

Elucidation of every molecular mechanism of cellular functions paves the way to devising tools that affect this mechanism for therapeutic purposes. GPCR desensitization via GRKs and arrestins, as well as arrestin-dependent signaling are no exceptions. Arguably, congestive heart failure is the most studied condition where GPCR desensitization plays a role in the pathology (reviewed in Lymperopoulos et al., 2012). Heart failure manifests itself as the loss of heart responsiveness to pro-contractile stimuli. This is primarily adrenalin, which acts via β-adrenergic receptors, both β1 and β2 subtypes. Both appear to undergo excessive desensitization mediated by GRK2. When GRK2 ability to suppress β-adrenergic signaling is reduced by overexpression of GRK2 C-terminus that outcompetes endogenous full-length kinase for the G protein βγ-subunit, which targets GRK2 to the plasma membrane where β-adrenergic receptors reside, heart failure is alleviated (Tevaearai et al., 2001). This does not appear to cause many side effects, in contrast to inhibitors of ubiquitously expressed GRK2 (even if selective ones were available), that would likely indiscriminately affect the desensitization of many GPCRs in various cell types. An alternative approach that has not been tested experimentally so far would be to express in the heart one of existing arrestin mutants that binds unphosphorylated β-adrenergic receptors (Gurevich et al., 1997; Kovoor et al., 1999; Celver et al., 2002). At least one of these mutants was shown to directly compete with GRK2 for the receptor and greatly facilitate receptor recycling back to the plasma membrane (Pan et al., 2003), where it can signal.

These "enhanced" phosphorylation-independent versions of non-visual arrestins were also proposed as tools to compensate for excessive GPCR signaling in diseases associated with activating receptor mutations and/or defects in receptor phosphorylation (Stoy and Gurevich, 2015). This approach was so far tested only in the visual system, where enhanced arrestin-1 improved photoreceptor performance and survival in mouse models with defective rhodopsin phosphorylation (Song et al., 2009b; Samaranayake et al., 2018). However, for this purpose enhanced mutants of non-visual arrestins must be rendered specific for particular GPCRs to avoid their effect on the signaling of perfectly normal GPCRs co-expressed in the same cell (Gurevich and Gurevich, 2017). The first attempts to develop receptor-specific non-visual arrestins appear promising, because as few as two mutations on the receptor-binding surface yielded mutants with ∼50–60-fold preference for some GPCRs over others (Gimenez et al., 2012b). Manipulation of the receptor-binding surface of arrestins was also shown to change their selectivity for particular functional forms of the receptor (Prokop et al., 2017). However, the ability of these receptor subtype-specific forms to selectively suppress signaling by certain GPCRs without affecting others co-expressed in the same cell still remains to be tested.

Another therapeutic approach that needs to be tested is the use of signaling-biased arrestins. WT non-visual arrestins have a lot of functions: they bind hundreds of different GPCRs and dozens of non-receptor signaling proteins, affecting multiple branches of cellular signaling (Hanson et al., 2006; Peterson and Luttrell, 2017). Thus, an increase or decrease of WT arrestin expression in cells cannot serve a specific function: too many things would change. However, quite a few arrestin mutants where individual functions are suppressed or destroyed were constructed: forms that do not bind GPCRs [KNC mutants (Breitman et al., 2012; Gimenez et al., 2012a, 2014)], mutants with disabled clathrin and/or AP2 binding sites (Kim and Benovic, 2002), arrestin-3 variant that does not facilitate JNK activation (Seo et al., 2011), arrestin-2 mutants that do not promote ERK1/2 activation due to reduced binding of upstream kinases MEK1 (Meng et al., 2009) or c-Raf1 (Coffa et al., 2011). One drawback is that in some cases mutations affect not only the intended function, but others, as well. For example, arrestin-3-KNC, despite normal or even enhanced binding to the kinases of ASK1-MKK4/7-JNK3 cascade, fails to facilitate the activation of JNK3, and even acts as a silent scaffold, suppressing JNK3 activation by WT arrestin-3 (Breitman et al., 2012). It is hardly practical to test every mutant for all the functions that corresponding WT arrestin fulfills, so these mutants might have limited therapeutic usability. In contrast, monofunctional elements extracted from multi-functional arrestin proteins hold greater promise. So far two of these were constructed. Separated arrestin-2 C-terminus carrying both clathrin and AP2 binding sites, effectively outcompetes the arrestin-receptor complexes in coated pits, suppressing arrestin-dependent GPCR endocytosis in cells (Krupnick et al., 1997b). Arrestin-3 N-terminal peptide (T1A, which is only 25 residues long and lacks most receptor-binding elements) serves as a scaffold for the ASK1/MKK4/7-JNK3 cascade, facilitating JNK3 activation *in vitro* and in cells (Zhan et al., 2016). Thus, at least two arrestin functions can be manipulated independently of the others. Of course, this is just the beginning, but these findings are encouraging.

All of the examples above imply gene therapy, which is currently in its infancy. Theoretically, identification of arrestin sites responsible for the binding of any particular partner enable the search for small molecules that can bind to the site and selectively inhibit an individual interaction. This avenue also needs to be explored, although it might yield fewer useful molecules than one would expect. Protein-protein interaction sites often involve relatively flat surfaces (Gurevich and Gurevich, 2014) or disordered protein elements (Gurevich et al., 2018), both of which are notoriously hard to target with small molecules.

To summarize, selective regulation of GRKs and arrestins appears promising as a therapeutic approach. Many avenues

of this regulation, involving both conventional small molecule therapeutics and gene therapy, must be explored.

AUTHOR'S NOTE

We use systematic names of arrestin proteins, where the number after the dash indicates the order of cloning: arrestin-1 (historic names S-antigen, 48 kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin).

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VG and EG wrote the manuscript.

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REVIEW
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G Protein-Coupled Receptor Kinase 2 (GRK2) as a Potential Therapeutic Target in Cardiovascular and Metabolic Diseases

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G protein-coupled receptor kinase 2 (GRK2) is a central signaling node involved in the modulation of many G protein-coupled receptors (GPCRs) and also displaying regulatory functions in other cell signaling routes. GRK2 levels and activity have been reported to be enhanced in patients or in preclinical models of several relevant pathological situations, such as heart failure, cardiac hypertrophy, hypertension, obesity and insulin resistance conditions, or non-alcoholic fatty liver disease (NAFLD), and to contribute to disease progression by a variety of mechanisms related to its multifunctional roles. Therefore, targeting GRK2 by different strategies emerges as a potentially relevant approach to treat cardiovascular disease, obesity, type 2 diabetes, or NAFLD, pathological conditions which are frequently interconnected and present as co-morbidities.

Keywords: GRK2, GPCR, cardiovascular, obesity, NAFLD, insulin resistance, inhibitors

INTRODUCTION

G protein-coupled receptor kinases (GRKs) were originally identified as key regulators of G protein-coupled receptor (GPCR) function. GPCRs are activated by a wide variety of stimuli and comprise the largest family of membrane receptors. GPCRs are crucially involved in a multitude of physiological process, and their dysregulation contributes to many diseases, having thus become the targets of ~35% of prescription drugs (Sriram and Insel, 2018). A fundamental characteristic of GPCR biology is that agonist-stimulated receptors, in addition to trigger activation of heterotrimeric G proteins and their classical downstream effectors, are specifically phosphorylated by GRKs, which in turn promotes binding of β-arrestin proteins to GPCRs thus inhibiting further interactions with G proteins, a process termed desensitization. Moreover, the fact that β-arrestins act as scaffold for proteins of the endocytic machinery and for many other signal transduction partners leads to transient receptor internalization and to a second wave of G protein-independent GPCR signaling cascades based on β-arrestin-orchestrated signalosomes. Therefore, GRK dosage and activity is a key feature that determines the extent of GPCR desensitization and internalization and the balance between G protein- and β-arrestin-dependent branches of GPCR signaling [reviewed in (Ranjan et al., 2016; Smith and Rajagopal, 2016; Chen et al., 2018)].

The mammalian GRK family entails seven members classified into three subfamilies: visual GRKs (GRK1, also known as rhodopsin kinase, and GRK7), the GRK2 subfamily (GRK2 and GRK3), and the GRK4 subfamily (GRK4, GRK5 and GRK6). GRK2, 3, 5, and 6 are ubiquitously expressed at varying levels, whereas GRK4 displays a more restricted tissue expression pattern and GRK1 and 7 are primarily present in specific retinal cells (Ribas et al., 2007; Mushegian et al., 2012; Watari et al., 2014). Importantly, accumulating evidence indicates that GRKs can act as signal transducers by themselves, displaying a repertoire of substrates and/or interacting partners beyond GPCR and also a more general regulatory role in a variety of signaling processes (Penela et al., 2010b; Mushegian et al., 2012; Watari et al., 2014; Nogues et al., 2017).

This multifunctionality is particularly evident for GRK2, which shows a complex array of non-GPCR substrates and cellular interactors, including receptor tyrosine kinases and their downstream molecules (EGFR, PDGFR, IRS1), signal transduction kinases (p38Mapk, AMPK, PI3K/Akt, MEK1), G protein subunits and modulators (Gαq, Gβγ, phosducin, RhoaA, RalA, EPAC1), transcription factors and their regulatory proteins (Smad2/3, IκBα), cytoskeletal proteins and modulators (ezrin, tubulin, GIT1, HDAC6), ubiquitin ligases (Mdm2, Nedd4–2), or different enzymes relevant for signaling (Pin1, eNOS) [reviewed in (Penela et al., 2010b; Evron et al., 2012; Mushegian et al., 2012; Nogues et al., 2017; Mayor et al., 2018)]. Moreover, altered GRK2 levels and functionality have been found in patient samples and/or in animal models of relevant pathological situations such as heart failure, hypertension, obesity and insulin resistance-related situations, non-alcoholic fatty liver disease (NAFLD), pain, inflammation, and some types of cancer, where anomalous GRK2 dosage or activity appears to contribute to disease progression *via* cell type and context-specific molecular mechanisms (Mushegian et al., 2012; Penela et al., 2014; Gurevich et al., 2016; Hullmann et al., 2016; Cannavo and Koch, 2018; Cruces-Sande et al., 2018; Mayor et al., 2018; Nogues et al., 2018; Wang et al., 2018). Interestingly, several cardiovascular diseases as well as obesity and type 2 diabetes-related disorders, clinical conditions often interrelated as co-morbidities, converge in displaying increased GRK2 levels, pointing at the inhibition of GRK2 as an attractive therapeutic target. We summarize in this review the physiopathological roles of GRK2 in cardiovascular and metabolic diseases and focus on potential strategies to downregulate GRK2 functions based on our current knowledge about the structural features and mechanisms of regulation of this protein.

MOLECULAR MECHANISMS CONTROLLING GRK2 ACTIVATION AND FUNCTIONALITY

As the rest of the GRK isoforms, GRK2 is a multidomain protein organized in several domains and regions. The elucidation of the structure of GRK2 alone (Lodowski et al., 2005) in complex with Gβγ subunits (Lodowski et al., 2003) or with both Gβγ and Gαq subunits (Tesmer et al., 2005) and the comparison with the available structures of other GRKs (Komolov and Benovic, 2018) has provided key insights into GRK2 activation mechanisms. All GRKs are serine/threonine kinases that belong to the large AGC kinase family and share a catalytic domain displaying the characteristic bilobular fold of protein kinases, with high similarity to other AGC members, such as PKA, PKB, and PKC (Pearce et al., 2010). This catalytic core is preceded by a domain displaying homology to RGS proteins (thus termed RH domain) that, in the case of GRK2 subfamily members, has been shown to specifically interact with Gαq/11 subunits, thus blocking its interaction with their effectors (Carman et al., 1999; Sanchez-Fernandez et al., 2016). The RH domain displays at its far N-terminus a N-terminal helix (αN) characteristic of GRKs and important for receptor recognition. The C-terminal region is more variable among GRKs, but in all cases it is key for the localization to the plasma membrane. The C-terminal region of GRK2 and GRK3 contains a pleckstrin homology domain (PH) that able to interact with membrane lipids such as the phospholipid PIP2 and also with free Gβγ subunits (Homan and Tesmer, 2014; Nogues et al., 2017) (**Figure 1**).

Importantly, GRKs show mechanisms of activation that are different to those of AGC kinases. In most AGC kinases, transitions from inactive to active conformations imply phosphorylation of regulatory motifs at the activation segment/loop located in the large kinase lobe and at the hydrophobic motif found C-terminal to the small kinase lobe. Phosphorylation of these sites directs the closure of catalytic lobes and stabilizes the active conformation of the critical αC helix (Pearce et al., 2010). However, such phosphorylated regulatory motifs are absent in GRK2, and this protein thus requires conformation-induced rearrangements to become active. GRK2 activation is based on the dynamic interactions of its αN-helix and the RH and PH domains among themselves and with activating partners such as agonist-occupied GPCR, Gβγ subunits, and PIP2, eventually leading to allosteric rearrangement of the functionally relevant AST loop and kinase domain closure (Homan and Tesmer, 2014; Nogues et al., 2017; Komolov and Benovic, 2018).

The recent co-crystallization of GRK5 with the β2AR (Komolov et al., 2017) indicates that GRKs would display high structural plasticity, with large conformational changes in the GRK5 RH/catalytic domain interface upon GPCR binding. In this model, the RH domain would serve as a docking site for GPCRs and help kinase activation *via* transient contacts of the RH bundle and kinase subdomains (Komolov and Benovic, 2018). Other studies support an important role for the RH domain of GRKs in GPCR interaction (Dhami et al., 2004; Baameur et al., 2010, 2014; He et al., 2017). In addition to the RH domain, the αN-helix is also relevant to stabilize the kinase domain closure in a process regulated by GPCR binding (Singh et al., 2008; Pao et al., 2009; Boguth et al., 2010; Beautrait et al., 2014). Additionally, other motifs located within the catalytic domain of GRK2 might also participate in GPCR binding (Gan et al., 2000, 2004).

The identification of residues and regions critical for receptor docking and/or allosteric kinase activation may help understand the preferential modulation of GPCR by certain GRKs and also guide the design of inhibitory strategies. On the other

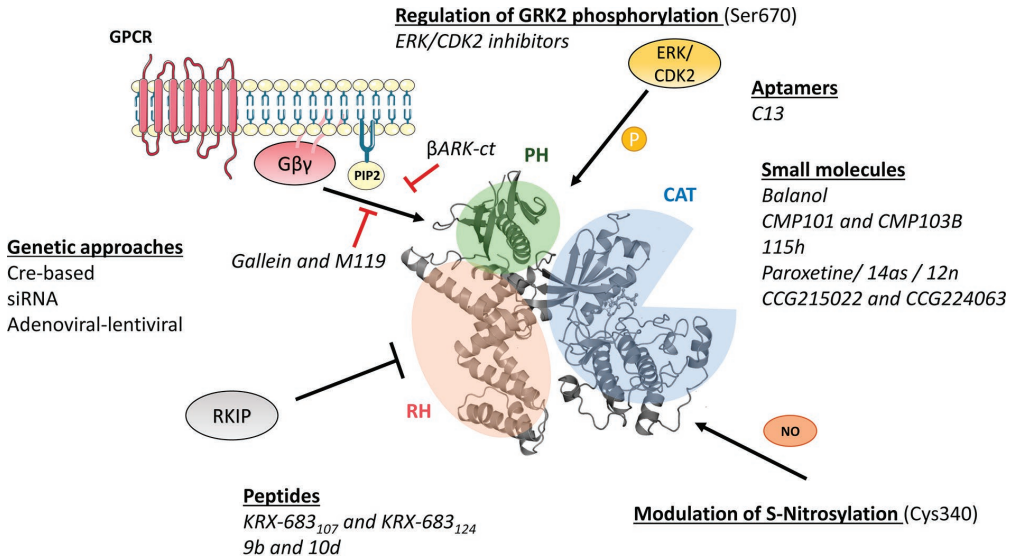

FIGURE 1 | Molecular mechanisms of GRK2 activation and functionality relevant for the design of therapeutic strategies. GRK2 dosage has been altered in different preclinical models by using global or tissue-specific Cre-based depletion methodologies, siRNA technology, and also adenoviral and lentiviral transfer of GRK2-specific silencing constructs. In addition to small molecule and aptamer compounds that able to keep the kinase in inactive conformations, other strategies to block GRK2 activation are based on the use of peptide sequences, fragments of its domains (βARKct), or small molecules (gallein, M119) in order to interfere with known GRK2 activators as GPCR and Gβγ subunits. Other strategies may be based on the interaction of GRK2 with inhibitory proteins such as RKIP, S-nitrosylation of specific residues in the catalytic domain, or *via* modulation of GRK2 phosphorylation at residues relevant for determining the substrate repertoire of GRK2. See text for details.

hand, although the current model explains how closure of the kinase domain and subsequent GRK2 activation is achieved in a GPCR and Gβγ/phospholipid-dependent manner, it is important to ascertain how GRK2 might be activated to trigger phosphorylation of the increasing and heterogeneous repertoire of non-GPCR and non-membrane GRK2 substrates.

In this regard, it has been shown that GRK2 functionality can be influenced by post-translational modifications in both the RH domain and the C-terminal region of the kinase (Penela et al., 2010a, 2014; Lafarga et al., 2012; Nogues et al., 2017). GRK2 can be phosphorylated by c-Src or EGFR on tyrosine residues located in the αN-helix (Tyr13) and within the RH region (Tyr-86 and Tyr-92), leading to enhanced catalytic activity toward both membrane receptors and soluble substrates (Sarnago et al., 1999; Penela et al., 2003; Chen et al., 2008; Sun et al., 2017), suggesting an allosteric effect on the kinase domain *via* contacts of these residues with the AST region in the kinase lobes (Beautrait et al., 2014). On the other hand, second messenger-governed kinases, such as PKA and PKC, respectively, phosphorylate GRK2 at Ser685 enhancing its ability to bind to Gβγ and the activated GPCR (Cong et al., 2001) or on Ser29 to increase GRK2 activity toward GPCR but not soluble peptides (Krasel et al., 2001).

Phosphorylation of GRK2 at Ser670 by different kinases such as ERK1/2 (Elorza et al., 2003) or CDK2 (Penela et al., 2010c) appears to play a very relevant modulatory role. This residue lies at the far C-terminus of GRK2 within the Gβγ-binding domain, and its phosphorylation impairs the interaction with Gβγ subunits, thus hindering GRK2 translocation to the plasma membrane and activity toward GPCR (Pitcher et al., 1999; Elorza et al., 2000). Strikingly, GRK2 Ser670 phosphorylation can promote changes in substrate specificity, association with partners and subcellular localization. Stimuli or context-specific ERK1/2-dependent GRK2 Ser670 phosphorylation is required to enable GRK2 to phosphorylate HDAC6 (Lafarga et al., 2012; Nogues et al., 2016), disrupts GRK2 interaction with GIT1 (Penela et al., 2008), or directs localization to the mitochondrial outer membrane *via* enhanced interaction with the chaperone Hsp90 (Chen et al., 2013). Thus, S670 phosphorylation plays a central role in the modulation of GRK2 functional features.

As for other mechanisms that able to control GRK2 activity, GRK2 can undergo S-nitrosylation of the Cys residue 340 within its core catalytic domain, obstructing kinase activity toward GPCRs (Whalen et al., 2007). On the other hand, GRK2 binds to clathrin, caveolin, or RKIP, which appear to participate in keeping GRK2 activity at bay at specific cellular locations (Gurevich et al., 2012; Schmid et al., 2015).

Finally, GRK2 expression levels are tightly regulated by a variety of mechanisms. GRK2 is rapidly degraded by the proteasome pathway in both basal and GPCR-stimulated conditions (Penela et al., 1998; Penela et al., 2003). GRK2 ubiquitination and turnover is enhanced by GPCR activation through complex mechanisms encompassing combined phosphorylation of GRK2 by c-Src and MAPK in a β-arrestin-dependent manner (Penela et al., 1998, 2001; Elorza et al., 2003), being Mdm2 a key E3 ligase involved in GRK2 proteolysis by the proteasome (Salcedo et al., 2006). While regulation of stability seems to be the main pathway

controlling GRK2 dosage, changes in mRNA transcription have also been reported in several pathological conditions, including hypertension, cardiac hypertrophy, and heart failure (Cannavo and Koch, 2018; Mayor et al., 2018). However, relatively little is known about the mechanisms governing promoter activity and transcript stability of GRK2 or regarding the participation of miRNAs in GRK2 regulation, although miR-K3, a Kaposi's sarcoma-associated herpesvirus (KSHV) miRNA, has been shown to repress GRK2 expression (Hu et al., 2015). A better knowledge of the mechanisms of modulation of GRK2 protein turnover and expression would help to design strategies to control GRK2 dosage in pathological settings.

PHYSIOPATHOLOGICAL ROLES OF GRK2

GRK2 levels and activity are reportedly increased in different tissues of patients and/or in preclinical models in cardiovascular and metabolic disease-related contexts, contributing to disease progression by a variety of mechanisms, whereas GRK2 inhibition plays a protective role (**Figure 2**).

GRK2 in Cardiac Function and Pathology

GRK2 has a relevant role in the control of cardiac function and enhanced GRK2 expression has been reported in the failing human hearts and in experimental models of heart failure (HF) in contexts of both chronic hypertensive and ischemic disease. Accordingly, genetic GRK2 deletion or pharmacological inhibition is cardioprotective in animal models recapitulating these pathological settings [reviewed in (Penela et al., 2006; Schumacher et al., 2015; de Lucia et al., 2018)].

Cardiac GRK2 mRNA and/or protein levels appear to be increased in HF patients with dilated or ischemic cardiomyopathy as a consequence of sympathetic nervous system hyperactivity in such situations. Augmented release of catecholamines is an early compensatory mechanism triggered in response to myocardial damage and dysfunction in order to maintain cardiac output *via* β-adrenergic-mediated effects in cardiac contractility. Enhanced GRK2 levels in the heart in

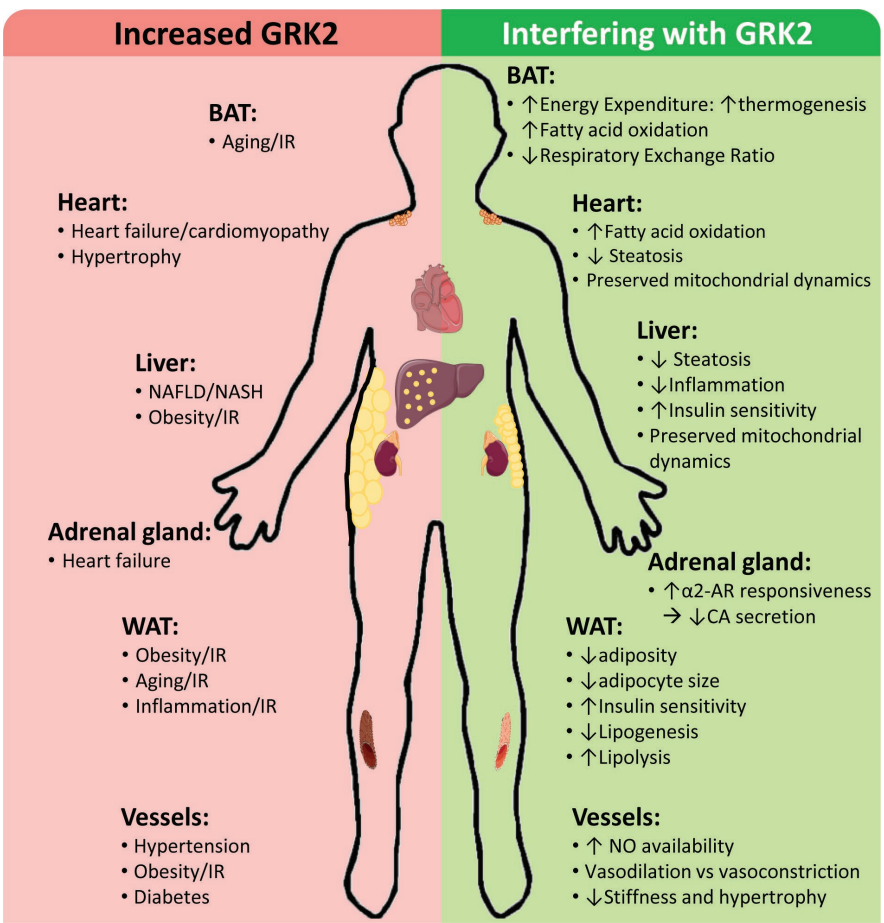

FIGURE 2 | Cardiovascular and metabolic disease-related contexts with increased GRK2 levels and effects of interfering GRK2 expression or functionality. Enhanced GRK2 levels have been reported in different tissues and cell types of patients and/or preclinical models of the indicated situations associated with cardiovascular and metabolic diseases. On the other hand, interfering GRK2 functionality in these settings in preclinical models can modulate different relevant cellular processes implicated in disease development and progression. See text for details.

such situations would initially help compensate such β-adrenergic overdrive. However, continued adrenergic stimulation and cardiac GRK2 overexpression are maladaptive, finally leading to decreased contractility and altered cardiac cell metabolism and survival (Ungerer et al., 1993; Sato et al., 2015b; de Lucia et al., 2018). Of note, in such contexts, the increased catecholamine release in adrenal glands triggers a "damaging cycle" by fostering GRK2 upregulation also in the chromaffin adrenal cells, which prevents α_2-adrenergic receptor-mediated feedback inhibition of adrenal catecholamine secretion thus perpetuating the neurohormonal overdrive and boosting GRK2- and β-adrenergic-mediated maladaptive actions (Lymperopoulos et al., 2007, 2016; Lymperopoulos, 2011).

Although such long-term maladaptive increase in GRK2 expression is well established in different experimental models, a decrease in GRK2 levels *via* proteasomal degradation has been reported in the subepicardial border and the infarct zone at 6 and 24 h after ligation of the coronary artery in dogs, and treatment with proteasome inhibitors shown to prevent GRK2 degradation and result in significant cardioprotection against malignant ventricular tachyarrhythmias (Yu et al., 2000, 2005; Huang et al., 2008). Therefore, a better knowledge of the time course of changes in cardiac GRK2 levels in pathological situations and its functional impact is required to better define the correct timing of therapeutic strategies targeting GRK2.

The functional role of GRK2 in cardiac tissue involves both modulation of GPCR and interactions with other signaling molecules. GRK2 overexpression in transgenic mice attenuates βAR signaling, whereas hemizygous GRK2 mice show an enhanced response to adrenergic agonists (Koch et al., 1995; Rockman et al., 1998). On the other hand, transverse aortic constriction and continued angiotensin II-mediated stimulation of Gq-coupled AT1 receptors cause increased GRK2 expression in the context of cardiac hypertrophy, and GRK2 has also been shown to modulate angiotensin II-mediated contraction by directly interacting with Gαq (Schumacher et al., 2016). GRK2 may also regulate the functionality of other relevant cardiac GPCRs such as GLP-1, adiponectin, k-opioid, or purinergic receptors (Heusch, 2015; Wang et al., 2015; Chen et al., 2017; Mayor et al., 2018).

In addition, GRK2 has emerged as an important modulator of cardiac insulin signaling, which is key for a balanced glucose and fatty acid metabolism in the heart and has cardioprotective features (Lucas et al., 2015; Hullmann et al., 2016; Riehle and Abel, 2016). Increased GRK2 levels worsen glucose uptake after ischemic injury *via* IRS1 phosphorylation, whereas cardiac-specific GRK2 knockout mice show enhanced heart glucose metabolism (Ciccarelli et al., 2011). Notably, a high fat diet (HFD) promotes cardiac GRK2 accumulation leading to impaired cardiac insulin sensitivity and reduced stimulation of the cardioprotective PI3K/Akt pathway, whereas this effect was prevented in 9-month-old hemizygous GRK2+/− mice, which showed a preserved insulin-dependent downstream signaling and an overall cardioprotective gene expression reprogramming (Lucas et al., 2014, 2015). Therefore, situations of neurohumoral activation and of obesity/insulin-resistance converge in promoting cardiac GRK2 upregulation, which emerges as a potential factor linking insulin-resistant pathological conditions and heart failure. In this regard, in a long-term obesity-induced cardiac remodeling model, reduced GRK2 dosage in hemizygous GRK2+/− mice preserves the activation of the PKA/CREB and AMPK pathways downstream of different cardiac GPCR, maintains the expression of key cardioprotective metabolic enzymes and mitochondrial proteins, and thus safeguards these animals from obesity-induced cardiomyocyte hypertrophy, fibrosis, and steatosis (Lucas et al., 2016). Also supporting a key role for GRK2 in insulin/GPCR pathways crosstalk, hyperinsulinemia associated to type 2-diabetes fosters β2AR phosphorylation leading to decreased βAR-regulated cardiac contractility, in a manner dependent on IRS1/IRS2, PKA, and GRK2 activity (Fu et al., 2014), this effect being potentiated upon GRK2 over expression and prevented upon GRK2 inhibition (Wang et al., 2017a).

These data point to a scenario in which GRK2 levels act as an integrating hub controlling both myocardial contractile function and cardiac metabolism. In fact, GRK2 levels appear to control such key aspects of cardiac function by molecular mechanisms additional to those already described. GRK2 localizes to cardiac mitochondrial fractions upon ERK1/2 phosphorylation in ischemic contexts, which favors GRK2/Hsp90 interaction and mitochondrial targeting. Upregulated mitochondrial GRK2 seems to be required for mitochondrial-dependent death pathway signaling and facilitates calcium-induced opening of the mitochondrial permeability transition pore (Chen et al., 2013). Moreover, the mitochondrial GRK2 pool appears to enhance superoxide levels and to diminish substrate utilization for energy production (Sato et al., 2015a), whereas enhanced kinase levels in cardiomyocytes potentiate oxidative stress and apoptosis (Theccanat et al., 2016) and contribute to impaired fatty acid uptake and oxidation (Pfleger et al., 2018). However, in other cell models, a positive role for GRK2 in mitochondrial biogenesis and ATP generation (Fusco et al., 2012) and a potential protective effect of GRK2 shuttling to the mitochondria by modulating fusion and recovery of this organelle in response to acute cell damage have been suggested (Sorriento et al., 2014; Franco et al., 2018). Therefore, more research is needed to define the role of mitochondrial GRK2 in both homeostasis and specific pathological contexts. Additionally, in transgenic models displaying increased cardiac GRK2, an impairment of the cardioprotective eNOS pathway and reduced NO bioavailability is observed in cardiac cells, thus promoting increased myocardial injury in ischemia/reperfusion mice models by mechanisms involving mutual inhibition of GRK2 and eNOS (Huang et al., 2013).

It is also worth noting that altered GRK2 levels in other cardiac cell types may contribute to heart dysfunction. An increase in GRK2 expression predominantly in endothelial cells was reported in the heart of rats 7 days after induction of myocardial infarction (Vinge et al., 2001). In addition, increased GRK2 levels in cardiac fibroblasts contribute to enhanced collagen synthesis and fibrosis and appear to favor maladaptive remodeling upon ischemia/reperfusion injury (Woodall et al., 2016), whereas inhibition of the Gβγ-GRK2 axis limits pathological myofibroblast activation after myocardial ischemia (Travers et al., 2017).

On the other hand, augmented GRK2 levels have been reported in peripheral blood samples in HF (Rengo et al., 2016) or acute myocardial infarction (Santulli et al., 2011) patients, but the potential pathophysiological impact of such altered dosage remains to be determined. Early and balanced recruitment of cells of the innate immune system (monocytes/macrophages, mast cells, and neutrophils) is key for initiating cardiac remodeling and repair after heart injury (Frangogiannis, 2014). Given the reported ability of GRK2 to regulate inflammatory responses and NFκB signaling (Sorriento et al., 2015; Steury et al., 2017) as well as chemokine and other GPCR involved in leukocyte trafficking (Grisanti et al., 2016), it is tempting to suggest that altered GRK2 levels in circulating cells may modulate the timing or intensity of the immune response triggered upon cardiac injury.

In sum, enhanced cardiac GRK2 dosage in pathological conditions is expected to simultaneously alter key pathways controlling cardiac physiopathology, including βAR and GPCR signaling, mitochondrial function, cardiac insulin signaling, and also metabolic and survival cascades or NO bioavailability, ultimately contributing to altered contractility, metabolic and energetic derangement, pathological gene expression reprogramming, maladaptive myocardial remodeling, and progression to heart failure.

These data put forward cardiac GRK2 inhibition as an attractive therapeutic strategy. In fact, cardiomyocyte-specific knockout of GRK2 in mice protects (Fan et al., 2013) injury promoted by ischemia/reperfusion while damage is increased in cardiac transgenic mice overexpressing the kinase (Brinks et al., 2010), by mechanisms reportedly related to proapoptotic signaling linked to increased GRK2 mitochondrial pools. Moreover, GRK2 deletion 10 days after myocardial infarction (Raake et al., 2008) or treatment with the GRK2 inhibitor paroxetine (see below) started 2 weeks after injury (Schumacher et al., 2015) improves cardiac function and reduces adverse ischemic remodeling in mice. On the other hand, adenoviral-mediated delivery of the C-terminal region of GRK2 (βARKct) also has a protective effect in heart failure and acute myocardial infarction settings in murine and pig models (reviewed in Sato et al., 2015b; Cannavo and Koch, 2018; de Lucia et al., 2018) by mechanisms likely involving prevention of the activation of GRK2 by Gβγ subunits (Rudomanova and Blaxall, 2017b).

A better knowledge of the interactome of GRK2 and its modulation in the different cardiac cell types, of the molecular mechanisms and stimuli leading to increased GRK2 expression in settings of cardiac hypertrophy, ischemia/reperfusion, and post-infarction remodeling, and of the detailed temporal pattern of such changes would help to fine-tune the therapeutic strategies targeting this protein.

Vasculature

Vascular tone is regulated by vasodilator and vasoconstrictor factors mainly released by endothelial cells (EC) in response to mechanical or chemical stimuli. The imbalance between these substances leads to the endothelial dysfunction and/or altered vasoconstrictor responses observed in cardiovascular diseases. Among the different systems involved in vascular dysfunction are effectors of the sympathetic nervous and renin angiotensin systems, endothelin-1, and prostanoids that can signal through specific GPCR leading to reduced NO bioavailability and endothelial dysfunction by different mechanisms (Vanhoutte, 2018). These include, among others, modulation of the expression/activity of the endothelial NO synthase (eNOS), eNOS uncoupling due to substrate or cofactor deficiency, and alterations in eNOS activation through its phosphorylation by different kinases including the PI3K/Akt pathway (Vanhoutte et al., 2016; Vanhoutte, 2018). In addition, activation of many GPCRs controls vascular smooth muscle cells (VSMC) proliferation and migration as well as extracellular matrix deposition (Althoff and Offermanns, 2015).

Changes in GRK2 levels and/or activity can mediate important effects in vascular function and structure that have been classically explained by GRK2-dependent desensitization of different GPCRs (Brinks and Eckhart, 2010) such as angiotensin II (AngII), endothelin, or adrenergic receptors. Overall, different studies describe that a partial deficiency or inhibition of GRK2 differentially alters vasoconstrictor responses to GPCR agonists (Rainbow et al., 2018), while vasodilator effects seem to be more generally increased. This apparently preferential desensitization by GRK2 of vasodilation versus vasoconstriction signaling has been invoked to explain the effect of upregulated GRK2 levels seen in human and murine hypertension (Eckhart et al., 2002; Izzo et al., 2008; Cohn et al., 2009; Santulli et al., 2013). In this line, studies in sinusoidal endothelial cells from injured livers demonstrated that Akt physically interacts with GRK2, which inhibits Akt activation and NO production (Liu et al., 2005). Thus, the increased expression of GRK2 observed in vessels from different mouse models of vascular or metabolic diseases (Taguchi et al., 2011a, 2012b) can be an underlying factor that decreases NO bioavailability and contributes to endothelial dysfunction (Lucas et al., 2015). For instance, chronic AngII treatment of C57Bl6 mice upregulates GRK2 in vessels, leads to inhibition of the Akt/eNOS pathway, and thus reduces NO production both basally and after AngII-infusion that finally results in endothelial dysfunction (Avendano et al., 2014). Interestingly, some of these differences appear to be gender-dependent (Taguchi et al., 2012a). More importantly, GRK2 inhibition or partial GRK2 deletion improved the endothelial dysfunction observed in obese/diabetic (Taguchi et al., 2011b, 2012c, 2013) or hypertensive (Avendano et al., 2014) animal models by restoring the impaired Akt/eNOS pathway and NO availability in a process in which glucose homeostasis may be implicated (Taguchi et al., 2017). Overall, these studies consolidate GRK2 as a genuine negative modulator of NO bioavailability. However, as mentioned above, recent evidence suggest that a reciprocal negative regulation exists between GRK2 and NO with increased NO bioavailability being an endogenous inhibitor of this kinase by S-nitrosylation of Cys340 of GRK2 [reviewed in (Cannavo and Koch, 2018)] pointing to a feedforward relationship between NO and GRK2. Interestingly, some recent reports have described that a more profound and global GRK2 knockdown may be detrimental

due to enhanced renin- and AT1R-mediated reactive oxygen species (ROS) production that can cause renal damage (Tutunea-Fatan et al., 2018) and the development of hypertension with age (Tutunea-Fatan et al., 2015). Together, these findings suggest that partial or local rather than generalized GRK2 targeting might be considered when increased NO levels and a correction of endothelial dysfunction need to be achieved.

Vessels from patients or animal models of obesity and metabolic syndrome frequently display hypertrophic remodeling with particular differences depending on the vascular bed (Briones et al., 2014). This vascular remodeling seems to be influenced by hemodynamic factors such as hypertension and most often can be reversed at least in part by pharmacological blockade of the renin-angiotensin-aldosterone or endothelin systems (Briones et al., 2014). The role of GRK2 in the structural and mechanical alterations in vessels is only beginning to be elucidated and might depend on the cell-specific location of GRK2 or on the experimental model. Thus, a partial overall GRK2 deletion prevents vascular hypertrophy and increased vessel stiffness observed in the AngII-induced hypertension model (Avendano et al., 2014). However, endothelial-specific depletion of GRK2 in Tie2-CRE/GRK2(fl/fl) mice leads to structural abnormalities that were reverted by a ROS scavenger (Ciccarelli et al., 2013), and an impaired angiogenic response with immature vessels was observed in these animals and in GRK2+/− mice (Rivas et al., 2013). The mechanisms responsible for these GRK2-mediated effects in vascular structure and development are not fully understood, but GRK2 and β-arrestins participate in the modulation of arterial smooth muscle purinergic signaling (Morris et al., 2011) and of agonist-stimulated VSMC migration through activation of proliferative and promigratory MAPK such as ERK1/2 (Morris et al., 2012). Conversely, a negative role for GRK2 in VSMC proliferation has also been shown (Peppel et al., 2000; Guo et al., 2009). In sum, the results presented in this section highlight a potentially novel strategy for the regulation of vascular dysfunction through GRK2 targeting.

Adipose Tissues

As a combination of different repositories, the adipose tissue can be considered a multi-depot organ located mainly in two compartments: subcutaneous and visceral (Cinti, 2005). Adipose depots can also be differentiated into white and brown adipose tissue according to their specific physiological roles and morphological appearance.

White Adipose Tissue

Classically considered as a mere energy store, the white adipose tissue (WAT) forms a large organ devoid of a specific shape and size but with a large physiological plasticity (Cinti, 2012) and important endocrine and homeostatic functions (Trayhurn and Beattie, 2001). Structural and/or functional variations in WAT together with changes of its fat load are key features correlating with metabolic alterations (Primeau et al., 2011; McLaughlin, 2012). In this regard, insulin is a critical regulator of the most important aspects of adipocyte biology. This hormone promotes triglyceride storage within WAT by promoting adipocyte differentiation and by stimulating glucose uptake and triglyceride synthesis while inhibiting lipolysis Thus, maintaining the integrity of insulin signaling is crucial for an adequate physiological role of adipocytes.

In this line, GRK2 has been unveiled as an important regulator of insulin signaling in different insulin-target tissues including WAT. In fact, GRK2 hampers insulin-mediated glucose uptake in 3T3L1 adipocytes by several mechanisms including interfering with Gαq/11 (Usui et al., 2005) and sequestering IRS1 in a process that is independent of its kinase activity (Garcia-Guerra et al., 2010). On the other hand, GRK2 can also act as a modulator of overall adiposity and fat mass accretion [reviewed in (Mayor et al., 2011)]. Aged or HFD-fed GRK2+/− mice show a decreased size of white adipocytes (Garcia-Guerra et al., 2010) and a reduced expression of enzymes involved in lipogenesis (Vila-Bedmar et al., 2012). Moreover, tamoxifen-induced GRK2 depletion during a HFD reduces adipose WAT mass and adipocyte size and increases markers of lipolysis as well as the *ex vivo* and *in vivo* lipolytic response of this tissue even after overweight and IR have already been established (Vila-Bedmar et al., 2015). Furthermore, insulin resistance, glucose levels, and GRK2 expression emerge as strongly associated variables in a homeostasis model assessment of adipose-derived stem cells obtained from lean and obese human patients (Ceperuelo-Mallafre et al., 2014). These data suggest that GRK2 can play a relevant role in the establishment of insulin-resistant states in murine and human WAT and that these states can be overturned by genetic ablation of GRK2, thus putting forward that interfering with GRK2 could be a good strategy to revert the damaging effects of a dysfunctional WAT.

Brown Adipose Tissue

Heat production through non-shivering thermogenesis occurs chiefly in the brown adipose tissue (BAT) in different organisms including humans. Therefore, BAT represents a natural target for increasing energy expenditure since thermogenesis relies on energy dissipation to maintain body temperature. This process depends on the specific expression in brown adipocytes of the uncoupling protein UCP1, a mitochondrial inner membrane protein that promotes dissipation of nutrient-derived energy in the form of heat [reviewed in (Cannon and Nedergaard, 2004)]. Therefore, although insulin regulates metabolism in both brown and white adipocytes, the role of both tissues in energy storage and utilization is quite different. Unlike WAT, BAT accumulates lipids not as a store for the excess of energy but as a source of molecules to be oxidized in mitochondria when thermogenesis is activated to produce heat (Guillen et al., 2013). Given the role of BAT as a sink for draining and oxidation of glucose and triglycerides from blood, enhancing BAT development/activity or promoting browning of WAT may contribute to a reduction in body weight and to the improvement of glucose tolerance.

GRK2 appears to play an important role in both BAT function and architecture, as well as in brown adipocyte differentiation (Vila-Bedmar et al., 2012). In keeping with this notion, the decreased age-induced weight gain observed in adult GRK2+/− mice seems to be due, at least in part, to a

preserved function of BAT in these animals. Accordingly, GRK2 hemizygous mice display higher energy expenditure and lower respiratory exchange ratio. This correlates with morphology of GRK2+/− BAT that is more consistent with an efficient thermogenic function (Vila-Bedmar et al., 2012). Moreover, decreasing GRK2 during a HFD through tamoxifen-induced genetic depletion prevents fat accumulation and increases the expression of fatty acid oxidation and thermogenic markers within BAT (Vila-Bedmar et al., 2015). Furthermore, BAT explants of these animals showed an enhanced *ex vivo* lipolytic response to the β-adrenergic agonist isoproterenol. Altogether, these data point to an increased capacity for fatty acid metabolism in mice with low GRK2 levels and might explain the absence of lipotoxicity observed in these animals despite the increased free fatty acid availability caused by enhanced WAT lipolysis. They also point toward GRK2 inhibition as a potential tool for the enhancement of brown fat activity that could have additive effects to the function of this kinase in the regulation of insulin signaling.

Liver

The liver plays a central role in the regulation of metabolic homeostasis. For instance, the synthesis of glucose from non-carbohydrate sources during nutrient deprivation or fasting occurs in the liver through gluconeogenesis. When refeeding allows glucose levels to rise, hepatic gluconeogenesis is inactivated and the liver stores ingested carbohydrates as glycogen. Insulin signals restore the normoglycemia after a meal chiefly by regulating these two processes, gluconeogenesis and glycogen synthesis, in the liver. However, in metabolic disorders, the liver develops IR leading to increased glucose output and decreased glucose clearance leading to sustained hyperglycemia that can bring about pathological consequences (Czech, 2017).

One of the most important complications related to hepatic IR is non-alcoholic fatty liver disease (NAFLD) that expands from simple steatosis to non-alcoholic steatohepatitis (NASH). NASH involves the establishment of inflammation, fibrosis, and cirrhosis in the liver, which can originate end-stage liver failure and eventually evolve into hepatocellular carcinoma (Malaguarnera et al., 2009). One major underlying cause of NASH is the development of hepatic IR, a feature that upregulates the levels of hepatic lipogenic transcription factors and underlies triglyceride accumulation in the liver thus causing hepatic steatosis (Malaguarnera et al., 2009; Smith and Adams, 2011; Cusi, 2012).

Interestingly, chronic insulin stimulation increases GRK2 levels and decreases insulin receptor expression in mouse liver FL83B cells (Shahid and Hussain, 2007). This is in accordance with the absence of an activation of glycogen synthesis following the defective phosphorylation status of IRS1 observed in these cells (Shahid and Hussain, 2007). In keeping with these results, our group has shown that GRK2 levels were increased in the liver of mice fed a HFD (Garcia-Guerra et al., 2010) or a methionine and choline-deficient diet (MCD), as a mouse model of NASH, and also in human patients diagnosed with NASH (Cruces-Sande et al., 2018). Also, Sprague-Dawley rats fed a HFD present increased hepatic plasma membrane-associated GRK2 (Charbonneau et al., 2007). Interestingly, insulin-mediated signaling is maintained in the liver of GRK2 hemizygous mice under different IR-inducing conditions (Garcia-Guerra et al., 2010). Moreover, decreasing GRK2 during a HFD by means of tamoxifen-induced genetic depletion prevents hepatic steatosis and shifts the expression of proinflammatory toward anti-inflammatory markers in the livers of these animals (Vila-Bedmar et al., 2015). These data uncover a role for GRK2 in the regulation of fat accumulation and inflammation in the liver. A very recent study also reveals that mice partially deficient for GRK2 are resistant to the development of NASH independently of obesity and IR (Cruces-Sande et al., 2018). These animals were diagnosed with simple steatosis and not NASH after an MCD feeding and present lower inflammation, a better handling of ER stress, preserved autophagy, and more active processes of mitochondrial biogenesis and dynamics after the MCD.

ORGAN CROSSTALK IN CARDIOVASCULAR AND METABOLIC DISEASES

It is worth noting that cardiovascular and metabolic diseases are often present as co-morbidities and the tissues and organs involved in these pathological situations are highly interconnected. Type-2 diabetes and obesity enhance the chance of developing heart failure independently of other risk factors (Riehle and Abel, 2016) by mechanisms involving altered systemic neurohormonal, metabolic, hormonal, and inflammatory mediators (enhanced catecholamine and angiotensin levels, hyperinsulinemia, hyperglycemia, augmented circulating free fatty acids (FFA), or altered adipokine and cytokine secretion), contributing to altered cardiac metabolism and signaling, and fostering the development of diabetic cardiomyopathy (Woodall et al., 2014; Jia et al., 2016). NAFLD has also been linked to increased risk of cardiovascular disease as a result of aberrant glucose, fatty acid and lipoprotein metabolism, oxidative stress, altered cytokine secretome, and endothelial dysfunction (Bhatia et al., 2012). In addition, emerging evidence points to additional crosstalk mechanisms from the heart to peripheral organs. The injured and remodeling heart secretes a variety of inflammatory cytokines and metabolic and lipid mediators that can in turn impact the functions of the kidney, adipose tissue, or the liver [reviewed in (Baskin et al., 2014; Fujiu et al., 2017)], thus perpetuating a pathological cycle among the heart and key metabolic tissues. The fact that different pathological triggers converge in promoting GRK2 upregulation in many of the tissues involved in such crosstalk reinforces the notion that simultaneous GRK2 inhibition in all the tissues implicated in a given pathology might have a synergic beneficial effect.

STRATEGIES TO TARGET GRK2

Genetic inhibition has been, so far, the most successful strategy to achieve a downregulation of GRK2 protein/function as a

tool to demonstrate the participation of this kinase in physiopathological processes and as a proof of concept of its potential use as a therapeutic target. A mounting amount of evidence describes the effects of GRK2 downregulation either in mouse models deficient for GRK2, tissue-specific and Cre-based inducible depletion, siRNA technology both in mice and in cultured cells, and also adenoviral and lentiviral transfer of GRK2-specific silencing or overexpression constructs. This has served to establish GRK2 as a target to tackle with important diseases, as recently reviewed elsewhere for cardiac (Cannavo et al., 2018), metabolic (Mayor et al., 2018), vascular (Schumacher and Koch, 2017), renal (Rudomanova and Blaxall, 2017b), and tumoral (Nogues et al., 2017) pathological contexts.

The genetic approaches to downregulate GRK2 levels, although not translatable to the clinical practice, can thus provide very useful information about the feasibility and potential drawbacks of GRK2 as a therapeutic target. In this regard, one particularly interesting model is the use of global hemizygous GRK2 mice, which would *bona fide* mimic the likely partial effects of pharmacological systemic inhibition of GRK2 and show the integrated effects of GRK2 inhibition in different tissues and cell types (Avendano et al., 2014; Lucas et al., 2014). On the other hand, inducible global GRK2 deletion models can help to determine whether systemic GRK2 downregulation can not only prevent but also revert certain diseases such as reported in the context of insulin resistance, overweight, and glucose (Vila-Bedmar et al., 2015). These approaches can also inform of potential disadvantages or possible side effects of GRK2 inhibition and differentiate between effects due to inhibition of kinase activity alone (by using kinase activity inhibitors as detailed below) or also to scaffold functions of this protein (only altered when decreasing protein levels). For instance, a recent publication suggests that a global GRK2 downregulation by using a silencing construct under the control of the U6 mouse polymerase III promoter may have detrimental outcomes in kidney function and development (Tutunea-Fatan et al., 2018), thus suggesting that therapeutic strategies that target GRK2 activity, not expression, could be safer in particular for the treatment of hypertension (Tutunea-Fatan et al., 2015).

More specific targeting strategies such as those reducing GRK2 expression in particular cell types can also provide useful information about possible cell type-specific drawbacks of chronic GRK2 inhibition. For instance, a reduction of GRK2 in myeloid cells increases the risk of septic shock in mouse models (Patial et al., 2011), although myeloid-specific GRK2 does not alter immune cell infiltration to the primary site of infection or bacterial clearance and does not significantly affect mortality in a cecal ligation puncture model of polymicrobial sepsis (Parvataneni et al., 2011). In microglia, GRK2-targeting can transform acute inflammatory pain into chronic hyperalgesia (Eijkelkamp et al., 2010), and decreased GRK2 levels in endothelial cells can cause reduced maturation of vessels (Rivas et al., 2013) and defects in vascular function and structure (Ciccarelli et al., 2013). In sum, although a reduction of GRK2 protein and/or activity is as a clear target for intervention in cardiovascular and metabolic pathologies, care should be taken when exploring the consequences of downregulating GRK2 in other diseases involving renal, myeloid, or endothelial cells.

In addition to genetic approaches, a variety of other potential strategies to inhibit GRK2 functions are being developed based on the structural features and mechanisms of regulation of this kinase and are described in the following sections.

Small Molecule Inhibitors of GRK2

The search for a potent and selective small molecule inhibitor of GRK2 has turned out to be a difficult quest. The first described compounds able to bind and negatively impact GRK2 activity were polyanionic and polycationic compounds such as heparin (Benovic et al., 1989). They were unable to cross the plasma membrane, but capable of inhibiting *in vitro* GRK2-dependent rhodopsin phosphorylation with IC50 values below μM.

It was not until the crystal structure of human GRK2 in complex with balanol, a fungal metabolite synthetized by *Verticillium balanoides,* was solved (Tesmer et al., 2010) that the mechanism of action of ATP-competitive inhibition of GRK2 was better elucidated and used in the search of compounds with increased selectivity for this kinase relative to other protein kinases and GRK family members [reviewed in (Guccione et al., 2016)]. The relative efficacy of balanol, as tested in *in vitro* kinase assays using tubulin and rhodopsin as substrates, showed certain selectively toward GRK2 and GRK3 (with IC50 in the low nM range for both isoforms) as compared with GRK5 and GRK7 with IC50 in the high nM range, similar to those reported for PKA (Tesmer et al., 2010). Balanol binds to a semi-closed inactive conformation of GRK2 (as compared with PKA) that could be exploited pharmacologically. Since balanol adopts different conformations when binding to GRK2 and to PKA, selectivity for GRK2 could be improved by designing molecules that adapt to the particular conformation of the catalytic site adopted by GRK2 when bound to this compound that could be different for different GRK isoforms.

The Takeda Pharmaceutical Company also identified several heterocyclic compounds that bound and inhibited GRK2, and they were subsequently co-crystallized with GRK2-Gβγ (Thal et al., 2011). Similar to balanol, these compounds (called CMPD101 and CMPD103A) stabilized GRK2 in a slightly closed non-catalytic conformation with a degree of closure that correlated with potency for each species. Their IC50 values in a rhodopsin phosphorylation assay were of 290 nM and 54 nM, respectively (Thal et al., 2011), although an IC50 value of 35 nM had been previously reported in the corresponding patent. CMPD103A and CMPD101 are remarkably more selective among GRK subfamilies than balanol since they inhibit GRK2 and GRK3 ("GRK2 subfamily") with IC50s in the nM rage for both isoforms (that share 92% identity in their kinase domain sequences), but do not have an effect on GRK1 or GRK5 isoforms when used in concentrations up to 125 μM (Thal et al., 2011). However, they inhibited PKA with an IC50 of 2 μM. Possibly because of bioavailability problems, these compounds have not yet reached clinical trials even when they show desensitization-blocking activity and good potency

in cellular assays [see (Rainbow et al., 2018) as an example]. Through a high throughput screening identification strategy using Ulight TopoIIα as an artificial substrate and subsequent SAR-guided development, the same company has more recently developed a novel class of GRK2 inhibitors with selectivity toward isoforms GRK1, 5, 6, and 7, but showing equipotent inhibition of GRK3 (Okawa et al., 2017). Crystal structures using human GRK2 show a ligand-binding pose and interactions similar to those previously observed (Thal et al., 2011). The best performing compound was named 115 h, has an IC50 for GRK2 of 18 nM, and blocks desensitization of the β-adrenergic receptor pathway in HEK293 cells, although these effects were shown only at 100 μM concentrations. The pharmacokinetic profile after oral administration was however not enough to show *in vivo* activity.

An emerging family of small molecules being developed as GRK2 inhibitors are based on the FDA-approved selective serotonin reuptake inhibitor (SSRI) paroxetine, which was first found to target GRK2 in an aptamer displacement assay (Thal et al., 2012). Paroxetine stabilizes a unique conformation of GRK2 that misaligns the small and large lobes of this kinase and thus represents a unique scaffold for the design of more selective GRK2 inhibitors (Thal et al., 2012; Homan and Tesmer, 2015). Even when the IC50 of paroxetine is in the μM range using the rhodopsin (≈20 μM) or tubulin (≈2 μM) *in vitro* phosphorylation assays, the selectivity toward other GRK isoforms was a great improvement. Paroxetine was 50 to 60 times more potent toward GRK2 than toward GRK1 and GRK5 and performed well in living cells with a 10- or 40-fold selectivity over PKA and PKC, respectively (Thal et al., 2012). Paroxetine was also shown to inhibit β-adrenergic receptor desensitization by blocking GRK2-mediated receptor phosphorylation and β-arrestin recruitment with an IC50 of ≈6 μM (Guo et al., 2017). *In vivo*, paroxetine has been described to improve LV function and structure and to reverse or even ameliorate several features related to cardiac dysfunction in a mouse model of myocardial infarct when compared with fluoxetine, a structural analog unable to inhibit GRK2 but with SSRI capacity (Schumacher et al., 2015). Also, in a rat model of limb ischemia-reperfusion (I/R) injury, decreased GRK2 expression levels were found in ipsilateral neurons of the superior cervical ganglion. However, in mice treated with paroxetine, GRK2 expression was preserved after I/R, thus linking GRK2 binding/inhibition with the stabilization of the protein (Tang et al., 2015). Moreover, in another rat model of collagen-induced arthritis, oral (gavage) administration of paroxetine showed *in vivo* effects by protecting the joints from inflammation and destruction by impairing T cell infiltration and activation (Wang et al., 2017b). Cytokine and chemokine levels in serum and synovial tissues were also reduced, as were the populations of CD4+ and CD8+ effector T cells with increased differentiation of Treg cells and an induction of immune tolerance. Altogether, these results show that this drug can have pharmacological effects in murine models even when using enteral administration.

When a library of known kinase inhibitors from the Structural Genomics Consortium at Oxford University was screened, several compounds targeting GRK2 with structures resembling paroxetine were found (Homan and Tesmer, 2015). The most active compound showed an IC50 below μM in the tubulin phosphorylation assay and was 100–1,000 times more potent toward GRK2 over GRK1 and GRK5 (Homan and Tesmer, 2015). A highly potent and selective GRK2 inhibitor called 14as has been more recently identified from a set of paroxetine-derivative compounds (Waldschmidt et al., 2016). 14as has an IC50 for GRK2 of 30 nM, shows more than 230-fold selectivity over GRK1, GRK5, PKA, and ROCK1, and performs two orders of magnitude better than paroxetine in cardiomyocyte contractility assays, although no data relative to the possible inhibition of the GRK3 isoform are shown or discussed. Co-crystal structures of three of the synthesized paroxetine derivatives revealed the establishment of hydrogen bonds that make GRK2 adopt a more open conformation relative to that achieved with other inhibitors, which probably underlies the high selectivity of these compounds. More interestingly, pharmacokinetic data indicate that its plasma concentration in mice after a single intraperitoneal administration is above its IC50 for over 7 h.

The molecule GSK180736A, a compound structurally similar to paroxetine that had been developed as a ROCK1 inhibitor, was shown to co-crystallize in the active site of GRK2 and is a potent and selective inhibitor of GRK2 with an IC50 of 770 nM and more than 100-fold selectivity over other GRK isoforms. This molecule was used to develop a library of hybrid inhibitors containing some features from the Takeda compounds, namely occupation of the hydrophobic binding site in the kinase domain of GRK2, together with others from the GSK180736A molecule (Waldschmidt et al., 2016). From this library, inhibitors that are highly selective for GRK2 as well as potent for both GRK2 and GRK5 emerged. In particular, the compound called 12n (CCG-224406) showed an IC50 of 130 nM for GRK2 and, remarkably, more than 700-fold selectivity over GRK1 and GRK5, although no comparison using GRK3 is shown in this study. Emerging from other two classes of GRK2-selective inhibitors, namely GSK180736A and paroxetine, a molecular design initiative led to the creation of a set of new hybrid compounds in which the benzodioxole ring of paroxetine was exchanged for an indazole (indazole-paroxetine hybrids) (Bouley et al., 2017). Crystal forms from these hybrid molecules showed that they not only form stronger interactions with the hinge of GRK2 but also stabilize a distinct conformation, compared with paroxetine analogs, of its kinase domain. Therefore, the binding modes of these two families of compounds to the active site of GRK2 are similar, but they use two different hinge-binding moieties: indazole and benzodioxole. Among them, the CCG224061 compound presents a 20-fold increase in potency for GRK2 (IC50 of 66 nM) over paroxetine. However, it also shows increased activity against GRK1 and 5, PKA, and ROCK1 relative to the latter compound, and the results relative to GRK3 inhibition are not shown. So, the indazole-paroxetine analogs were more potent than benzodioxole derivatives, however at the cost of poorer selectivity, and possibly with a particular pharmacokinetic profile that may include renal clearance.

A very recent study has utilized some of the most active among the formerly presented GRK2 inhibitors to test GRK2-mediated desensitization of different arterial vasoconstrictor stimuli using *ex vivo* approximations (Rainbow et al., 2018). Paroxetine, Takeda's CMPD101, and two amide derivatives of GSK180736A (Waldschmidt et al., 2016) termed CCG215022 and CCG224063 were used. Each of these compounds attenuated desensitization toward angiotensin II- or UTP-mediated contractile responses in myograph assays using rat arterial rings, as well as in $P2Y_2$ and H_1 histamine-mediated PLCβ signaling assays, in MSMC and ULTR cells with an IC50 in the low μM range (CCG224063 in the low nM). Altogether these and other functional and structural data are unveiling interesting molecular insights into the binding modes, kinase selectivity and pharmacological features of these families of GRK inhibitors.

Aptamers

Since the first RNA-based aptameric molecule that acts as an *in vitro* inhibitor of GRK2 activity was described (Mayer et al., 2008), several efforts to improve the potency and selectivity of this class of compounds have been pursued. This C13 molecule can stabilize a unique inactive conformation of GRK2 through different interactions within and outside the kinase domain and reorganizes certain regions of the kinase to establish interactions with its polyanionic phosphodiester backbone (Mayer et al., 2008). C13 achieves inhibition of rhodopsin phosphorylation by GRK2 at low nM concentrations with a 20-fold selectivity over GRK5, and no appreciable effects detected in a panel of 14 other kinases (Mayer et al., 2008). However, possible effects on a cellular setting have not yet been reported, and this is of particular concern since these types of molecules are not bioavailable when administered orally, may not be stable inside the organism due to endoribonuclease-driven cleavage, and do not readily cross cell membranes. So, medicinal chemistry efforts should be implemented in order to overcome these difficulties. They have however been successfully used as a method of selection to screen for better drug-like inhibitory molecules by aptamer displacement assays as described above.

Peptides

Other strategy to inhibit GRK2 activity is based on using peptide sequences to interfere with the interface between the kinase and its substrates or activators. Peptides corresponding to the β2-adrenergic receptor sequence reported to interact with GRK2 have been identified and subsequently modified (Benovic et al., 1990) to reduce kinase activity with an IC50 in the low μM range but showing varying degrees of selectivity among GRK isoforms. Interestingly, these peptides do not seem to interact broadly with the kinase domain of GRK2, but rather mimic the first intracellular loop of the receptor and establish only one contact point with the kinase. One of them shows activity in cells, impairs β2-adrenergic receptor desensitization, and enhances receptor signaling in the human A431 cell line. Other peptides derived from residues of the substrate-kinase interaction domain in GRK2 and GRK3 have been tested in several animal models of type 2 diabetes. Acylated glycine derivatives of these short peptides, such as $KRX\text{-}683_{107}$ and $KRX\text{-}683_{124}$, can reduce plasma glucose concentrations possibly through GRK2-mediated inhibition of insulin signaling and display a systemic antidiabetic effect (Anis et al., 2004; Cipolletta et al., 2009). Another group of peptides was identified from a library of cyclic peptides designed using the GRK2 crystal structure, in particular the HJ loop inside the catalytic fragment of this kinase (Winstel et al., 2005). Peptides 9b and 10d showed sub μM values of IC50 when tested in an *in vitro* rhodopsin phosphorylation assay with poor inhibition of GRK5 and also presented activity in cells increasing basal and isoproterenol-stimulated cAMP production in HEK-293 cells overexpressing β2-adrenoreceptors.

Rational design of inhibitory peptides taking advantage of allosteric intramolecular interactions in GRKs has also been studied. For instance, N-terminal interactions as well as those emerging from an interface able to stabilize the closed active conformation of GRK2 (where receptor binding is proposed to activate GRK2) have been explored (Huang et al., 2009). A peptide encompassing the first 14 residues of GRK2 decreases receptor phosphorylation with an IC50 of 50 μM and at the same time enhances binding to phospholipids in what appears to be a membrane-specific effect since it has no impact on the kinase activity toward the soluble substrate tubulin (Pao et al., 2009).

Targeting the Gβγ-GRK2 Interface

The overexpression of a C-terminal fragment of GRK2 called GRK2ct or βARKct has been used as a strategy for GRK2 inhibition, since this peptide should inhibit endogenous GRK2 binding to Gβγ subunits and thus subsequent activation and translocation to the plasma membrane. Importantly, transgenic mouse models expressing βARKct constructs in the heart or adenoviral-mediated delivery of this construct in mice and pig models has shown to be cardioprotective in both acute and chronic models of HF. Since these aspects have been recently reviewed (Rudomanova and Blaxall, 2017b; Campbell and Smrcka, 2018; Cannavo et al., 2018), we will only discuss herein potential future developments of this strategy and also some caveats regarding its functional impact.

Since viral-based gene delivery remains a daunting therapeutic methodology, efforts have been made to identify small molecule Gβγ interactors as a more viable alternative. Consequently, a virtual screening was performed on a National Cancer Institute chemical library to identify small molecules capable of binding Gβγ subunits, using as an assay the ability to displace Gβγ binding to SIGK, a peptide that has been co-crystallized with the dimer, and thus identifying a surface critical for Gβγ interaction with effectors (Campbell and Smrcka, 2018). Among those, one termed M119 (cyclohexanecarboxylic acid [2-(4,5,6-trihydroxy-3-oxo-3H-xanthen-9-yl)-(9Cl)]) demonstrated high apparent affinity for Gβγ dimers and inhibited Gβγ-SIGK binding *in vitro* (Bonacci et al., 2006). The compound M119 and its highly homologous and more stable analog gallein have been successfully used ever since both in cultured cells and *in vivo*. They are able to impair Gβγ-dependent GRK2 recruitment to the plasma

membrane in response to GPCR in different cell types. Moreover, they can hamper HF progression and improve cardiac function in different murine models where they halt fibrosis, hypertrophic gene expression, and inflammation and also reduce myocardial infarct size [reviewed in (Rudomanova and Blaxall, 2017a)]. Also, in this context, a group has recently reported the development of nanobodies that able to specifically inhibit Gβγ-dependent signaling (Gulati et al., 2018).

It is worth noting that either GRK2ct or these new types of compounds would not only affect Gβγ-GRK2 activation but also block other Gβγ effectors downstream GPCR activation, which may cause off-target effects that need to be considered when evaluating their therapeutic roles. Although the notion of selective delivery of Gβγ inhibitors to specific target tissues using viral-guided approaches is attractive, the possible clinical application of this kind of strategies faces many regulatory and technical issues that need to be solved.

Emerging Strategies to Target GRK2 Functionality

The in-depth characterization of GRK2 modulatory mechanisms and the identification in recent years of a variety of new cellular partners of this kinase help envisage novel targeting strategies based on processes that able to modulate GRK2 activity/expression or to control specific functions of GRK2.

One type of approach is based on the modulation of GRK2 activation and translocation to the plasma membrane. Similar to the already discussed inhibitory effect caused by disruption of the interaction between GRK2 and Gβγ subunits, the GRK2$^{K567E/R578E}$ mutation, which eliminates anionic phospholipid binding, also ablates receptor phosphorylation in cells (Carman et al., 2000). In fact, efficient GRK2-mediated phosphorylation of activated GPCRs is dependent not only on its recruitment to the membrane by Gβγ subunits but also on the presence of phosphatidylinositol 4′,5′-bisphosphate (PIP2), a highly negatively charged lipid that not only helps recruit GRK2 but also orients the GRK2-Gβγ complex so that it is better able to phosphorylate activated GPCRs (Yang et al., 2016). This result is in contrast to what occurs with the GRK5 isoform, which adopts similar orientations on lipid bilayers whether or not they contain PIP2 (Yang et al., 2013). Therefore, isoform-selective inhibitors could be designed to target the GRK2-phospholipid interaction and thus impair or redirect kinase activity toward defined substrates (e.g., soluble vs membrane bound). However, other studies have described the ability of phosphatidylserine (PS) to enhance GRK2-dependent phosphorylation of purified and reconstituted human m2 mAChR twofold to threefold while PIP2 strongly inhibited this reaction (DebBurman et al., 1995). A possible explanation for this apparent discrepancy may be that neither Gβγ nor phospholipid interactions appear to play a major role in GPCR phosphorylation by GRK2 *in vitro*, possibly because high concentrations of the kinase and/or receptor can be used to drive the reaction by mass action and thus lead to apparently contradictory results. Since this type of GRK2-plasma membrane *in vivo* interactions may not be accurately mimicked by *in vitro* systems, cell-based assays should be utilized in order to develop inhibitors based on the ability to interfere with lipid-dependent GRK2 activity. In any case, the fact that GRK2 can be considered a lipid-dependent kinase that may be both upregulated and downregulated by phospholipids is an interesting feature that should not be overlooked when suggesting new strategies for the regulation of its activity.

Other strategies may rely on the interaction of GRK2 with inhibitory proteins. RKIP, a multifaceted kinase modulator belonging to the conserved family of phosphatidylethanolamine-binding proteins (PEBPs), is a small 21 kDa protein that able to regulate different signaling cascades and physiological processes. Phosphorylation of RKIP S153 by PKC appears to reorganize RKIP domains from a structure that binds and inhibits Raf-1 into a conformation that associates and blocks GRK2 by binding to its N-terminus domain [reviewed in (Skinner and Rosner, 2014)]. The inhibitory mechanism possibly requires RKIP dimer formation (Deiss et al., 2012). In fact, overexpression of phosphomimetic (RKIP$^{SK153/7EE}$) or dimeric (RKIP$^{\Delta143-146}$) RKIP peptides impairs phosphorylation of βARs by GRK2 in HEK293 cells as detected using antiphosphoserine antibodies and also reduces GRK2-mediated rhodopsin phosphorylation *in vitro* (Deiss et al., 2012). This study also shows that, at least in murine hearts, RKIP exists mostly in the S153-phosphorylated form and is thus predominantly bound to GRK2. In keeping with these data, interfering with RKIP by using specific antibodies, antisense, or RNAi plasmids enhances β-adrenergic signaling and its related contractile activity in cardiomyocytes (Lorenz et al., 2003). Moreover, a modest overexpression of this protein in a transgenic mouse model produces an increase in cardiac contractility achieved by the simultaneous activation of the β_1AR and β_2AR subtypes of adrenergic receptors that is thus well-tolerated and persistent (Schmid et al., 2015). These results open the possibility to use phosphorylated/dimeric RKIP to interfere with GRK2 functions in manners that could be alternative or additive not only to the use of pharmacological GRK2 blockade but also to study new modes to achieve GRK2 functional downregulation. However, given the multifaceted role of this peptide in the control of other important kinases and pathways such as Raf, MEK, ERK, NFκB, GSK3β, among others, the selectivity of such strategies could be compromised and should be carefully examined. Finally, since other proteins such as caveolin1, actinin, or clathrin have been reported to bind to GRK2 and help maintain pools of inactive kinase at defined subcellular locations (Penela et al., 2010a), it is tempting to suggest that peptides derived from these regulatory partners (or small molecules mimicking those) may modify the catalytic activity of GRK2 and/or its subcellular distribution, thus selectivity affecting certain GRK2 substrates.

On the other hand, post-translational mechanisms activating/inhibiting GRK2 or switching its partner repertoire could potentially be used to interfere with GRK2-dependent cellular actions. As previously mentioned, GRK2 activity is regulated in opposing ways by different covalent modifications, such as those entailing tyrosine phosphorylation of GRK2 within the N-terminus and the RH domain, Ser670 phosphorylation, or cysteine-nitrosylation in the catalytic domain. In particular, the S-nitrosylation of GRK2 limits the ability of this kinase to phosphorylate and desensitize the β2AR [recently reviewed in

(Cannavo and Koch, 2018)]. Accordingly, mice carrying a point mutation that substitutes the Cys340 in GRK2 for a serine ($GRK2^{C340S}$) are less resistant to cardiac ischemic injury and lose responsiveness of their myocardium to increased or decreased NO (Huang et al., 2013). Moreover, a water-soluble N-nitrosamine able to nitrosylate GRK2 without generating NO has been shown to impair isoproterenol-mediated β2AR-desensitization *via* GRK2 inhibition (Makita et al., 2013), thus providing a proof of concept that S-nitrosylation of GRK2 can be used as a therapeutic strategy.

Modulation of upstream kinases may also affect GRK2 functionality in specific contexts. In particular, our group has reported that GRK2-mediated HDAC6 phosphorylation requires the previous phosphorylation of GRK2 in S670 (see previous sections), which apparently provokes a switch in GRK2 substrate usage (Lafarga et al., 2012). $GRK2^{S670A}$ showed a noticeably reduced ability to phosphorylate HDAC6 as compared with WT GRK2 but did not show any defects in phosphorylating other GRK2 canonical substrates. This discovery opens the possibility for redirecting GRK2 activity toward defined substrate subsets. For instance, the use of compounds or cellular strategies that impair GRK2 phosphorylation in S670, such as ERK or CDK2 inhibitors, would either hamper or completely impair phosphorylation of HDAC6, and thus, GRK2-dependent effects downstream this deacetylase such as growth factor signaling, proliferation, and anchorage-independent growth of breast cancer cells (Nogues et al., 2016). Although not specifically targeted to GRK2, such ERK or CDK2 inhibitors would indirectly rewire the GRK2 substrate repertoire by affecting its Ser670 phosphorylation status. In this context, it would be of interest to identify small molecules that differentially inhibit the activity of unphosphorylated and Ser670-phosphorylated GRK2 toward distinct cellular substrates. Other possible approach to impair GRK2 activity toward given substrates might be the use of peptides (or small molecules) targeting the specific domains of GRK2 (or of its partner) involved in their recognition. Also, the substrate preference of GRK2 would depend on its particular subcellular localization in a given cellular setting. For instance, GRK2 can be localized to the mitochondria by way of its interaction with Hsp90 proteins triggered by S670 phosphorylation (Chen et al., 2013), and an important pool of microsomal-bound GRK2 has been described in mouse liver and neuronal tissues as well as in cultured cells (Murga et al., 1996). The consequences of the existence of different subcellular populations of GRK2 in terms of accessibility to defined substrates and the possibility that they may cause different final downstream effects are only beginning to be studied and should be fully elucidated.

A better knowledge of the stimuli and mechanisms controlling GRK2 expression may help to develop approaches to directly decrease GRK2 expression. As discussed in previous sections, diverse stimuli (catecholamines, angiotensin, high-fat diet) appear to converge in promoting enhanced GRK2 expression in different tissues and cell types in the context of given diverse cardiovascular and metabolic diseases. Thus, it is tempting to suggest that altering such stimuli would help to prevent pathological GRK2 accumulation. In fact, beta-blockers as well as exercise have been described to reduce myocardial and vascular GRK2 levels [reviewed in (Hullmann et al., 2016; Cannavo et al., 2018; Mayor et al., 2018)]. On the other hand, the mostly unexplored field of GRK2 expression modulation by miRNAs may also identify tools for decreasing kinase levels in a tissue-specific way. Finally, the well-established degradation of GRK2 *via* the ubiquitin-proteasome pathway may also allow for strategies aimed at increasing GRK2 downregulation in particular cellular settings such as proteasome activators or the use of emerging techniques to target specific proteins for degradation, such as the use of the so-called Proteolysis Targeting chimeric molecules (PROTACs) (Gu et al., 2018).

CONCLUSIONS

The GRK2 signaling hub is important in signaling pathways and processes related to very relevant cardiovascular pathological conditions (heart failure, cardiac hypertrophy, hypertension) and to diseases related to altered metabolic homeostasis (obesity metabolic syndrome, type 2 diabetes, NAFLD). These data along with the increased GRK2 expression reported in preclinical models of these pathologies and in samples from patients put forward this kinase as a promising therapeutic target. Moreover, the fact that these pathological conditions are frequently interconnected suggests that inhibiting GRK2 may have common beneficial effects when such situations concur as co-morbidities. In fact, genetic approaches have established the importance of GRK2 as a potential therapeutic target in some of these pathological processes. Strategies aimed to target GRK2 are beginning to yield some fruits, and several small molecules, peptides, and inhibitory constructs have been developed that effectively inhibit GRK2 activity and have been shown to display effects in cells and even in animal models. However, there are many challenges that need to be addressed. In addition to attaining specificity toward other GRKs and other kinases and off-targets, GRK2 inhibitors with acceptable *in vivo* potency and pharmacokinetic profiles are still awaited. On the other hand, the potential drawbacks of pathological effects of chronic GRK2 inhibition in defined cell types and tissues need to be carefully considered in order to identify therapeutic windows. In addition, the identification of novel GRK2 substrates and partners and the existence of different subcellular pools open the possibility of specifically targeting the interaction of GRK2 with specific subsets of signaling proteins. A better knowledge of the dynamic structural events leading to GRK2 activation, of the interfaces involved in its interaction with given partners, and of the molecular mechanisms involved in the modulation of GRK2 expression, activity, and localization would help to develop or improve novel strategies for GRK2 inhibition. This would be very important not only for advancing in the path for therapeutic application but as advanced research tools to dissect the precise contribution of GRK2 to physiological and pathological processes.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Therapeutic Potential of Targeting ß-Arrestin

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ß-arrestins are multifunctional proteins that modulate heptahelical 7 transmembrane receptors, also known as G protein-coupled receptors (GPCRs), a superfamily of receptors that regulate most physiological processes. ß-arrestin modulation of GPCR function includes termination of G protein-dependent signaling, initiation of ß-arrestin-dependent signaling, receptor trafficking to degradative or recycling pathways, receptor transactivation, transcriptional regulation, and localization of second messenger regulators. The pleiotropic influence ß-arrestins exert on these receptors regulates a breadth of physiological functions, and additionally, ß-arrestins are involved in the pathophysiology of numerous and wide-ranging diseases, making them prime therapeutic targets. In this review, we briefly describe the mechanisms by which ß-arrestins regulate GPCR signaling, including the functional cellular mechanisms modulated by ß-arrestins and relate this to observed pathophysiological responses associated with ß-arrestins. We focus on the role for ß-arrestins in transducing cell signaling; a pathway that is complementary to the classical G protein-coupling pathway. The existence of these GPCR dual signaling pathways offers an immense therapeutic opportunity through selective targeting of one signaling pathway over the other. Finally, we will consider several mechanisms by which the potential of dual signaling pathway regulation can be harnessed and the implications for improved disease treatments.

Keywords: ß-arrestin, ß-arrestin signaling, G-protein-coupled receptors, biased signaling, 7TMR

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ß-ARRESTIN STRUCTURE AND FUNCTION

The arrestin family of proteins includes four members and a variety of splice variants. There are two visual arrestins, found only in the retina (Smith et al., 2000), and two ubiquitously expressed arrestins named ß-arrestin1 (arrestin-2) (Lohse et al., 1990) and ß-arrestin2 (arrestin-3) (Attramadal et al., 1992). The beta prefix is because their first documented receptor substrate was the $ß_2$-adrenergic receptor ($ß_2$AR) and the "arrestin" term was because their major function is in terminating (or 'arresting') signaling *via* G proteins (Benovic et al., 1987). ß-arrestin proteins have functional and structural domains that allow them to bind to receptors as well as biochemical intermediates, events that are key to their function (Shenoy and Lefkowitz, 2011; Peterson and Luttrell, 2017). Although ß-arrestins interact with several different types of cell surface receptors (Shenoy and Lefkowitz, 2011), this review will focus on their role in modulating heptahelical receptors.

Heptahelical receptors, also known as seven-transmembrane receptors (7TMRs), are characterized by seven membrane-spanning domains and constitute the largest family of cell surface receptors known to date. The 7TMR superfamily is responsible for transducing a wide variety of extracellular signals into intracellular functions. More than three decades ago, it was shown that ligand activation of 7TMRs resulted in intracellular signaling through receptor coupling to heterotrimeric guanine nucleotide or G proteins (Rodbell et al., 1971; Northup et al., 1983). Thus, 7TMRs are more commonly known as G protein-coupled receptors (GPCRs). Therapeutically, these GPCRs are a very important class of receptor since they mediate almost all physiological processes, and their signaling is the target of roughly 40% of all prescribed drugs (Wise et al., 2002).

Numerous *in vitro* studies show that ß-arrestins terminate G protein-mediated cell signaling by translocating and binding to GRK-phosphorylated serine and threonine residues in the GPCR third intracellular loop or C terminal tail. Once bound, ß-arrestins sterically prevent further receptor-G-protein coupling (Benovic et al., 1987). This termination of G protein signaling is the canonical role for ß-arrestin. Later it was found that in addition to terminating G protein signaling, ß-arrestins also initiate ß-arrestin-dependent cell signaling by acting as scaffold proteins that couple the receptor to a growing list of signaling intermediates, many of which are kinases (Luttrell et al., 1999; Lefkowitz and Shenoy, 2005). This receptor-ß-arrestin-kinase complex is internalized *via* endocytic vesicles thereby becoming an intracellular "signalosome". Recently, this concept has been broadened to show that endocytic vesicles lacking the receptor, but containing receptor-activated ß-arrestin, can also internalize and activate cell signaling (Eichel et al., 2018). In this newly discovered mechanism, the interaction of translocated ß-arrestin with the GPCR is temporary but sufficient to change the conformation of ß-arrestin thereby activating it. The activated ß-arrestin is able to bind to membrane phosphoinositides that link it to the cell membrane for endocytosis into signaling vesicles.

Internalization of signaling vesicles is mediated by interactions between the C terminal tail of GPCR-activated ß-arrestin and the cell membrane endocytic proteins, clathrin and AP-2 (adapter protein-2) (Goodman et al., 1996; Laporte et al., 1999). This adaptor function of ß-arrestin is crucially important to not only receptor internalization but also ß-arrestin-dependent signaling. ß-arrestins bind to E3 ubiquitin ligases and deubiquitinases that direct GPCR-ß-arrestin vesicles to degradative or recycling pathways within the cell, thus modulating receptor cell surface expression (Shenoy et al., 2001; Shenoy, 2007). This trafficking function of ß-arrestins is yet another way by which these multifunctional proteins modulate GPCR signaling and cellular responses. Furthermore, ß-arrestins are also able to dampen G protein-mediated second messenger generation (ie., cAMP) by binding to second-messenger degrading enzymes, such as phosphodiesterases (PDE), and translocating them to the ligand-activated receptor (Perry et al., 2002). Taken together, ß-arrestins are the predominant modulators of GPCR signaling. The activated conformation, and thus function, of ß-arrestin is influenced by the GPCR type to which it has translocated as well as the conformation of the activated receptor (reviewed in (Peterson and Luttrell, 2017)). The ß-arrestins are structurally flexible proteins which allow them to bind to a broad spectrum of partners and mediate a wide variety of functions (Scheerer and Sommer, 2017). Because biased ligands influence different conformational states of GPCRs, they indirectly influence ß-arrestin-dependent signaling, making them key therapeutic molecules. Additionally, biasing the structure/function of ß-arrestins to impact signal transduction has also been proposed as a novel therapeutic strategy (Chen et al., 2018). Our review does not focus on the finer points of structural data and complex equilibria of conformational states as these have recently been the topics of several excellent reviews (Peterson and Luttrell, 2017; Scheerer and Sommer, 2017; Chen et al., 2018).

With the development of mice deficient in either ß-arrestin1 or ß-arrestin2 (Conner et al., 1997; Bohn et al., 1999b), evidence supported the *in vitro* experimental conclusions that ß-arrestins play an important role in regulating normal physiological and pathophysiological responses. Despite the lethality resulting from elimination of both ß-arrestins, single ß-arrestin-knockout (ß-arrestin-KO) mice are quite normal and perturbations of homeostasis are often required to observe an effect of the absence of ß-arrestin expression.

The first published study examining normal physiologic responses in ß-arrestin2-KO mice involved exogenous opioid administration as the homeostatic perturbation (Bohn et al., 1999a). Bohn et al. showed the immediate anti-nociceptive effect of opioids was enhanced in ß-arrestin2-KO mice, and the negative side effects of opioids, such as respiratory depression and diminished gastrointestinal motility, were reduced. Although signaling pathways were not measured, the absence of depressed respiration and gut motility in ß-arrestin2-KO mice suggested that ß-arrestin-dependent signaling promoted those responses. Conversely, the enhanced analgesia pointed to a physiologically relevant role for ß-arrestin2-mediated desensitization of opioid receptor G protein-dependent signaling in the mechanism of pain relief.

The first study demonstrating a role for ß-arrestin in disease pathogenesis used a murine model of asthma (Walker et al., 2003). This study, and our subsequent work, showed ß-arrestin2-dependent phospho-p38 mitogen-activated protein kinase (Pp38) signaling to be crucial for T helper type 2 (Th2) cell chemotaxis (Lin et al., 2018). Furthermore, protection from developing the asthma phenotype in ß-arrestin2-KO mice is associated with significant inhibition of CD4+ Th2 cell chemotaxis to the lung and marked reductions in airway epithelial cell mucin secretion and airway inflammation (Walker et al., 2003).

ß-ARRESTIN MODULATION OF PHYSIOLOGY AND PATHOPHYSIOLOGY

ß-arrestins trigger physiological responses through scaffolding of cell signaling proteins such as extracellular signal-regulated kinases 1 and 2 (ERK1/2), proto-oncogene tyrosine protein kinase Src (c-Src family tyrosine kinases), phosphoinositide 3-kinases, protein kinase B (AKT), c-Jun N-terminal kinases (JNK3) and elements

of nuclear factor κb (NF κb) (Shenoy and Lefkowitz, 2011; Peterson and Luttrell, 2017).

Although convenient to characterize GPCR signaling as two distinct signaling pathways, one that is G-protein dependent/ß-arrestin-independent and the other that is ß-arrestin-dependent/G protein-independent, the reality is more complex. We recently showed that P-p38 signaling, and associated chemotaxis, of T helper type 2 cells is partially dependent on ß-arrestin2 and that this ß-arrestin2-dependent signaling is downstream of Gαi (Lin et al., 2018). In that paper, we described the dual chemotaxis signaling pathways as ß-arrestin dependent and ß-arrestin independent; of course, both pathways were G protein-dependent. Work from Grundmann et al. (2018) has provided further evidence of G protein-dependency for ß-arrestin-mediated signaling. They used groundbreaking CRISPR/Cas9 technology to produce HEK293 cells that either do not express arrestins or lack all G proteins except Gαi, which was pharmacologically inhibited using pertussis toxin (PTX). Using these cell lines, dubbed "zero arrestin" and "zero functional G," respectively, they showed that ß-arrestin-dependent signaling is downstream of G proteins for several GPCRs including some canonical receptors where this was not previously believed to be the case. Although the receptor and cell types were limited in their study, the results have important implications for drug discovery and suggest G proteins as the "genuine drivers of GPCR-mediated signal transduction." However, whether or not ß-arrestin is a signaling molecule or a scaffolding protein, or is dependent or independent of G protein signaling, is immaterial to its extremely important regulation of cellular function (Gutkind and Kostenis, 2018). Thus, the lexicon used to describe GPCR signaling could benefit from expansion and clarification where descriptors of both the G protein and ß-arrestin involvement are listed (G protein-dependent/independent and ß-arrestin-dependent/independent).

As predicted by experimental findings, the ß-arrestin-dependent signaling pathway, like its G protein (ß-arrestin-independent) counterpart, regulates a wide variety of important cellular responses including cell development, growth and survival, immune cell function, protein translation, and neuronal signaling (Gu et al., 2015). The ability of ß-arrestins to desensitize G protein-dependent and mediate ß-arrestin-dependent GPCR signaling pathways uniquely positions these proteins to exert a major influence on physiology and pathophysiology.

The discovery that a single GPCR subtype can couple to different transduction proteins and produce multiple cellular responses has led to development of ligands that can preferentially "bias" the receptor toward one pathway. Some of these "biased ligands" preferentially target the ß-arrestin pathways (by either activation or inhibition) and have shown promise in drug development. These advances will be discussed in the section titled *ß-arrestin versus G-protein signaling in disease*.

ß-ARRESTIN EXPRESSION IN DISEASE

Consistent with the broad range of physiological processes modulated by ß-arrestins, the upregulation of ß-arrestin expression is associated with many diseases. Whether or not changes in expression are adaptive or maladaptive remain to be determined. For example, in a mouse model of cardiac dysfunction, enhanced cardiac ß-arrestin2 expression mitigated adverse cardiac remodeling (Grisanti et al., 2018); whereas in murine asthma, T cell and lung structural cell overexpression of ß-arrestin2 is maladaptive (Walker et al., 2003; Chen et al., 2015; Sharma and Parameswaran, 2015). Elevated ß-arrestin expression and concomitant anti-apoptotic effect is associated with fibrotic diseases (reviewed in (Gu et al., 2015) and in multiple sclerosis (MS) where CD4+ T cells from patients have a higher expression of ß-arrestin1 (Shi et al., 2007). These results implicate increased ß-arrestin1 as mediating the survival of CD4+ T and promoting disease pathogenesis (Shi et al., 2007). Another study reported similar upregulation of ß-arrestin1 expression in the brains of MS patients and in an animal model of experimental autoimmune encephalomyelitis (EAE), a commonly used model of human inflammatory demyelinating disease (Tsutsui et al., 2008). Other diseases that implicate ß-arrestin in pathophysiology include Alzheimer's disease (AD), cystic fibrosis (CF) and meningitis. In AD, brain protein and mRNA levels of ß-arrestin1 and ß-arrestin2 are elevated (Liu et al., 2011; Jiang et al., 2013). In CF, patient nasal epithelial cells, as well as CF model cells, overexpress ß-arrestin2 (Manson et al., 2008). With respect to immunity, expression of ß-arrestin2 was shown to be elevated in PBMCs of patients with cryptococcal meningitis (Bochaton-Piallat et al., 2016). Up and down-regulation of ß-arrestin proteins is clearly associated with pathology. Interestingly, disease-associated mutations in either ß-arrestin subtype have, so far, not been found.

EFFECTS OF ß-ARRESTIN ON DISEASE

Inappropriate modulation of cell survival can lead to cancer and fibrotic diseases, while abnormalities in immune cell function have implications for autoimmunity, infection, and the inflammatory component of many diseases. For an in depth review of the role for ß-arrestins in disease, please refer to the work by Sharma and Parameswaran (2015). Below we present examples of how ß-arrestin modulation of cellular events can become pathophysiological.

Once adhered to human brain endothelial cells, the meningococcus bacterium promotes endothelial cell $ß_2$AR-ß-arrestin2 signaling to cSrc-mediated cytoskeletal reorganization (Coureuil et al., 2010). This delocalizes endothelial junction proteins resulting in destruction of the blood brain barrier tight junctions and enhanced brain infection. Cytoskeletal changes also promote bacterial adhesion to endothelial cells (Coureuil et al., 2010). ß-arrestin2 also hinders bacterial killing through reducing peripheral blood mononuclear cell cytotoxic activity, decreasing serum levels of interferon-gamma (IFN-γ), an anti-bacterial cytokine, and increasing the serum level of IL-10, an anti-inflammatory cytokine (Bochaton-Piallat et al., 2016). Similarly, ß-arrestin2 inhibits the antiviral response by

reducing the killing effectiveness of natural killer (NK) cells (Yu et al., 2008). ß-arrestin2 transgenic mice were shown to have higher organ viral loads following murine cytomegalovirus; additionally, NK cells from these mice displayed reduced cytotoxicity. Conversely, ß-arrestin2 knockout mice and their NK cells displayed lower tissue viral titers and enhanced cytotoxicity, respectively.

Studies have also shown that ß-arrestins are involved in the initiation, development, and metastasis of many types of cancer (Song et al., 2018). In a murine model of chronic myelogenous leukemia, in which mice were transplanted with diseased hematopoietic stem cells, mice devoid of ßarrestin-2 did not succumb to the disease following transplantation of the diseased cells. In contrast, control mice died within 2 months of the transplantation of diseased cells. The study showed ß-arrestin2 promotes signaling *via* the wnt/beta-catenin pathway to promote cancer stem cell maintenance (Fereshteh et al., 2012). Similarly, in a murine model of myelofibrosis, a myeloproliferative neoplasm, mice transplanted with donor ß-arrestin2-KO hematopoietic stem cells infected with a myelofibrosis retrovirus did not develop the disease, whereas controls uniformly succumbed to disease. Abolition of ß-arrestin2-mediated promotion of anti-apoptosis prevented ß-arrestin2-KO cells from repopulating long-term and decreased self-renewal of infected ß-arrestin2-KO cells (Rein et al., 2017).

The role of ß-arrestins in ovarian, prostate, brain, gastric, lung, and breast cancers has been well established (Sobolesky and Moussa, 2013). One study showed ß-arrestin1 could be used as a plasma biomarker to differentiate certain types of lung cancers (El-Khoury et al., 2018). Ovarian cancer metastasis has also been reported to be mediated by ß-arrestin1 (Purayil and Daaka, 2018). Studies also suggest ß-arrestin2 as a prognosis marker for colorectal cancer (Ren et al., 2018) and a promoter of lymph node metastasis in non–small cell lung cancer (Cong et al., 2017). Both ß-arrestin1 and 2 have been reported as promoters of prostate cancer albeit through different mechanisms (Kong et al., 2018). Numerous GPCRs and their downstream effectors are envisaged to provide many targets and novel strategies in hepatocellular carcinoma prevention and treatment (Peng et al., 2018). Interestingly, the ß-arrestin biased ß-blocker carvedilol, a ligand that activates $ß_2$AR-ß-arrestin2 signaling while inactivating canonical $ß_2$AR-Gs signaling, has been found to be beneficial in cancer prevention by virtue of blocking a key step in carcinogenesis, i.e., ERK translocation into the nucleus (Wisler et al., 2007; Cleveland et al., 2018).

Fibrosis is defined as the accumulation of excess extracellular matrix (ECM) components which are mainly derived from fibroblasts and myofibroblasts (Cox and Erler, 2011; Bochaton-Piallat et al., 2016). If highly progressive, the fibrotic process leads to organ malfunction and death (Gu et al., 2015). Renal fibrosis can result in many kidney diseases (diabetic nephropathy, uronephrosis and polycystic kidney) and often leads to chronic kidney disease. ß-arrestin-deficient murine models under unilateral ureteral obstruction show attenuated renal interstitial fibrosis. Moreover, mice and human kidney tissue samples morphologically consistent with nephropathy show increased expression of ß-arrestin1 (Xu et al., 2018). However, it is unknown what receptor is responsible for such a response. Data have implicated AT_1 receptor-mediated ß-arrestin signaling as responsible for the observed increased extracellular matrix synthesis resulting in renal fibrosis (Wang et al., 2017b). Mice with unilateral ureteral obstruction had increased collagen I and fibronectin that correlated with the tubulointerstitial fibrosis and ß-arrestin upregulation. When ß-arrestin signaling was stimulated in a rat renal fibroblast cell line (NRK-49F cells) using [Sar(1), Ile(4), Ile(8)] AngII (SII), an AT_1 receptor ß-arrestin biased ligand, increased collagen I and fibronectin expression was observed, while silencing ß-arrestin expression had the opposite effect (Wang et al., 2017b). Expression of ß-arrestin2 is upregulated in liver biopsies from patients with hepatitis B and C, suggesting ß-arrestin2 promotes liver fibrosis (Gu et al., 2015). Taken together, the above data suggest ß-arrestin signaling promotes fibrosis in some tissues such as kidney, liver, heart, and lung (Lovgren et al., 2011; Gu et al., 2015). These results support the development of G-protein biased ligands that may shut down the pro-fibrotic actions of ß-arrestin in disease.

The range of diseases in which ß-arrestins play a role is very broad. For example, ßarrestin plays a role in brain function. A positive correlation between brain ß-arrestin (ß-arrestin1 and 2) levels (both protein and mRNA) and Alzheimer's disease diagnosis, severity, and amyloid burden has been demonstrated by several investigators (Liu et al., 2011; Thathiah et al., 2012; Liu et al., 2013). Also, G protein-independent signaling downstream of brain β2AR, delta opioid receptor, and orphan G protein-coupled receptor 3 promote cleavage of amyloid precursor protein (APP) by γ-secretase (Jiang et al., 2013) and production of amyloid-β peptide, a defining pathological feature of AD. In a murine model of AD, neurons lacking ß-arrestin2 demonstrate reduced amyloid-ß peptide secretion in culture and detection in the hippocampus and cortex (Thathiah et al., 2012). In brief, ß-arrestin 2 promotes the pathogenesis of AD through GPCR-initiated regulation of γ-secretase activity, which results in elevated levels of amyloid-ß peptide (Jiang et al., 2013). In other cell types, ß-arrestin can have a positive impact on brain function. For example, ß-arrestin2 promotes cofilin translocation to dendritic spines in response to N-methyl-D-aspartic acid (NMDA) receptor activation, and cofilin promotes dendritic spine remodeling which is needed for normal learning and memory (Pontrello et al., 2012).

There are also examples where the canonical role for ß-arrestins is involved in neuropathophysiology. In Parkinson's disease (PD), a neurodegenerative illness where loss of dopaminergic neurons from the nigrostriatal system affects locomotion, chronic treatment with L-DOPA, a dopamine precursor, induces dyskinesias by D_1 receptor overactivation (Urs et al., 2015). These abnormal involuntary movements are mediated by Gs signaling as noted by rodent and nonhuman primate models of PD. In ß-arrestin2-KO mice treated with L-DOPA, such movements increased after L-DOPA treatment compared to controls and were prevented after ß-arrestin2 overexpression in both mice and monkeys (Urs et al., 2015). Using PD animal models (rats and macaques),

others have demonstrated that using Gs-biased ligands for D_1 receptors that decrease βarrestin-2 recruitment and associated desensitization of G protein signaling results in sustained locomotive activity (Gray et al., 2018), supporting a canonical role for ß-arrestin in regulating PD dyskinesias.

ß-ARRESTIN VERSUS G-PROTEIN SIGNALING IN DISEASE

As discussed above, data from the last 2 decades have shown that a single GPCR subtype can couple to different transduction proteins and produce multiple cellular responses. These observations have resulted in a new era of pharmacology where, relative to the endogenous hormone or neurotransmitter, ligands can selectively or at least preferentially activate one of the diverse responses produced by a single GPCR subtype. This phenomenon has been termed "ligand-directed trafficking of receptor stimulus" or "biased signaling." In many cases, the therapeutic effect of a ligand is mediated by one pathway and the adverse effects by another pathway. Thus, rational development of biased ligands has become an active part of modern drug discovery.

To date, the diseases with the most clinical data in support of the development of biased drugs are: the use of ß-adrenergic receptors (ßAR) and angiotensin AT_1 receptor ligands in congestive heart failure (CHF) (Barrese and Taglialatela, 2013; Lymperopoulos and Aukszi, 2017); μ-opioid receptor (μOR) ligands in the management of pain; and to a lesser extent, possibly $ß_2$AR ligands in asthma (Dickey et al., 2010; Forkuo et al., 2016; Joshi et al., 2017; Nguyen et al., 2017). There is also strong preclinical evidence supporting the use of biased ligands for other diseases, such as D_1 and D_2 receptor ligands for Parkinson's disease and schizophrenia, respectively (Park et al., 2016; Gray et al., 2018), sphingosine 1P (S1P) receptor ligands for multiple sclerosis (Dhar et al., 2016), and adenosine A_1 receptor ligands for ischemic heart disease (Baltos et al., 2016), among others. It is important to emphasize that neither the canonical signaling pathway associated with the G-protein nor signaling *via* the ß-arrestin-dependent pathway (or any pathway for any receptor) can be termed as universally beneficial or detrimental. The (patho)-physiological effects of any pathway will always be disease-specific, time-dependent, and indeed often cell-specific.

For example, in CHF and asthma, the pathways mediating the beneficial versus adverse effects are currently believed to be the opposite for each disease. In CHF, there are data to support ß-arrestin signaling as anti-apoptotic and cardioprotective (Rojanathammanee et al., 2009; Carr et al., 2016b). This is not only true for ß-arrestin activation by the FDA-approved "ß-blocker," carvedilol, in CHF, but also ß-arrestin activation in other GPCRs such as the angiotensin AT_1 receptor (Kim et al., 2012; Monasky et al., 2013). In CHF, the angiotensin AT_1 receptor can activate both its canonical G-protein pathway (in this case, Gq) and the ß-arrestin-dependent pathway (Noor et al., 2011; Monasky et al., 2013; Teixeira et al., 2017). Considerable data implicated a clear division between the detrimental effects of AT_1 receptor signaling via Gq and the protective effect of signaling via ß-arrestin and led to the development of the biased antagonist, TRV-120027 that preferentially inhibits Gq signaling (Boerrigter et al., 2011; Boerrigter et al., 2012). Thus, the ideal ligand profiles for both the $ß_2$AR and AT_1 receptor, when used in CHF, appear to be ligands that antagonize the G-protein pathway (Gs and Gq respectively), and either stimulate, or at least not antagonize the receptor conformation that promotes ß-arrestin-dependent signaling. Perhaps the most compelling evidence for the advantage of this ligand profile as ideal is the observed therapeutic advantage of carvedilol in CHF (Wisler et al., 2007). In this regard, it is important to point out a study using a mutated $ß_2$AR ($ß_2AR^{TYY}$) where the mutation renders the $ß_2$AR unable to bind G proteins; carvedilol was the only beta-blocker that retained the ability to activate ERK1/2 (Shenoy et al., 2006). However, more recent findings show that initiation of ERK1/2 activation by $ß_2$AR involves a signaling route that is independent of ß-arrestins (O'Hayre et al., 2017). Examination of the role for ß-arrestins in $ß_1$AR signaling to ERK1/2 shows that all ERK1/2 signaling downstream of the $ß_1$AR requires Gαi protein activation (Wang et al., 2017a), including that induced by carvedilol, but that loss of ß-arrestin2 results in reduced ERK1/2 signaling (Eichel et al., 2016). Future studies are required before we fully understand how carvedilol's superior clinical efficacy may be related to its unique signaling profile and the role for ß-arrestins. For now, O'Hayre et al. posit that re-interpretation of original findings with respect to the impact of the scaffolding function of ß-arrestins, which may control the localized activation of ERK, versus the ß-arrestin-promoting activation of ERK1/2 may explain some discrepancies (O'Hayre et al., 2017).

In the management of pain, μ opioid receptors (μOR) mediate pain relief through Gi/o activation, while ß-arrestin-dependent signaling induces respiratory depression and constipation (Bohn et al., 1999b; Raehal et al., 2005; DeWire et al., 2013; Altarifi et al., 2017). For example, compared to their wild type controls, ß-arrestin2-KO mice treated with morphine, a μOR agonist, exhibited increased antinociception using a tail-flick and hotplate models of pain (Bohn et al., 1999b). In addition, the ß-arrestin2-KO mice exhibited less of a reduction in gastrointestinal transit, and no respiratory suppression was observed compared to wild type mice (Raehal et al., 2005). These and other findings led to the development of TRV130, also known as oliceridine, a Gi/o-biased ligand (DeWire et al., 2013; Altarifi et al., 2017) for the μOR. Clinical trials using TRV130 for phase II (Viscusi et al., 2016; Singla et al., 2017) and III studies (APOLLO-1 and -2) have been completed, and while the results are not yet published, TRV-130 displayed greater analgesia and less gastrointestinal and respiratory side effects compared to morphine (Fossler et al., 2018). Currently, an open-label safety study is underway (ATHENA trial).

Thus, clinical development of biased ligands is now an active area of research by several pharmaceutical companies and academic laboratories. However, to date, no new chemical entities have made it to FDA-approval, and some have failed in Phase II trials (Pang et al., 2017). While this may cause diminished enthusiasm that pursuing the development of biased ligands may only work theoretically, it is important

to emphasize that biased ligands such as carvedilol have already shown superior clinical efficacy (Wisler et al., 2007). There are also data showing, famotidine, a histamine H_2 receptor antagonist used to reduce gastric acid secretion in acid-peptide disorders, may have greater therapeutic efficacy compared to other H_2 receptor antagonists (Campoli-Richards and Clissold, 1986). A suggested explanation is that famotidine, besides working as a G protein signaling antagonist by decreasing cAMP, also stabilizes the H_2 receptor conformation that induces desensitization, likely through ß-arrestin (Alonso et al., 2015).

The second generation antipsychotic cariprazine, a dopamine D_2 and D_3 receptor partial agonist in most systems, was recently approved for the treatment of schizophrenia and exhibited increased safety and tolerability profiles compared to first generation antipsychotics (Durgam et al., 2015). Although not a selective drug, biased signaling towards Gi has been suggested to confer cariprazine with less side effects (i.e. cognitive impairment, hyperprolactinemia, weight gain) (Solmi et al., 2017). Conversely, preclinical data also suggest that ß-arrestin biased ligands for the D_2 receptor can also contribute to schizophrenia treatment by resetting the balance of the excitation inhibition in the prefrontal cortex (Urs et al., 2016). Therefore, both signaling pathways are highly important in the therapeutics for this pathology and reinforce the idea that both targets are equally valuable in drug discovery.

As noted above, some recent attempts to rationally develop biased ligands have failed at various stages of development. However, this should not be interpreted as a failure that biased ligands can be developed. Indeed, one of the major societal and scientific benefits of the pharmaceutical industry's development of dozens of generic drugs for major diseases and symptoms, is that biased ligands have already been developed. For example, the scientific community has already thoroughly screened, using *in vitro* assays, dozens of ß$_2$AR and μOR ligands already in clinical use for their activity at several pathways (Wisler et al., 2007; Molinari et al., 2010; Stallaert et al., 2012; Vezzi et al., 2013; van der Westhuizen et al., 2014); and as described above, other drugs are now retroactively being implicated as being biased ligands as a means of explaining their different therapeutic outcome from other members of the same class of drugs (Wisler et al., 2007; Shonberg et al., 2013; Alonso et al., 2015).

To further develop the hypothesis that biased ligands can be rationally designed, it may be useful to view the strategy as not dissimilar from one previously used to produce dozens of therapeutically improved ligands. The last half of the 20th century saw a proliferation of the discovery of receptor subtypes. In many ways, biased ligand synthesis can be viewed as analogous to the development of more receptor subtype selective ligands. The different conformational states that are thermodynamically required to activate different pathways can be viewed in the same way as the different receptor conformations associated with different receptor subtypes.

Alternative or complementary ways to bias GPCR signaling include allosteric modulation of GPCR as well as the use of pepducins and/or nanobodies. Nanobodies, camelid antibody fragments, have been developed that preferentially bind to and stabilize the human ß$_2$AR in various conformations (Rasmussen et al., 2011; Staus et al., 2016). Rasmussen et al. produced nanobody 80 (Nb80) that, when bound to receptor, stabilizes the conformation of the receptor producing Gs signaling. Staus *et al* developed four families of nanobodies that stabilized active or inactive ß$_2$AR conformations and found biased inhibition of either G protein activation or ß-arrestin recruitment (Staus et al., 2014). They identified Nb60, a negative allosteric nanobody, to modulate and stabilize inactive β2AR state (Staus et al., 2016). As nanobodies can modulate biased ß$_2$AR signaling, they can be potential therapeutic agents regulating various pathological processes involving ß$_2$AR. Interestingly, Martin et al. synthesized a set of peptidomimetics which are structurally similar to the complementarity-determining region 3 (CDR3) of the nanobody Nb80, and inhibit ß$_2$AR-G protein coupling (Martin et al., 2017).

Another approach to biasing receptor signaling involves pepducins, first developed by Covic et al. (2002a,b). Pepducins are the lipid-peptide conjugates with sequences derived from the intracellular loops of the targeted GPCR (Carr and Benovic, 2016). By penetrating cells, pepducins can access receptor conformations not accessible to extracellular ligands that must rely on extracellular receptor binding (Carr et al., 2016b). Pepducins regulate the activity of GPCRs by allosteric modulation (Quoyer et al., 2013). Pepducins for several GPCRs have been reported in the last decade (Covic et al., 2002a; Licht et al., 2003; Remsberg et al., 2007; Tchernychev et al., 2010; Carr et al., 2014, 2016b). For example, intracellular loop1–9, a ß-arrestin–biased pepducin for the β$_2$AR, has been reported to be completely ß-arrestin–biased in primary adult murine cardiomyocytes, possibly enhancing cardioprotective effects for CHF therapy (Carr et al., 2016b). Intracellular loops 3–9, a pepducin modulator of G_s-biased ß$_2$AR signaling, has been shown to be a potential asthma therapy candidate as ß-arrestins are believed to be responsible for the symptoms associated with asthma (Walker et al., 2003; Dickey et al., 2010; Thanawala et al., 2013; Carr et al., 2014; Lin et al., 2018). Another G_i-biased pepducin, ATI-2341, has been developed for the chemokine receptor CXCR4 (Quoyer et al., 2013). It is noteworthy that successful use of pepducins *in vivo* is currently constrained because pepducins lack a targeting mechanism in multilayered tissues and thus are limited to cells in close proximity to the circulating pepducins (Carr et al., 2016a). However, pepducins can potentially share sequences in the intracellular loops of closely-related GPCRs and may have enhanced therapeutic effects mediated by various GPCRs ("polypharmacology") (Carr and Benovic, 2016).

CONCLUDING REMARKS

Given the functional versatility of ß-arrestins, they are aptly suited to effectively and broadly regulate cell signaling and resultant physiological and pathophysiological processes. The therapeutic potential of targeting ß-arrestins is enormous, since disease-specific treatments could increase the safety

and efficacy of GPCR-targeted therapeutics. On the other hand, given that a single ß-arrestin subtype can modulate dozens of GPCRs, this may pose problems in drug discovery. The search for ß-arrestin modulators will not be easy given the complexity of GPCR signaling pathways and the pleiotropy of ß-arrestin functions making precise targeting of paramount importance. Despite these challenges, there are several ligands preferentially targeting ß-arrestin signaling now in clinical trials and more in development. Thus, we will hopefully soon have answers as to the impact of these ligands in future therapies.

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JW and RB contributed to the conceptualization of the subject matter. JW, RB, ELG-R, and AH reviewed the literature and wrote sections of the manuscript.

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Aldosterone Jeopardizes Myocardial Insulin and β-Adrenergic Receptor Signaling *via* G Protein-Coupled Receptor Kinase 2

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Hyperaldosteronism alters cardiac function, inducing adverse left ventricle (LV) remodeling either *via* increased fibrosis deposition, mitochondrial dysfunction, or both. These harmful effects are due, at least in part, to the activation of the G protein-coupled receptor kinase 2 (GRK2). In this context, we have previously reported that this kinase dysregulates both β-adrenergic receptor (βAR) and insulin (Ins) signaling. Yet, whether aldosterone modulates cardiac Ins sensitivity and βAR function remains untested. Nor is it clear whether GRK2 has a role in this modulation, downstream of aldosterone. Here, we show *in vitro,* in 3T3 cells, that aldosterone impaired insulin signaling, increasing the negative phosphorylation of insulin receptor substrate 1 (^{ser307}pIRS1) and reducing the activity of Akt. Similarly, aldosterone prevented the activation of extracellular signal-regulated kinase (ERK) and the production of cyclic adenosine 3′,5′-monophosphate (cAMP) in response to the β_1/β_2AR agonist, isoproterenol. Of note, all of these effects were sizably reduced in the presence of GRK2-inhibitor CMPD101. Next, in wild-type (WT) mice undergoing chronic infusion of aldosterone, we observed a marked GRK2 upregulation that was paralleled by a substantial β1AR downregulation and augmented ^{ser307}pIRS1 levels. Importantly, in keeping with the current *in vitro* data, we found that aldosterone effects were wholly abolished in cardiac-specific GRK2-knockout mice. Finally, in WT mice that underwent 4-week myocardial infarction (MI), we observed a substantial deterioration of cardiac function and increased LV dilation and fibrosis deposition. At the molecular level, these effects were associated with a significant upregulation of cardiac GRK2 protein expression, along with a marked β1AR downregulation and increased ^{ser307}pIRS1 levels. Treating MI mice with spironolactone prevented adverse aldosterone effects, blocking GRK2 upregulation, and thus leading to a marked reduction in cardiac ^{ser307}pIRS1 levels while rescuing β1AR expression. Our study reveals that GRK2 activity is a critical player

downstream of the aldosterone signaling pathway; therefore, inhibiting this kinase is an attractive strategy to prevent the cardiac structural disarray and dysfunction that accompany any clinical condition accompanied by hyperaldosteronism.

Keywords: aldosterone, mineralocorticoid receptor, GRK2, insulin, β-adrenergic receptor, heart failure

INTRODUCTION

Aldosterone is a corticosteroid hormone synthesized by the adrenal cortex mainly in response to renin–angiotensin system (RAS) activation and by high potassium (K^+) levels (Hu et al., 2012; Cannavo et al., 2018a). Importantly, aldosterone activities were initially perceived as confined to a few organs, especially to the distal tubule and collecting duct of the nephrons in the kidney (Spät and Hunyady, 2004; Marney and Brown, 2007). However, the demonstration of specific aldosterone binding to its cytoplasmic/nuclear mineralocorticoid receptor (MR) in disparate areas of the cardiovascular system has widened the range of aldosterone targets (Marney and Brown, 2007; Schrier et al., 2010; Dooley et al., 2012; Verhovez et al., 2012). It is now consolidated, for instance, that this hormone controls the function and the growth, as well as the metabolism of several cell types, including cardiomyocytes, fibroblasts, and vascular cells (Brilla et al., 1993; Stockand and Meszaros, 2003; Hitomi et al., 2007; Cannavo et al., 2016; Cannavo et al., 2018a). This evidence has advanced aldosterone and its receptors to a new frontline for treating heart failure (HF), i.e., to prevent its onset or to arrest its progression (Brilla et al., 1993; Stockand and Meszaros, 2003; Hitomi et al., 2007; Cannavo et al., 2016; Cannavo et al., 2018a). Consistent with this new scenario, high aldosterone levels, and the subsequent MR hyperactivation, have been associated to the onset of insulin resistance, which is a well-recognized risk factor of cardiovascular disease (CVD) (Hitomi et al., 2007; Wada et al., 2009). More in detail, in vascular smooth muscle cells (VSMCs) and in adipocytes, aldosterone has been shown to inhibit insulin receptor substrate-1 (IRS1) that is considered a second messenger of the insulin receptor, thus preventing Akt activation and glucose uptake (Hitomi et al., 2007; Wada et al., 2009). Moreover, recent studies have demonstrated that, in fibroblasts, MR inhibition is able to prevent pro-fibrotic βAR activation (Reddy et al., 2015; Hori et al., 2017) and, as previously seen in the heart, aldosterone can both augment the hypertrophic response and myocardial apoptosis and fibrosis (Cannavo et al., 2016; Cannavo et al., 2018a). In this context, we have recently reported that, downstream of the aldosterone/MR system, there is the activation of G protein-coupled receptor kinase 2 (GRK2) and GRK5 (Cannavo et al., 2016). Accordingly, both *in vitro* and *in vivo*, we have found that aldosterone stimulation induces cardiac GRK2 activation, followed by increased cell death and mitochondrial dysfunction. Conversely, aldosterone-mediated GRK5 activation results in an increased pathological hypertrophy (Cannavo et al., 2016). Of note, GRK2 has been discovered as a regulator of cardiac contractility *via* β-adrenergic receptor (βAR) phosphorylation and subsequent desensitization in response to catecholamine stimulation (Cannavo et al., 2018b). However, in HF, a condition characterized by increased sympathetic nervous system activation (SNS), GRK2 activity/expression is upregulated, and this elevation in GRK2 activity has many adverse effects in the cardiovascular system because it induces massive βAR downregulation, with consequent left ventricular (LV) dysfunction (Cannavo et al., 2018b). Intriguingly, in addition to these canonical effects, GRK2 inhibits also IRS1, *via* direct binding and subsequent phosphorylation at the serine in position 307 thus leading to an impaired Akt activity (Garcia-Guerra et al., 2010; Ciccarelli et al., 2011; Mayor et al., 2011). Despite all this evidence, it remains to be determined still if aldosterone influences cardiac insulin signaling and βAR function and if this eventual modulation requires GRK2. Hence, in the present study, we tested the impact of aldosterone stimulation, and the role of GRK2, on both insulin and βAR signaling *in vitro*, in fibroblasts (3T3 cells), and *in vivo,* in mice undergoing chronic aldosterone infusion or surgical-induced myocardial infarction (MI), two models of hyperaldosteronism (Cannavo et al., 2016).

MATERIALS AND METHODS

Agonists and Inhibitors

Aldosterone (A9477), spironolactone (S3378), insulin (I2643), and isoproterenol (ISO) (I5627) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CMPD101 (HB2840) was purchased by Hello Bio (Bristol, UK).

Cell Culture and Stimulation Conditions

3T3-L1 fibroblasts were purchased from ATCC (ATCC® CL-173™) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% P/S. Prior to stimulation, all the cells were starved with serum-free media. A group of cells was serum-starved for 12 h and then was stimulated with aldosterone (1 μM) at different time points in fresh serum-free media. Another group of cells was serum-starved for 1 h and then, under this condition, was pretreated with aldosterone (1 μM) and/or CMPD101 (3 μM) for additional 12 h, as previously done by us (Esposito et al., 2011). Then, the cells have been stimulated with insulin (100 nm) or ISO (10 μM) dissolved in fresh serum-free media. Control unstimulated cells were maintained in serum-free media.

Immunoblots

Cells (fibroblasts) and left ventricular (LV) samples were lysed in a RIPA buffer with protease (cOmplete-Roche, USA) and phosphatase inhibitor (PhosSTOP-Roche, USA) cocktail

(Roche). Protein samples were quantified using a DC™ Protein Assay (Bio-Rad) and read at 750 nm using an iMark microplate reader (Bio-Rad). Then proteins (40 μg) were separated by 4–20% Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (Invitrogen) and were transferred to PVDF membrane (Bio-Rad). After blocking, with milk 5% in TBS-Tween 0.1%, the membranes were incubated and probed with the first antibody at 4°C overnight according to manufacturer's instructions. Then, the proteins were probed with a corresponding secondary antibody, followed by the visualization of the proteins with a chemi-doc XRS system (Bio-Rad), and quantitative densitometric analysis was performed using the chemi-doc XRS imaging software. Protein levels of a) GRK2 (sc-562, C-15, Santa Cruz Biotechnology, 1:1,000; 05-465, Millipore, 1:1,000); b) GAPDH (sc-32233, 6C5, Santa Cruz Biotechnology, 1:2,000); c) phosphor-ERK (extracellular signal-regulated kinase) 1/2 (#9106, Cell Signaling, 1:1,000), total ERK 1/2 (#9102, Cell Signaling, 1:1,000); d) phospho-Akt (pAkt, sc-514032, Santa Cruz Biotechnology, 1:1,000); e) total Akt (sc-8312, Santa Cruz Biotechnology, 1:1,000); f) phospho-IRS1 (^{Ser307}pIRS1, #05-1087, Millipore, 1:1,000); g) total IRS1 (tIRS1, sc-515017, Santa Cruz Biotechnology, 1:1,000); and h) β-1 adrenergic receptor (β1AR, PA1-049, Invitrogen, 1:1,000) were assessed.

Cyclic Adenosine 3′,5′-Monophosphate (cAMP) Assay

cAMP levels were assessed using a commercial kit (Cayman chemical—501001), following the manufacturer's instructions. Briefly, 3T3-L1 fibroblasts were plated in six-well multi-well, and after stimulation, the cells were lysed in 250 μl of 0.1-M HCl. Then, after centrifugation (1,000 g for 10 min), the resulting supernatants were quantified with a DC™ Protein Assay (Bio-Rad) to assess equal protein concentration. Then, the plate was read at 415 nm using an iMark microplate reader (Bio-Rad). Fifty microliters of the lysate were used to perform the enzyme-linked immunosorbent assay (ELISA) assay.

Animal Models

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University Lewis Katz School of Medicine. We used wild-type (WT) mice, mice bearing floxed grk2 (grk2-fl/fl), and cardiac-specific GRK2 knockout (KO) mice, generated breeding αMHC-Cre mice with grk2-fl/fl (Cannavo et al., 2016). All animals (males and females, 9–10 weeks) were maintained on a C57Bl/6 background.

Experimental Procedure: *Pump implantation*—Mice were anesthetized with isoflurane [2.5% (vol/vol)] and mini-osmotic pumps (1004, ALZET, DURECT Co., Cupertino, USA), loaded with Phosphate-buffered saline (PBS) and 5% ethanol (vehicle); aldosterone (2 μg·mouse·day^{-1}) and spironolactone (20 mg·kg^{-1}·day^{-1}) were implanted subcutaneously through a subscapular incision, which was then closed using 3.0 silk suture (Ethicon), as previously described (Cannavo et al, 2016; Tian et al., 2009). After 4 weeks of infusion, transthoracic echocardiography was performed, and then blood samples were collected by puncturing the heart, and after euthanasia, the heart was explanted and excised for pathological examination and immunohistochemistry.

Myocardial Infarction (MI)—Surgically induced MI was performed in WT mice (C57BL6, 9-week-old both females and males) as previously described (Gao et al., 2010). One-day post-MI, mice were randomly assigned to one of the following groups: MI and MI + spironolactone (MI + Spiro). Sham-operated animals were used as control.

Echocardiography

Transthoracic echocardiography was performed in all experimental groups (vehicle and aldosterone infused; sham-operated, MI, and MI + Spiro) to assess cardiac structure and function at baseline and at the end of the study period (4 weeks), using a VisualSonics VeVo 2100 system (VisualSonics, Toronto, Canada), as previously described (Cannavo et al., 2016). No gender-dependent differences were observed in terms of basal, post-MI, or post-aldosterone cardiac function.

Histology

Cardiac specimens were fixed in 4% formaldehyde and embedded in paraffin. After deparaffinization and rehydration, 5-μm-thick sections were prepared and mounted on glass slides.

Picro-Sirius Red Staining—Cardiac sections were stained with 1% Sirius Red in picric acid (Sigma-Aldrich, St. Louis, Missouri) to detect interstitial fibrosis and to calculate infarct size (Cannavo et al., 2017). The percentage of fibrosis and the infarct size were quantified using a software (ImageJ). Cardiac fibrosis images were acquired using a BA410 microscope (Motic®). For each of the samples, five to six fields (~400–900 cells for field) were acquired to detect fibrotic areas.

TUNEL staining—Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on fixed paraffin-embedded LV sections using a commercial kit (Roche), and the assay was performed according to manufacturer's instructions. Finally, cardiac sections were mounted with Fluoroshield with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, Missouri). Fluorescent images were acquired using a ZOE™ Fluorescent Cell Imager (Bio-Rad). For each of the samples, five to six fields (~400–900 cells for field) were acquired.

ANALYSIS AND STATISTICS

All experimental procedures (*in vitro* and *in vivo*) were performed in a blinded fashion. Data are expressed as means ± SE. Statistical significance was determined by a Student *t*-test or Mann–Whitney *U* exact test when the sample size was <10. For multiple comparisons, one-way analysis of variance with Dunnet's or Tukey's *post hoc* tests was performed. All data were analyzed using GraphPad Prism software version 8 (GraphPad Software, La Jolla, California). Statistical significance was accepted at $p < 0.05$.

RESULTS

GRK2 Signaling Downstream of Aldosterone/MR System Impairs Insulin Signaling in 3T3 Fibroblasts

In adipocytes and in VSMCs, aldosterone blocks insulin signaling, contributing to insulin resistance (Hitomi et al., 2007; Wada et al., 2009). Yet, whether GRK2 has a role in this chain of events remains untested. Therefore, to fill this gap in knowledge, we stimulated 3T3 cells with aldosterone (1 μM) at different time points (15 min, 30 min, 6 h, and 12 h) to analyze the expression levels of GRK2. Surprisingly, GRK2 protein levels were increased as early as 15 min after the aldosterone stimulation initiation, and they remained elevated at 30 min, 6 h, and 12 h, compared with those in unstimulated cells (NS) that exhibited no change in the expression of this kinase between these same time points (**Figures 1A, B**). Of note, mRNA transcript levels for GRK2 were not elevated over control levels 15–30 min or 6 h after aldosterone stimulation (**Supplementary Figure 1**). A marked elevation in GRK2 mRNA levels was evident only after 12 h of aldosterone stimulation (**Supplementary Figure 1**). This evidence is entirely consistent with previous reports from Cipolletta and coworkers (Cipolletta et al., 2009) who demonstrated that, following acute ISO or insulin stimulation, GRK2 is markedly upregulated, thus corroborating the notion that this kinase is critical for the regulation of physiological pathways governed by either GPCR- or non-GPCR-related factors. Furthermore, we have previously reported that 30-min stimulation with aldosterone is sufficient to phosphorylate GRK2 at the serine 670 site (^{s670}pGRK2), inducing the translocation of the kinase to the mitochondria. On these grounds, we next analyzed the phosphorylation levels of GRK2 and found that these levels varied with time (**Figure 1C**). More in detail, from 15 to 30 min after aldosterone stimulation initiation, ^{s670}pGRK2 amounts were markedly elevated over control levels. However, already 6 or 12 h after aldosterone superfusion, the extent of ^{s670}pGRK2 sharply declined (**Figure 1C**). In aggregate, this set of data suggests that, upon aldosterone stimulation, GRK2 is able to deploy both its

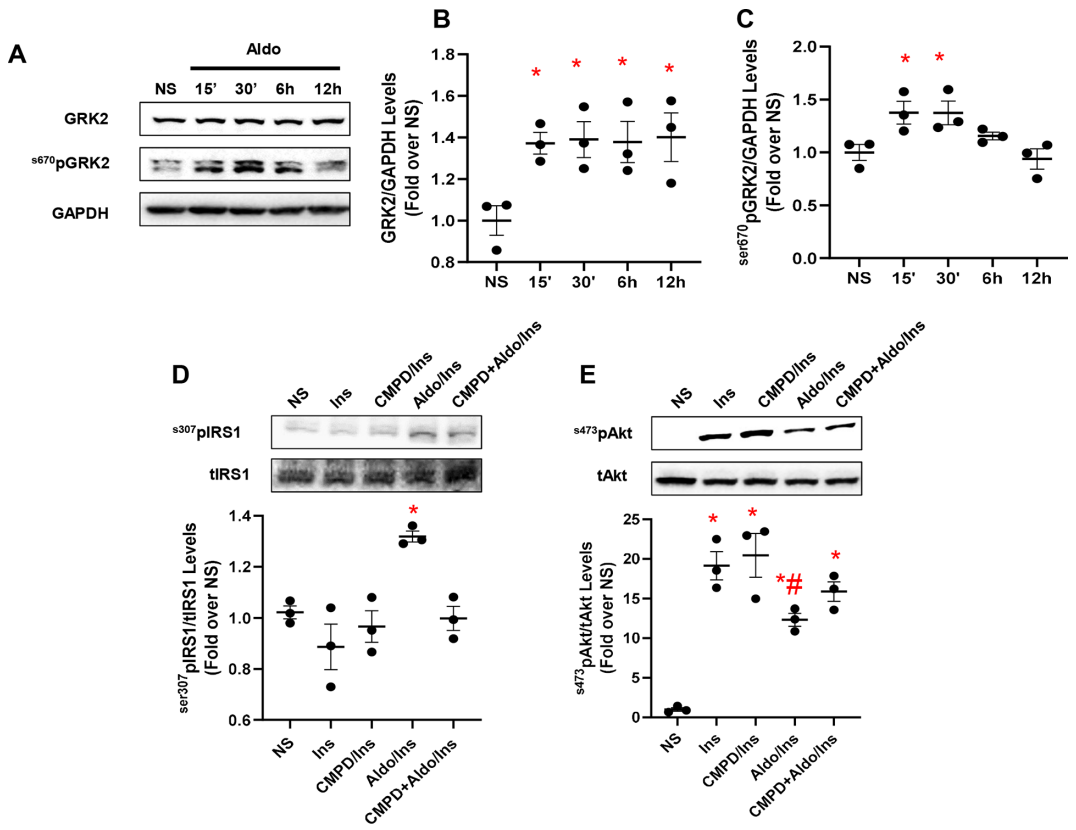

FIGURE 1 | Aldosterone impairs insulin signaling *in vitro* in fibroblasts. Representative immunoblots **(A)** and densitometric quantitative analysis **(B–C)** of multiple independent experiments (n = 3) to evaluate GRK2 phosphorylation (Ser670) and expression levels, in 3T3 fibroblasts. Shown is a time course (15 min to 12 h) of aldosterone (Aldo, 1 μM) treatment; GAPDH was used as loading control; Dunnet's *post hoc* test. *p < 0.05 vs unstimulated cells (NS). **(D)** Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple independent experiments (n = 3) to evaluate IRS1 phosphorylation levels (^{s307}pIRS1) as a ratio of inactivated IRS1 to total IRS1 (tIRS1). The cells were either NS or stimulated with Aldo (1 μM) and/or CMPD101 (3 μM) for 12 h. After Aldo and/or CMPD101 treatment, cells were stimulated with insulin (Ins, 100 nM) for 15 min. Tukey's *post hoc* test. *p < 0.05 vs NS. **(E)** Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple independent experiments (n = 3) to evaluate Akt phosphorylation (^{s473}pAkt) as a ratio of activated Akt to total Akt (tAkt), in cells either NS or stimulated with Aldo (1 μM) and/or CMPD101 (3 μM) for 12 h. Then, cells were stimulated with Ins (100 nM) for 15 min. Tukey's *post hoc* test. *p < 0.05 vs NS; #p < 0.05 vs Ins.

more canonical effects, such as regulating plasma membrane receptor function, i.e., βAR signaling, and noncanonical actions, such as those due to its translocation to mitochondria where the kinase can bind to non-GPCR-related molecular entities (Pitcher et al., 1999; Cannavo et al., 2018b).

Based on the previously reported findings, we next set out to evaluate the impact of aldosterone/GRK2-dependent signaling on insulin and βAR receptor-dependent pathways. To this end, a group of cells was pretreated with the GRK2 inhibitor, CMPD101 (3 μM) (Lowe et al., 2015; Bouley et al., 2017), in the presence or absence of aldosterone (1 μM). Then, these cells were stimulated with insulin (100 nM, for 15 min) and tested for the phosphorylation levels of IRS1, at the serine 307 (^{s307}pIRS1). This is a well-consolidated site targeted by GRK2 that, upon phosphorylation, shuts down the insulin signaling (Ciccarelli et al., 2011). We also analyzed the activation of Akt by monitoring its phosphorylation at serine 473 (^{ser473}pAkt). As shown in **Figures 1D, E**, as compared with unstimulated cells (NS), insulin did not increase the amount of ^{s307}pIRS1 but induced a sustained activation of Akt. In stark contrast, aldosterone treatment markedly elevated ^{s307}pIRS1 levels while reducing Akt activity of Akt in response to insulin (**Figures 1D, E**). Of relevance, the detrimental effects exerted by aldosterone on insulin signaling were blunter by pretreating cells with the GRK2 inhibitor, CMPD101 (**Figures 1D, E**). It is worth stressing that neither CMPD101 nor aldosterone (taken singularly) had any influence on the mediators (Akt) of insulin signaling (**Supplementary Figures 2A, B**).

Finally, we tested the impact of spironolactone, an MR antagonist, previously found to block the upregulation of GRK2 as well as its deleterious effects in response to the activation of aldosterone/MR system (Cannavo et al., 2016). To this aim, a group of 3T3 cells was pretreated with spironolactone (10 μM for 30 min) prior to aldosterone and/or insulin treatment. Insulin stimulation induced a robust Akt activation, and the treatment with the mineralocorticoid hormone blunted this effect (**Supplementary Figure 3A**).

Aldosterone Alters βAR Signaling in 3T3 Fibroblasts *via* GRK2 Activation

A correlation exists between aldosterone and βAR activation (Reddy et al., 2015; Hori et al., 2017). More in detail, in experimental models of LV dysfunction based on the chronic infusion of ISO, either spironolactone or eplerenone effectively prevents ISO-triggered collagen deposition (Reddy et al., 2015; Hori et al., 2017). Interestingly, none of these studies, however, have tested whether MR antagonism also prevents βAR dysfunction due to chronic ISO administration as well as whether chronic MR activation has an impact on βAR function and GRK2 upregulation. To fill these critical gaps in knowledge, we examined the effect of aldosterone on βAR function *in vitro,* using 3T3 cells, assessing the activation status of the ERK that is a useful marker of βAR signaling (Cannavo et al., 2018b). To this end, the cells, either unstimulated (NS) or stimulated with ISO (10 μM for 10 min), were pretreated with aldosterone (1 μM for 12 h) and/or with CMPD101 (3 μM). As shown in **Figure 2A**, in response to ISO, we observed a robust ERK

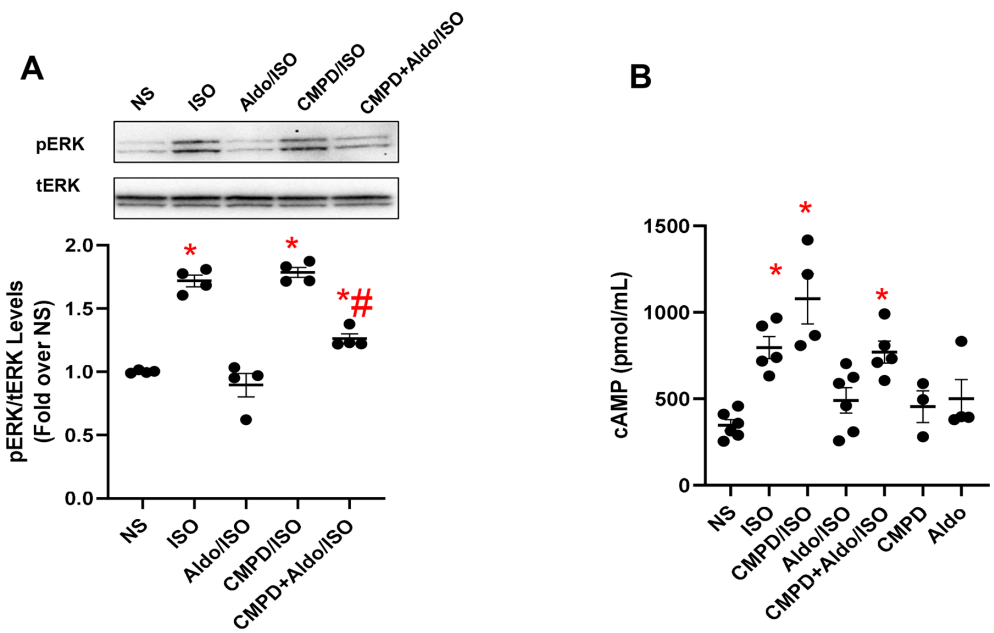

FIGURE 2 | Aldosterone dysregulates βAR signaling *in vitro* in fibroblasts. **(A)** Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) of multiple independent experiments ($n = 4$) to evaluate extracellular signal-regulated kinase (ERK) 1/2 phosphorylation (pERK) as a ratio of activated ERK to total ERK (tERK). The cells were either NS or stimulated with aldosterone (Aldo, 1 μM) and/or CMPD101 (3 μM) for 12 h. After Aldo and/or CMPD101 treatment, cells were stimulated with isoproterenol (ISO, 10 μM) for 15 min. Tukey's *post hoc* test. *$p < 0.05$ vs NS; #$p < 0.05$ vs ISO. **(B)** Dot plots showing levels of cyclic adenosine 3′,5′-monophosphate (cAMP, pmol/ml) in 3T3 fibroblasts NS or stimulated with ISO (10 μM) for 15 min. Prior ISO stimulation some groups of cells were pretreated with Aldo (1 μM) and/or CMPD101 (3 μM) for 12 h. Tukey's *post hoc* test. *$p < 0.05$ vs NS.

activation (pERK) compared with NS cells, which was blocked entirely by aldosterone pretreatment. Nonetheless, pretreatment of cells with CMPD101 significantly abolished the effects of aldosterone and resulted in enhancement of ERK activation in response to ISO. Next, we analyzed the effects of aldosterone on the production of cAMP that is typically elevated after βAR stimulation (Cannavo et al., 2018b). Importantly, we observed that ISO induced a significant rise in cAMP levels, but this effect was abolished by aldosterone pretreatment (**Figure 2B**). Further, the inhibition of GRK2 had a positive influence on the cAMP formation, thus preventing the harmful effects of aldosterone on βARs. Interestingly, neither CMPD101 nor aldosterone alone had a sizable impact on ERK phosphorylation (**Supplementary Figures 4A, B**) and cAMP formation/accumulation (**Figure 2B**). These data support the notion that aldosterone impairs βAR function and that GRK2 is a crucial component of aldosterone-mediated adverse effects.

Chronic *in Vivo* Infusion of Aldosterone Upregulates GRK2 in the Heart

Hyperaldosteronism is a condition that usually precedes or occurs after MI, precipitating LV dysfunction and remodeling (Weber 2001; He et al., 2011; Cannavo et al., 2016). Importantly, chronic (4 weeks) administration of aldosterone (2 μg·mouse·day^{-1}) results in a significant increase in myocyte GRK2 expression and mitochondrial activity, along with augmented cell death and fibrosis, as recently shown by our group (Cannavo et al., 2016). Yet, whether Aldo impairs insulin signaling and βAR function in the mouse heart, *in vivo*, remains to be directly tested. Thus, to translate our *in vitro* findings to *in vivo* clinically relevant situations, we subjected Normal Littermate Control (NLC) (WT) and cardiac-specific GKR2 knockout (cGRK2KO) mice to a 4-week continuous infusion of aldosterone. A group of mice was infused with vehicle solution as a control. We found that in NLC mice, this intervention elicited a marked rise in GRK2 protein levels in LV lysates, as compared with those in vehicle-treated animals (**Figure 3A**). This change was accompanied by a prominent surge in cardiac ^{ser307}pIRS1 levels and by marked downregulation of β1AR density (**Figures 3B, C**). Importantly, the deleterious effects associated with aldosterone stimulation were utterly blunted in cGRK2KO mice (**Figure 3**). Indeed, in the presence of low GRK2 expression (**Supplementary Figure 5**), aldosterone stimulation failed to either increase ^{ser307}pIRS1 levels or reduce β1AR density. Thus, these data attest that GRK2 is chiefly involved in the *in vivo* signaling effects elicited by chronic aldosterone stimulation.

MR Antagonism by Spironolactone Prevents Postischemic Cardiac Insulin Resistance and β1AR Dysfunction Thus Preventing HF Onset

MR blockade, either *via* spironolactone or eplerenone treatment, prevents Aldo-induced increment in GRK2 expression and activity (Cannavo et al., 2016). However, whether spironolactone exerts its protective, anti-ischemia effects, at least in part, through the inhibition of GRK2, and thus restoring cardiac insulin and/or β1AR sensitivity, is currently unknown.

To address these questions, MI was surgically induced in mice *via* coronary artery ligation (Gao et al., 2010). One-day after MI, mice were treated with spironolactone (20 mg·kg·day^{-1}) for the entire study period (4 weeks). Sham-operated animals were used as controls. The echocardiographic analysis was performed at baseline and 4 weeks post-MI (**Figure 4A**).

As shown in **Figure 4B**, MI-inflicted mice displayed a marked deterioration of LV function compared with Sham animals, as indexed by percentage fractional shortening (%FS). Conversely, spironolactone-treated MI mice had significantly ameliorated LV dysfunction and reduced infarct size (**Figures 4B, C**). In a consistent manner, at the tissue level, myocytes from untreated MI mice harbored prominent cardiac fibrosis, assessed by picro-sirius red staining (**Figure 4D**), and augmented myocardial cell death, as per TUNEL staining (**Figure 4E**). It is worth noting that all these adverse effects were significantly blunted in MI mice receiving

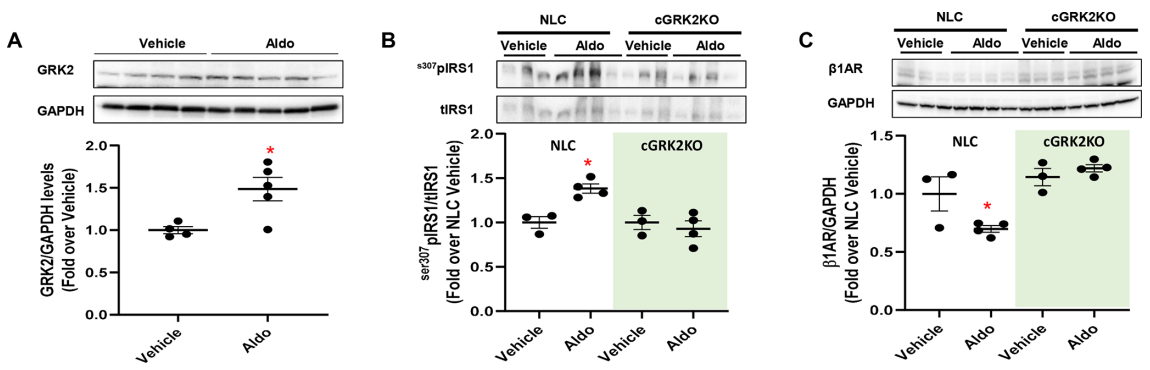

FIGURE 3 | *In vivo* effects of chronic aldosterone infusion on murine myocardium. **(A)** Representative immunoblots (upper panels) and densitometric quantitative analysis (lower panel) showing levels of GRK2 in total cardiac lysates from mice treated with Aldosterone (Aldo, 2 μg·mouse·day^{-1}; $n = 5$) or Vehicle (PBS and EtOH 5%; $n = 4$) for 4 weeks. GAPDH levels were used as loading control; Mann–Whitney test. *$p < 0.05$ vs Vehicle. **(B–C)** Representative immunoblots (upper panels) and quantitative data showing levels of **(B)** phosphorylated IRS1, at serine 307 (^{s307}pIRS1) and **(C)** β1-adrenergic receptor (β1AR) in total cardiac lysates from NLC and cardiac GRK2KO mice either treated with Aldo (2 μg·mouse·day^{-1}; $n = 4$) or Vehicle (PBS and EtOH 5%; $n = 3$) for 4 weeks. GAPDH levels were used as loading control; Dunnet's *post hoc* test. *$p < 0.05$ vs NLC Vehicle.

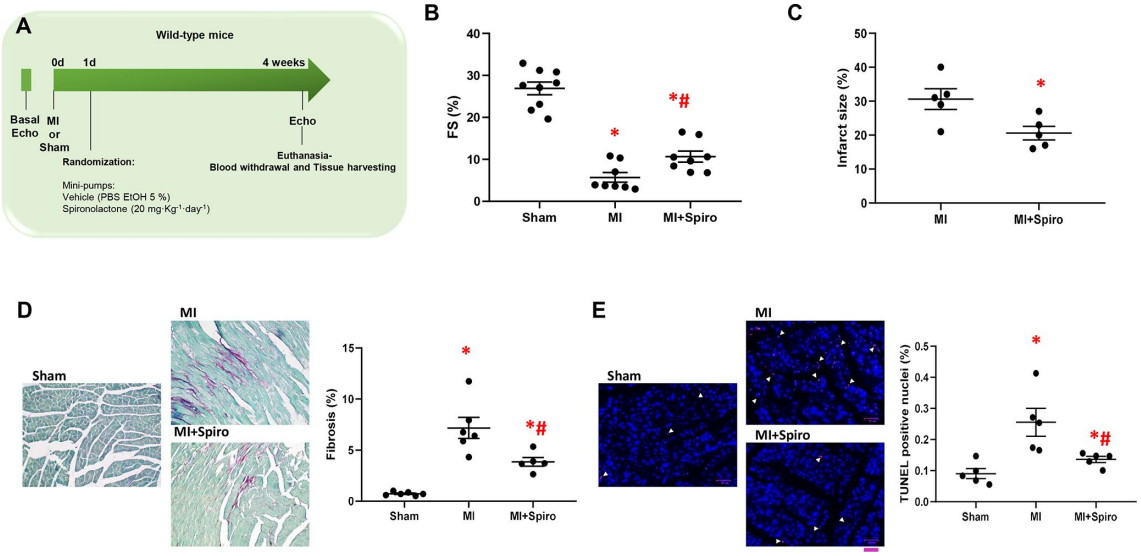

FIGURE 4 | Spironolactone attenuates cardiac dysfunction and prevents adverse remodeling in post-ischemic murine hearts. **(A)** Schematic representation of 4-week study of myocardial infarction (MI) in mice. Echocardiography was performed at day 0 (0d), and MI were surgically induced. Sham-operated mice were used as controls. After 1-day (1d) post-MI, mice were randomized for treatments: MI (controls), MI + Spironolactone (Spiro, 20 mg·kg^{-1}·day^{-1}). Four week post-MI, echocardiography was performed and animals (n = 8–9 per group) euthanized for tissue harvesting. **(B)** Dot plots showing measurements for fractional shortening (FS, %). Tukey's *post hoc* test. *p < 0.05 vs Sham; #p < 0.05 vs MI. **(C)** Dot plots showing percentage of infarct size in MI, MI + Spiro mice (n = 5 mice per group). Mann–Whitney test. *p < 0.05 vs MI. **(D–E)** Representative images and quantitative data showing percentage of **(D)** cardiac fibrosis (Picro-Sirius red staining, magnification 20×) and **(E)** cell death (TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)/DAPI staining, scale bar: 50 μM) in cardiac sections from Sham, MI, and MI + Spiro mice (n = 4–6 mice per group). Tukey's *post hoc* test. *p < 0.05 vs Sham; #p < 0.05 vs MI.

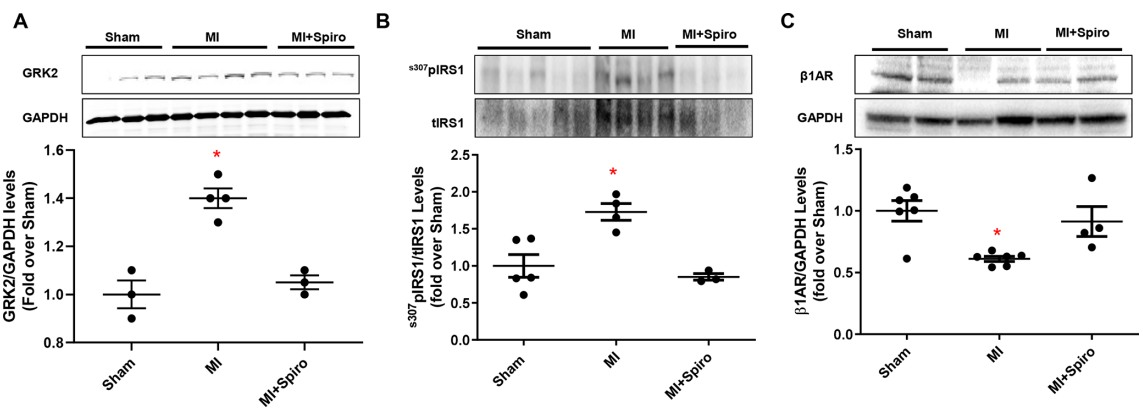

FIGURE 5 | Aldosterone/mineralocorticoid receptor (MR) blockade prevents GRK2 upregulation in postischemic failing hearts and abolishes Insulin and βAR signaling downregulation. **(A–C)** Representative immunoblots (upper panels) and quantitative data (lower panels) showing levels of GRK2 **(A)**, ^{ser307}pIRS1 **(B)** and β1AR **(C)** in total cardiac lysates of Sham, MI, and MI + Spiro mice (n = 3–6 mice per group). tIRS1 and GAPDH levels were used as loading control; Tukey's *post hoc* test. *p < 0.05 versus Sham.

spironolactone (**Figures 4D, E**). Intriguingly, at the molecular level, the marked MI-dependent GRK2 upregulation was prevented by this MR antagonist (**Figure 5A**). Consistently, augmented levels of ^{ser307}pIRS1 and reduced β1AR expression, accompanying the increased GRK2 activity and attesting insulin signaling dysfunction and β1AR downregulation, were also significantly attenuated by spironolactone (**Figures 5B, C**). Thus, MR antagonism by spironolactone ameliorates post-MI LV dysfunction, improving, among other possible beneficial effects, insulin resistance and β1AR dysfunction.

DISCUSSION

SNS and RAS are well-recognized primary pathogenic drivers of HF (Swedberg et al., 1990). Accordingly, most of the agents that block the activation of these systems are part of the current HF therapeutic

armamentarium [i.e. β-blockers, Angiotensin II receptor (AT1R) blockers, ACE inhibitors, and MR antagonists] (Cannavo et al., 2013; D'Addio et al., 2017; Komici et al., 2017). However, since not all patients can benefit from these drugs, there is a keen interest in the identification of additional, specific strategies able to block the hyperactivity of these noxious systems (Shafiq and Miller, 2009). Importantly, MR antagonists have shown to be therapeutic and safe in a wide range of HF patients with reduced EF, both symptomatic (in NYHA class III and IV) (Pitt et al., 1999) and in asymptomatic or mildly symptomatic (in NYHA class I and II) (Zannad et al., 2011). Indeed, these therapeutic agents are currently indicated in the majority of HF patients; thus, the interest in the mechanism beyond MR inhibition is continuously growing.

In this context, we have recently revealed novel mechanisms whereby aldosterone may affect cardiac function (Cannavo et al., 2016). More in details, we have found that the adverse effects of aldosterone on cardiomyocytes are not exclusively mediated by MR activation but also mediated by GRK2 (Cannavo et al., 2016). Importantly, this kinase is centrally involved in MR-mediated cardiac toxicity that is negatively affecting mitochondrial function and survival of cardiomyocytes. Moreover, in the present study, our data add new important pieces to the mosaic of how aldosterone/GRK2 system impacts on cardiac function and remodeling (**Figure 6**). In this context, we have found that aldosterone negatively affects insulin and β1AR function both *in vitro* in 3T3 cells and *in vivo* in the hearts of two murine models of hyperaldosteronism (aldosterone-infused or infarcted mice). Moreover, we have demonstrated that such mechanism appears to be orchestrated by GRK2. More in detail, we have shown in 3T3 cells that soon after (15 and 30 min) aldosterone stimulation initiation, GRK2 becomes upregulated and phosphorylated at s670, which is in line with our previous report in cardiomyocytes (Cannavo et al., 2016). However, GRK2 expression levels remain high even only up to 12 h. After that, the amount of the kinase phosphorylation comes back to control levels. This evidence suggests that, upon phosphorylation, GRK2 is able to both translocate to mitochondria, following phosphorylation, and to bind plasma membrane receptor when unphosphorylated, as previously reported (Pitcher et al., 1999; Cannavo et al., 2016; Cannavo et al., 2018b). In the same vein, here we demonstrate that, after chronic aldosterone treatment of 3T3 cells, a prominent inhibition of insulin signaling occurs, as documented by the increased ^{s307}pIRS1 levels and a subsequent reduction in ^{s473}pAkt levels, followed by the marked impairment of the βAR function with a consequent decrease of ERK activation and cAMP production in response to ISO stimulation. Interestingly, the usage of the GRK2 inhibitor, CMPD101, markedly blunted the effects of aldosterone, preventing both insulin and βAR signaling dysfunction.

Next, we aimed at confirming the *in vitro* findings in an *in vivo* model of hyperaldosteronism, such as the chronic (4 weeks) continuous infusion of aldosterone in mice, as done previously (Cannavo et al., 2016). With this tool in hand, we demonstrated that aldosterone increased cardiac ^{s307}pIRS1 levels and caused a massive

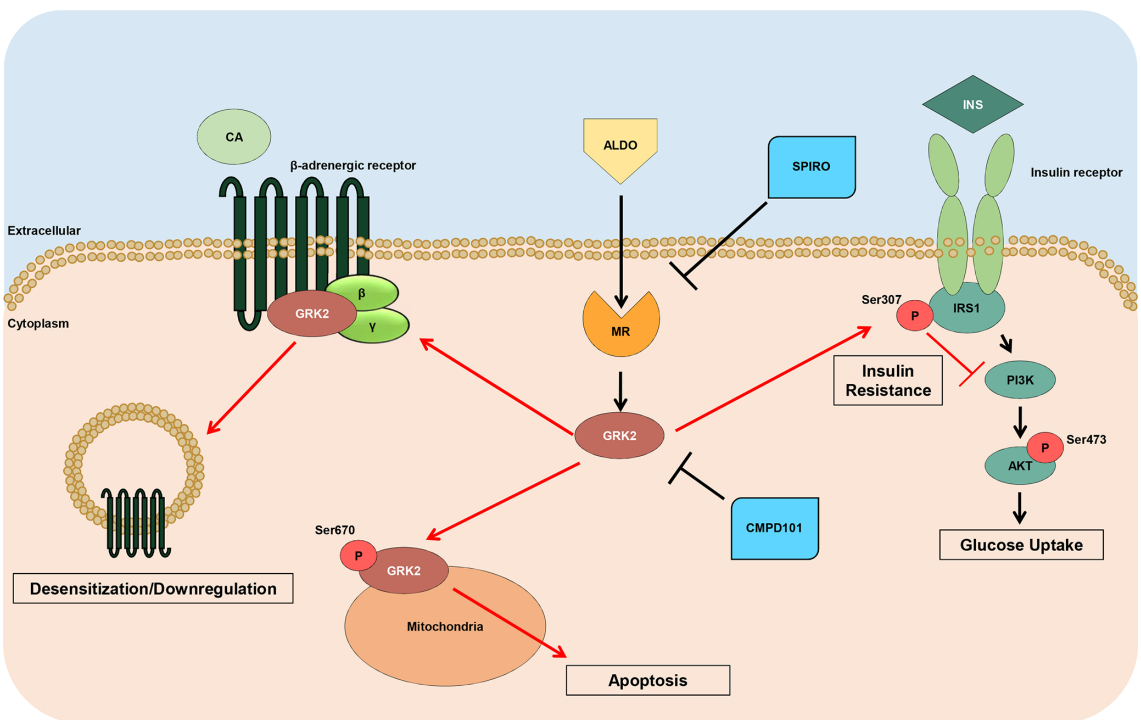

FIGURE 6 | Schematic representation of GRK2 noxious effects downstream Aldosterone. High levels of Aldosterone induce the hyperactivation of MR leading to the upregulation of GRK2, which in turn, induces the negative phosphorylation of IRS1 (^{ser307}pIRS1), with a consequent impaired response to insulin (INS) and reduced Akt activation, and downregulates βARs that do not response to catecholamine (CA) stimulation. Further, this kinase, when phosphorylated at ser670, is able to translocate to mitochondria, where it increases myocyte apoptosis. Of note, either spironolactone or CMPD101 (GRK2-inhibitor) are able to block the expression and noxious effects of GRK2 downstream aldosterone.

β1AR downregulation in NLC mice but not in cGRK2KO mice. This evidence goes hand in hand with previous findings showing that cGRK2KO hearts were functionally and structurally protected against hyperaldosteronism (Cannavo et al., 2016).

Finally, our results that were obtained in infarcted mice with the MR antagonist, spironolactone, which has previously been attested as a blocker of aldosterone-dependent GRK2 upregulation, consolidate the idea of the potential GPCR dependence into the pathological effects induced by aldosterone.

More in details, our data demonstrate that following MI, GRK2 levels are significantly increased while insulin and β1AR signaling pathway are impaired. Conversely, spironolactone treatment is able to mitigate such noxious aldosterone activities and to prevent both IRS1 negative phosphorylation, β1AR downregulation, and GRK2 upregulation. Interestingly, all the beneficial effects of spironolactone correlated with the preserved cardiac function and better remodeling parameters markedly deteriorated by MI.

Limitations

One limitation of our study is the lack of a randomization parameter for the MI studies. One day after MI, we randomly assigned the mice to spironolactone or vehicle treatment, performing an echocardiographic analysis only at baseline (before MI) and at the end of the study period. Although we did not check the degree of the infarct size in the two groups, at 1-day post-MI, we are confident that the infarct area was homogeneous between the animals. Indeed, we have long-standing expertise on *in vivo* MI, and our method has been proven to inflict a very reproducible infarct size (Gao et al., 2010). Moreover, in studies in which we checked for cardiac function after randomization, we did find a statistically significant difference among post-MI randomized groups (Cannavo et al., 2017). Therefore, the effects seen with the different treatments are not due to an unequal initial magnitude of the infarct lesion. Another limitation of the present study is the lack of data regarding the effects of aldosterone on the β2AR signaling pathway. However, β1AR signaling is the major isoform expressed in cardiomyocytes, liable for the main actions of the βAR system on the cardiac function and tightly regulated by GRK2. Future, in-depth studies shall address the impact of aldosterone on the β2AR signaling in cardiomyocytes, fibroblasts, and endothelial cells. Indeed, this receptor subtype is much less expressed at cardiomyocyte level, but it is also targeted by GRK2; therefore, aldosterone may also alter β2AR-dependent cardiac cell functions.

CONCLUSION

Previous evidence has attested that an association exists between aldosterone and insulin signaling dysfunction. However, no studies have assessed the relevance of this cross talk on heart function. Nor have studies clarified whether GRK2, a primary cardiac regulator of IRS1, has any role in the way aldosterone influences the insulin signaling and also in βAR-mediated modulation of myocardial function and response to ischemic stress. Here, we fill these gaps in knowledge by demonstrating, for the first time, that canonical and noncanonical actions of GRK2 account for aldosterone-triggered attenuation of insulin- and βAR-mediated effects at the heart levels. Several clinical cardiovascular disorders are characterized by a persistently elevated "aldosterone tone"; see, for instance, the hypertensive or the HF syndrome. In light of this, the current evidence showing that, in infarcted mice, the MR blocker, spironolactone, offsets aldosterone-induced cardiac insulin-signaling dysfunction and β1AR downregulation by blunting canonical (and likely noncanonical) effects mediated by GRK2 levels suggests the unique opportunity of combining the benefits of a direct MR antagonism to those elicited by the pharmacological inhibition of GRK2, a novel, highly translational perspective that warrants further, in-depth investigation.

ETHICS STATEMENT

All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University Lewis Katz School of Medicine.

AUTHOR CONTRIBUTIONS

ACa, FM, and GR designed, analyzed data, and wrote the manuscript. ACa, LB, FM, AE, DL, GG, and CP performed the experiments. AR, ACi, NF, NP, and WK revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2019.00888/full#supplementary-material

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