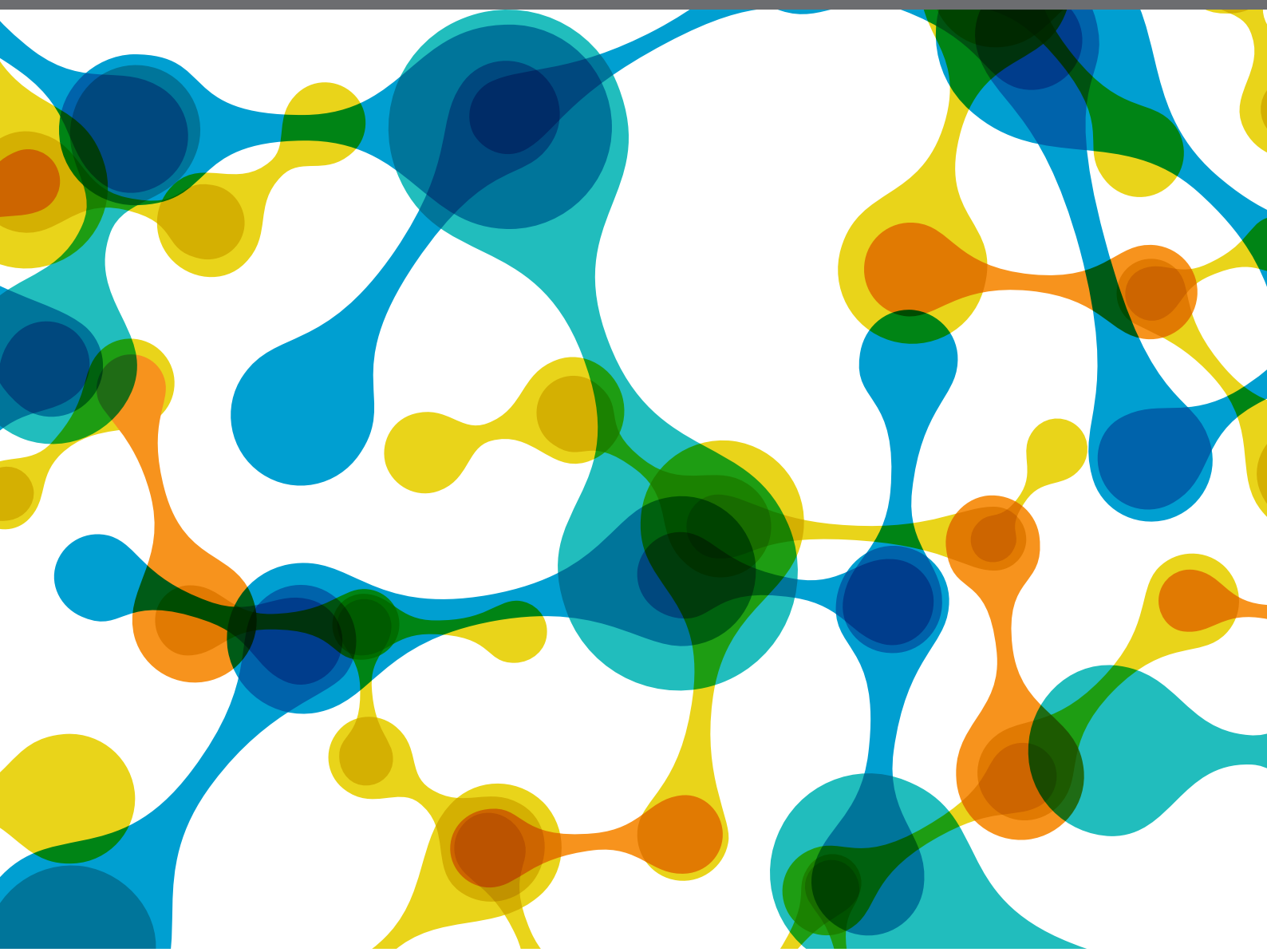
NATRIURETIC PEPTIDES IN CARDIOVASCULAR PATHOPHYSIOLOGY

EDITED BY: Massimo Volpe, Speranza Rubattu, Amie Moyes, Adrian J. Hobbs, S. Jeson Sangaralingham and John C. Burnett

PUBLISHED IN: Frontiers in Physiology



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ISSN 1664-8714
ISBN 978-2-88976-321-4
DOI 10.3389/978-2-88976-321-4

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NATRIURETIC PEPTIDES IN CARDIOVASCULAR PATHOPHYSIOLOGY

Topic Editors:
Massimo Volpe, Sapienza University of Rome, Italy
Speranza Rubattu, Mediterranean Neurological Institute Neuromed (IRCCS), Italy
Amie Moyes, Queen Mary University of London, United Kingdom
Adrian J. Hobbs, Queen Mary University of London, United Kingdom
S. Jeson Sangaralingham, Mayo Clinic, United States
John C. Burnett, Mayo Clinic, United States

Citation: Volpe, M., Rubattu, S., Moyes, A., Hobbs, A. J., Sangaralingham, S. J., Burnett, J. C., eds. (2022). Natriuretic Peptides in Cardiovascular Pathophysiology. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88976-321-4

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frontiers | Frontiers in Physiology

EDITORIAL
published: 18 May 2022
doi: 10.3389/fphys.2022.890158

Editorial: Natriuretic Peptides in Cardiovascular Pathophysiology

Massimo Volpe[1]* *and Speranza Rubattu*[1,2]*

[1]Department of Clinical and Molecular Medicine, School of Medicine and Psychology, Sapienza University of Rome, Rome, Italy, [2]IRCCS Neuromed, Pozzilli, Italy

Keywords: natriuretic peptides, cardiovascular diseases, heart failure, prevention, therapy

Editorial on the Research Topic

Natriuretic Peptides in Cardiovascular Pathophysiology

The natriuretic peptides (NPs) family includes a class of hormones and their receptors needed for the physiological control of cardiovascular functions. Over the last 40 years, several experimental and clinical findings have clarified the fundamental contribution of these hormones to the physiological regulation of blood pressure and of cardiac, vascular, brain and renal functions. The present article collection includes a series of relevant papers discussing old and new concepts on the pathophysiological implications of NPs in cardiovascular diseases, the currently available NP-based drugs for the treatment of heart failure (HF), as well as the new molecules which will be soon tested in the clinical setting. Furthermore, an update is provided on a novel, molecular genetic-based approach aimed at the development of NP-based therapeutic applications in the treatment of major cardiovascular diseases (CVDs).

As shown by Cerrudo et al., increases of plasma atrial natriuretic peptide (ANP) without a concomitant increase of plasma B-type natriuretic peptide (BNP) indicate a state of atrial hemodynamic overload independently of ventricular hypertrophy. In fact, the ventricular re-expression of ANP is mainly induced in volume-overloaded deoxycorticosterone acetate (DOCA)-salt treated rats whereas BNP is induced in pressure-overloaded rats exposed to renovascular hypertension. The ANP/type A natriuretic peptide receptor (NPRA) axis is predominantly responsible for regulating the renal hemodynamic and Na + excretory responses to intravascular blood volume expansion. Notably, the effects of the NP systemic hormones are reinforced by a local renal NP system provided with the machinery for synthesis, action and degradation (Choi and Fernández). As strengthened in the article by Pandey, the ANP/NPRA axis provides cardiac protective mechanisms against maladaptive cardiac disorders, remodeling of CVDs and metabolic disorders. From a mechanistic point of view, an enhanced ANP-BNP/NPRA signaling protects the heart by inhibiting ventricular expression of NF-κB, a master regulator of proinflammatory cytokines. Within the endothelium, NPs exert several beneficial functions including anti-inflammatory and anti-thrombotic effects that support similar protective actions of the arm of the RAAS led by the ACE2-Ang (1-7)-MAS receptor. The combined beneficial vascular properties of NPs and ACE2-Ang (1-7)-MAS could reveal useful in several pathological conditions, even in the setting of the deleterious vascular consequences of the COVID-19 infection, as discussed in the article by Rubattu et al.

In an experimental model of cardio-renal syndrome, the aorto-caval fistula, an abundant expression of both corin and Proprotein Convertase Subtilisin/Kexin Type 6 (PCK6) is found in both cardiac and renal tissues as a possible compensatory mechanism able to enhance ANP/BNP actions and to counteract the development of cardiac hypertrophy, pulmonary congestion and renal dysfunction in this model of volume expansion (Khoury et al.).

OPEN ACCESS

Edited and reviewed by:
Johannes Van Lieshout, University of Amsterdam, Netherlands

***Correspondence:**
Massimo Volpe
massimo.volpe@uniroma1.it
Speranza Rubattu
speranzadonatella.rubattu@uniroma1.it

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *05 March 2022*
Accepted: *02 May 2022*
Published: *18 May 2022*

Citation:
Volpe M and Rubattu S (2022) Editorial: Natriuretic Peptides in Cardiovascular Pathophysiology. Front. Physiol. 13:890158. doi: 10.3389/fphys.2022.890158

Noteworthy, the review article by Miyoshi et al. shows evidence that the NP level assessed in umbilical cord blood and amniotic fluid may serve as a diagnostic biomarker of HF in fetuses with congenital heart disease and/or arrhythmia. This interesting result expands the common knowledge on the role of NPs as biomarkers for diagnosis of HF.

An intertissue communication between adipose and cardiac tissues has been documented in previous works. First, NPs play a lipolytic effect. Furthermore, the known inverse relationship between circulating BNP and body mass index (BMI), termed as the "natriuretic handicap," is at least in part related to the increased expression of type C natriuretic peptide receptor (NPR-C) leading to enhanced degradation of BNP in adipose tissue. Moving from this evidence, the article by Egom develops the concept that the NPR-C pathway may play on its own a pathophysiological role, particularly in the context of obesity-related HF with preserved ejection fraction (HFpEF). In fact, it is worth mentioning that NPR-C plays biological functions other than the known role of clearance receptor. The signaling pathway underlying its biological properties may be the inhibition of adenylyl cyclase (AC) through a pertussis toxin–sensitive inhibitory G protein (Gi) or activation of phospholipase C (PLC) through Gi protein, therefore reducing adenylyl cyclase activity and intracellular cAMP levels. The author proposes the concept that the increased expression of NPR-C may in part explain the "obesity paradox" (that refers to the fact that obese patients with established HF tend to have better long-term prognosis than non-obese patients). Within the specific condition of obesity-related HFpEF, low NPR-C activity may promote cardiac fibrosis and remodeling, leading to diastolic dysfunction, the major cardiac functional deficit in HFpEF. Therefore, as pointed out by Egom, an enhanced NPR-C mRNA levels in various cardiometabolic disorders may represent a compensatory response to low NPR-C activity with the goal of re-establishing cardiometabolic function. The NPR-C pathway may represent a novel therapeutic target in cardiometabolic disorders, including but not limited to obesity and insulin resistance, in addition to HFpEF. Enhancing the NPR-C pathway may also represent an attractive therapeutic strategy to reduce body wasting, increase the ability to tolerate higher HF therapeutic doses of neurohumoral inhibitors, and improve HF outcomes.

In the context of hypertension and of anti-hypertensive therapy, the availability of agents enhancing the natriuretic biological functions has been intensively pursued over the last 2 decades. The recent design of MANP, a mimetic peptide, represents a promising strategy that fulfils the expectancy. In fact, MANP reduces blood pressure levels through the promotion of increased natriuresis, diuresis and aldosterone suppressing properties, similarly to native ANP. The first human trial confirmed the role of MANP as a valuable anti-hypertensive agent (Cannone and Burnett). The proANP 31-67 peptide, derived from the amino terminal fragment of NT-proANP, is another promising molecule. It shows cardiorenal protective actions in preclinical models and can be supported as a therapeutic strategy to counteract hypertensive and diabetic organ damage, renal diseases, obesity and HF (da Silva et al.).

Neprilysin/AT1R blockade (sacubitril/valsartan, S/V) is a novel therapeutic strategy introduced for the treatment of HF with reduced EF (HFrEF). As discussed by Gallo et al., the benefits of a S/V-based strategy have been demonstrated along most of the HF continuum, in which the neurohormonal dysfunction has a pivotal role in the development and progression of the disease. In fact, S/V appears to provide consistent benefits in a left ventricular EF (LVEF) range between 25 and 50%. This evidence suggests that HF patients with mid-range EF (HFmrEF) could be a reasonably successful target for S/V-based treatment, thus extending the current recommendations for this drug. As highlighted by the authors, a dichotomous vision of clinical presentations of HF, based on the LVEF values, should be dismissed in guidelines and trial designed clinical practice to define borders and boundaries of patient classification. Instead, the current evidence with S/V supports the notion that HF should be rather viewed as a continuous variable reflecting the whole spectrum of the properties of the LV.

Several possible therapeutic targets exist within the NP system beyond Neprilysin, all with the promise of improving HF treatment (Gidlof). In the specific, we know about the existence of epigenetic mechanisms actively regulating the NP system at multiple levels. Many of these mechanisms are active in the failing myocardium and they could potentially be exploited for therapeutic NP augmentation. They include several potential RNA targets such as miR-425 and miR-155. Furthermore, miR-100 and miR-143 could constitute potential RNA-based targets to achieve an increased level of circulating NPs. Currently, work is ongoing to elucidate the therapeutic benefit of NPPA-AS1 knock-down, a natural antisense transcript with potential regulatory capacity, in models of HFpEF and HFrEF. Finally, NPR-C silencing could result in increased circulating levels of ANP and reduced cardiac hypertrophy and fibrosis in HF.

Overall, this is an extremely interesting and expanding research field that needs many experimental and clinical studies addressed to meet the expectancies and ultimately to provide the next generation of NP-based therapy.

AUTHOR CONTRIBUTIONS

MV and SR contributed to conception and writing of the editorial, and approved the submitted version.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Physiology

MINI REVIEW
published: 18 March 2021
doi: 10.3389/fphys.2021.654356

Significance of Atrial and Brain Natriuretic Peptide Measurements in Fetuses With Heart Failure

Takekazu Miyoshi[1,2], Hiroshi Hosoda[1] and Naoto Minamino[3]*

[1]*Department of Regenerative Medicine and Tissue Engineering, National Cerebral and Cardiovascular Center, Suita, Japan,* [2]*Clinical Research Center, National Center for Child Health and Development, Tokyo, Japan,* [3]*Omics Research Center, National Cerebral and Cardiovascular Center, Suita, Japan*

Fetal heart failure is mainly caused by congenital heart defect and arrhythmia. It is difficult to appropriately diagnose the severity of fetal heart failure simply by ultrasonography because the development of a fetal heart in fetoplacental circulation and how well the fetal myocardium can adapt to postnatal cardiopulmonary circulation are challenging to assess. In adult cardiology, natriuretic peptides (NPs) are the most useful biomarker of heart failure; however, studies investigating NP levels in the fetuses and amniotic fluid are quite limited. Furthermore, little is known about their production and metabolism. This review summarized the most relevant findings on NP levels in the umbilical cord blood and amniotic fluid. The findings can then extend their use as a diagnostic biomarker of heart failure in fetuses with congenital heart defect and/or arrhythmia.

Keywords: natriuretic peptide, fetal heart failure, congenital heart defect, arrhythmia, placenta, metabolism, prenatal diagnosis, ultrasonography

OPEN ACCESS

Edited by:
Speranza Rubattu, Istituto Neurologico Mediterraneo Neuromed (IRCCS), Italy

Reviewed by:
Massimo Volpe, Sapienza University of Rome, Italy
Mariann Gyöngyösi, Medical University of Vienna, Austria
Seethalakshmi R. Iyer, Mayo Clinic, United States

****Correspondence:***
Hiroshi Hosoda
hosoda.hiroshi.ri@ncvc.go.jp

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *16 January 2021*
Accepted: *02 March 2021*
Published: *18 March 2021*

Citation:
Miyoshi T, Hosoda H and Minamino N (2021) Significance of Atrial and Brain Natriuretic Peptide Measurements in Fetuses With Heart Failure. Front. Physiol. 12:654356. doi: 10.3389/fphys.2021.654356

INTRODUCTION

Prenatal diagnosis of complex congenital heart defects (CHDs) is critical to predict the need for emergent postnatal interventions and facilitates a more rapid stabilization of postnatal circulation (Levey et al., 2010; Holland et al., 2015). The pathophysiology of CHDs, including Ebstein's anomaly, is associated with a high perinatal mortality caused by the progression of heart failure *in utero* (Freud et al., 2015). Sustained tachyarrhythmia or bradyarrhythmia is associated with the progression of fetal heart failure (Schmidt et al., 1991; Naheed et al., 1996). Therefore, transplacental antiarrhythmic therapy for fetal tachyarrhythmia is highly recommended during preterm gestation (Donofrio et al., 2014). An accurate prenatal diagnosis of structural heart abnormalities or arrhythmias and an assessment of heart failure *in utero* are important in providing appropriate management for fetuses with CHD and/or arrhythmia (Miyoshi et al., 2019c).

The development of a fetal heart in fetoplacental circulation and how well the fetal myocardium can adapt to postnatal cardiopulmonary circulation are difficult to assess. Thus, fetal heart failure is still challenging to diagnose. Recent studies have shown that the cardiovascular profile score is a comprehensive echocardiographic marker of fetal heart failure (Falkensammer et al., 2001; Huhta, 2005; Hofstaetter et al., 2006). This method is based on a composite scoring system for grading the severity of fetal heart failure using five echocardiographic parameters that are as follows: fetal effusion, arterial and venous Doppler findings, heart size,

and cardiac function. The cardiovascular profile score may be useful in the baseline and serial evaluations of fetuses at risk of myocardial dysfunction. However, there are some issues associated with the use of echocardiography for evaluating cardiac function in fetuses with CHD and/or arrhythmia (Wieczorek et al., 2008; Miyoshi et al., 2019c). For example, the severity of tricuspid valve regurgitation is not always easy to evaluate accurately (Neves et al., 2014). The gap in timing between atrial contraction and atrioventricular valve closure due to arrhythmias leads to abnormal venous Doppler findings. Therefore, it is important to identify objective biomarkers that can reflect the severity of fetal heart failure.

Natriuretic peptides (NPs) are established as biomarkers of heart failure in adult cardiology (Böhm et al., 2011; McMurray et al., 2012); however, studies investigating NP levels in the fetuses and amniotic fluid are quite limited. Furthermore, little is known about their production and metabolism. This review summarized the most relevant findings on NPs in the umbilical cord blood and amniotic fluid. This finding can extend their use as a diagnostic biomarker of heart failure in fetuses with CHD and/or arrhythmia.

CLINICAL SIGNIFICANCE OF NPs AS BIOMARKERS OF FETAL HEART FAILURE

Umbilical Cord Blood NP Levels

Our previous study showed that plasma NP levels in the umbilical cord blood were correlated with the severity of heart failure in fetuses with CHD and/or arrhythmia (Miyoshi et al., 2018b; **Table 1**). The plasma concentrations of atrial NP (ANP), brain NP (BNP), and N-terminal fragment of pro-brain NP (NT-proBNP) in the umbilical cord blood had similar profiles in heart failure. Several studies showed that fetuses with CHD have significantly higher NT-proBNP levels in the umbilical cord blood than controls (Lechner et al., 2009; Merz et al., 2012; Bae et al., 2015; Lee et al., 2016). The NT-proBNP levels in the umbilical cord blood of fetuses with a single ventricular physiology are significantly higher than those with a biventricular physiology. Fetuses with a ventricular outflow tract obstruction and an intact interventricular septum have significantly higher NT-proBNP levels than those with other types of CHD. Hence, a high ventricular pressure leads to increase NP levels (Merz et al., 2012; Bae et al., 2015). We analyzed in detail the association between heart failure severity and NP concentrations. Results showed that, compared with other types of CHD, right heart defects with moderate or severe tricuspid valve regurgitation, including Ebstein's anomaly, are associated with lower cardiovascular profile scores and higher NP levels (Miyoshi et al., 2018b). Conversely, fetuses with a right heart defect but no or mild tricuspid valve regurgitation, which does not lead to high right ventricular pressure, had low NP levels. These findings strongly support the notion that elevated NP levels are mainly attributed to a high central venous pressure, rather than morphological abnormality itself.

Fetal tachyarrhythmia or bradyarrhythmia was strongly correlated with high NP levels (Miyoshi et al., 2018b). Abnormal venous Doppler sonography findings were more common and severe in fetuses with tachyarrhythmia or bradyarrhythmia than in those with CHD. Elevated NP levels are closely associated with abnormal venous Doppler findings, which indicate an increase in central venous pressure (Johnson et al., 1992). Elevated wall stress leads to cardiac remodeling and hypertrophy, which increases myocardial oxygen consumption and aggravates myocardial function. To overcome reduction in ventricular compliance, end-diastolic filling pressure, and hydrostatic central venous pressure increase to maintain cardiac output, thereby resulting in a higher release of NP from the fetal heart (Harada et al., 1998; Gardiner, 2005). Furthermore, our previous study found that NP levels in the umbilical cord blood reflect the severity of fetal arrhythmia and responses to fetal therapy. In the fetuses with tachyarrhythmias, NP levels in the responders of fetal therapy decreased to the levels similar to normal fetuses. Thus, NP concentrations can be used as biomarkers for the efficacy of fetal therapy (Miyoshi et al., 2019a). Similar to adults, damage to the ventricular wall in fetal tachyarrhythmia is reversible *in utero* (Gopinathannair et al., 2015). Meanwhile, the group with no indications for fetal therapy had significantly lower cardiovascular profile scores than the control group and had similar NP levels in the umbilical cord blood. Thus, NP levels complement echocardiographic assessment, and they may be useful in determining whether fetal treatment for arrhythmia is indicated.

Preterm birth and fetal acidemia are associated with high NP levels in fetuses with CHD and/or arrhythmia (Miyoshi et al., 2018b). Earlier studies have shown that gestational age is not an important determinant of ANP levels in fetuses and newborns (Kingdom et al., 1992; Ville et al., 1994). Plasma ANP levels in the umbilical cord blood were actually higher in fetuses with hydrops than in controls. Therefore, preterm birth caused by fetal heart failure or hydrops may contribute to high NP levels. A previous study has revealed that umbilical cord vein ANP levels were inversely correlated to umbilical artery pH (Kingdom et al., 1992). Maternal hypertensive disorder and fetal acidemia during labor stimulate fetal ANP production (Mäkikallio et al., 2001). In a recent research, high umbilical cord blood BNP levels and low pH might be associated with adverse outcomes in fetuses with CHD (Sahin-Uysal et al., 2020). Therefore, NP levels in the umbilical cord blood may be a useful surrogate marker of fetal maturation and antenatal stress (Kanbe et al., 2009).

Amniotic Fluid NP Levels

In the amniotic fluid, NT-proBNP levels increase in a stepwise fashion with the severity of fetal heart failure in fetuses with CHD and/or arrhythmia (Miyoshi et al., 2018a). In contrast, ANP and BNP concentrations in the amniotic fluid are extremely low; hence, they are not good markers of fetal heart failure (**Table 1**). Although NT-proBNP is released from cardiomyocytes in equimolar amounts of BNP, it is not metabolized by the NP receptor C (NPR-C). Moreover, the

Abbreviations: NP, Natriuretic peptide; CHD, Congenital heart defect; ANP, Atrial natriuretic peptide; BNP, Brain natriuretic peptide; NT-proBNP, N-terminal fragment of pro-brain natriuretic peptide; NPR-C, NP receptor C.

TABLE 1 | ANP, BNP, and NT-proBNP in the umbilical cord blood and amniotic fluid in fetuses with CHD/arrhythmia.

	ANP	BNP	NT-proBNP
Detection specificity	Mature ANP proANP	Mature BNP proBNP	NT-proBNP proBNP
Umbilical cord blood			
Concentration, median (minimum–maximum)*	42 (6–1,975) pg/ml	18 (0.2–1,276) pg/ml	636 (140–24,921) pg/ml
Major molecular form	Mature ANP	proBNP	NT-proBNP
Major sites of metabolism	Placenta and umbilical vessels	Not identified	Not identified
Mechanism of metabolism	NPR-C NEP digestion	NPR-C NEP digestion	Protease digestion
Stability	Unstable	Less stable	Stable
Use as a biomarker of fetal heart failure	Useful	Useful	Highly useful
Factors affecting concentrations	Fetal heart failure, tachyarrhythmias or bradyarrhythmias, right heart defects with moderate or severe TR, ventricular outflow tract obstruction without interventricular septum, preterm birth, and acidemia	Fetal heart failure, tachyarrhythmias or bradyarrhythmias, right heart defects with moderate or severe TR, ventricular outflow tract obstruction without interventricular septum, preterm birth, and acidemia	Fetal heart failure, tachyarrhythmias or bradyarrhythmias, right heart defects with moderate or severe TR, ventricular outflow tract obstruction without interventricular septum, preterm birth, and acidemia
Amniotic fluid			
Concentration, median (minimum–maximum)#	0.3 (0.2–9.8) pg/ml	3.9 (0.2–15.3) pg/ml	48 (7–1,329) pg/ml Approximately 1/30 of the umbilical vein level
Stability	Unstable	Unstable	Stable
Use as a biomarker of fetal heart failure	Not applicable	Not applicable	Extremely useful
Factors affecting concentrations	Not identified	Not identified	Fetal heart failure, tachyarrhythmias or bradyarrhythmias, right heart defects with moderate or severe TR, and gestational age

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CHD, congenital heart defect; NEP, neutral endopeptidase; NPR, natriuretic peptide receptor; NT-proBNP, N-terminal fragment of pro-brain natriuretic peptide; proANP: pro-atrial natriuretic peptide; proBNP; pro-brain natriuretic peptide; and TR, tricuspid valve regurgitation.

**Umbilical vein plasma (Miyoshi et al., 2018a).*

#Amniotic fluid (Miyoshi et al., 2018b).

half-life of NT-proBNP is significantly longer than that of BNP (McMurray et al., 2012). The glycosylation of NT-proBNP may further prevent metabolism *via* protease digestion in the amniotic fluid, similar in the blood (Hammerer-Lercher et al., 2008). Amniotic fluid NT-proBNP levels were strongly correlated with umbilical vein plasma NT-proBNP levels. Preterm birth, fetal tachyarrhythmias or bradyarrhythmias, and right heart defects with moderate or severe tricuspid valve regurgitation were associated with high amniotic fluid NT-proBNP levels, similar to the umbilical vein plasma NT-proBNP levels (Miyoshi et al., 2018b). Amniotic fluid NT-proBNP levels in fetuses with fetal tachyarrhythmias or bradyarrhythmias and right heart defects with moderate or severe tricuspid valve regurgitation were median 230 (range, 50–539) pg/ml and median 231 (range, 132–1,329) pg/ml, respectively, which were significantly higher than those of median 33 (range, 1–185) pg/ml in normal fetuses.

There are few data on the association between amniotic fluid NPs and fetal heart failure. Previous studies have reported a good correlation between amniotic fluid NT-proBNP levels and the severity of twin-twin transfusion syndrome in monochorionic diamniotic twin pregnancies (Bajoria et al., 2002; Delabaere et al., 2010; Habli et al., 2010; Van Mieghem et al., 2010). Both donor and recipient twins develop heart failure in severe twin-twin transfusion syndrome. However, BNP release is affected by factors such as fetal hypoxemia and renin transfer involving placental shunting from the donor to the recipient twin. Moreover, whether BNP production in the amniotic membrane is affected by polyhydramnios or oligohydramnios due to the twin-twin transfusion syndrome remains unclear. Our study focused on singletons with CHD and/or arrhythmia and systematically compared the association between amniotic fluid NP levels and fetal heart failure. We concluded that amniotic fluid NT-proBNP levels can reflect the severity of fetal heart failure (Miyoshi et al., 2018a).

Several studies have investigated NP levels in the umbilical cord blood and amniotic fluid upon delivery. The effects of maternal blood or vaginal secretion cannot be completely eliminated during amniotic fluid NP measurements, even though the mode of delivery and labor were not associated with NP concentrations (Miyoshi et al., 2018a). Percutaneous umbilical blood sampling or amniocentesis is required to provide real-time information and to identify therapeutic strategies with NPs in fetuses with CHD and/or arrhythmia. Ultrasonography is a non-invasive and repeatable investigation, while percutaneous umbilical blood sampling and amniocentesis are invasive and have several medical restrictions. However, amniocentesis is a common obstetric procedure that uses a hollow needle inserted into the uterus for screening chromosomal abnormalities in a fetus. Compared with percutaneous umbilical cord blood sampling, amniocentesis has a lower rate of complications and is technically easier to perform (Bigelow et al., 2016; Salomon et al., 2019). Our results will help to optimize the design of prospective studies using cardiovascular profile scores, and the

measurement of NP concentrations in amniotic fluid samples collected *via* amniocentesis should be planned to identify the proper timing of delivery and improve the prognosis of fetuses with CHD and/or arrhythmia. Amniotic fluid NT-proBNP measurements are expected to complement the inadequate points of echocardiography in fetuses with CHD and/or arrhythmia.

PRODUCTION AND METABOLISM OF NPs IN THE FETOPLACENTAL CIRCULATION

Molecular Forms and Metabolism of NPs in the Fetoplacental Circulation

A recent study has found the differential metabolism of ANP and BNP in the fetoplacental circulation (Miyoshi et al., 2019b; **Figure 1**). After passing through the placenta, the ANP levels in the umbilical vein plasma decreased to approximately one-half of the levels in the umbilical artery plasma in fetuses with CHD and/or arrhythmia and in controls. Thus, the placenta and umbilical vessels may be the major sites of ANP metabolism. Interestingly, previous reports showed that ANP, but not BNP, is expressed in the human placenta, particularly in cytotrophoblast cells (Lim and Gude, 1995; Graham et al., 1996). In our study cohort, there were several cases in which the ANP levels were higher in the umbilical vein than in the umbilical artery plasma. Hence, ANP may be secreted from the placenta locally or into the fetoplacental circulation (Miyoshi et al., 2019b) and may play a pivotal role in the regulation of fetoplacental hemodynamics.

By contrast, plasma BNP levels did not almost decrease after passing through the placenta, regardless of the type or presence of fetal heart disease. In adult cardiology, the half-life of BNP in the plasma is 10-fold longer than that of ANP (Mukoyama et al., 1991). Binding and internalization by the NPR-C and enzymatic degradation are the two main pathways involved in the clearance of circulating NPs (Nussenzveig et al., 1990; Norman et al., 1991). NPR-C-mediated degradation is the major mechanism responsible for the clearance of NPs from the circulation (Matsuo et al., 2019). The binding affinity of ANP to NPR-C is greater than that of BNP to NPR-C (Koller et al., 1991; Suga et al., 1992). Delayed BNP metabolism also reflects relative resistance to neutral endopeptidase, which is a major peptidase responsible for NP degradation (Smith et al., 2000; Walther et al., 2004). Therefore, the lower affinity of BNP to NPR-C and its resistance to neutral endopeptidase can make BNP more stable in the fetoplacental circulation.

Reverse-phase high-performance liquid chromatography revealed that in the fetoplacental circulation, ANP and BNP mainly comprised the mature and precursor forms, respectively (Miyoshi et al., 2019b; **Table 1**). In the adult circulation, ANP

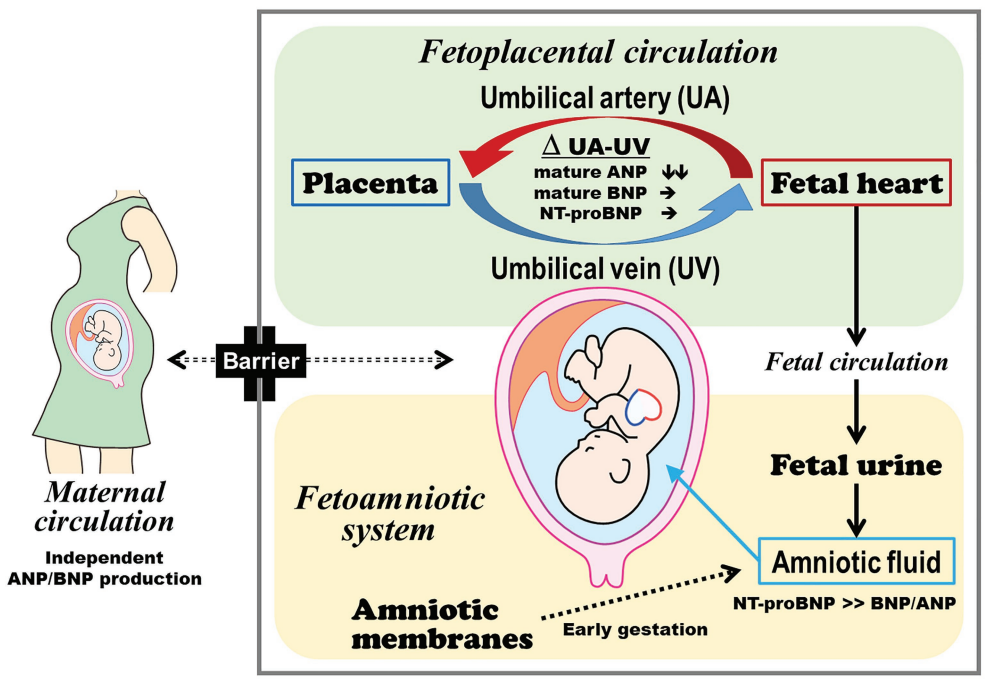

FIGURE 1 | Production and metabolism of ANP, BNP, and NT-proBNP in the fetoplacental circulation and amniotic fluid. The fetus and mother independently produce and metabolize ANP and BNP. Fetal plasma ANP comprises the mature form, and the placenta and umbilical vessels can be the major sites of ANP metabolism. Fetal plasma BNP predominantly consists of the precursor form, which may reduce BNP metabolism in the fetoplacental circulation in addition to its lower affinity to natriuretic peptide receptor C. The plasma ANP, BNP, and NT-proBNP levels in the umbilical cord blood reflect the severity of heart failure. Amniotic fluid NT-proBNP derived from the fetal heart *via* fetal urine can be used to assess fetal heart failure at late preterm gestation or thereafter. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; and NT-proBNP, N-terminal fragment of pro-brain natriuretic peptide.

circulates as a mature alpha-ANP with full bioactivity, and BNP in the blood mainly comprises mature, fully active BNP-32 and weakly active its precursor proBNP (Matsuo et al., 2019). Recent studies have shown that glyco-proBNP – a glycosylated precursor – is a major circulating component, which results from impaired processing events by the glycosylation of threonine-71 of proBNP, in adults (Liang et al., 2007; Seferian et al., 2007; Semenov et al., 2009; Miller et al., 2011). ProBNP is highly glycosylated, and its properties are different from those of simple 108-residue proBNP peptide. The presence of highly glycosylated proBNP in the circulation may help to reduce BNP metabolism in the placenta and umbilical vessels since glycosylation generally provides protective effects against proteases (McCarthy et al., 2014). Differences in the circulating molecular forms are also responsible for the different properties in the metabolic clearance between ANP and BNP in the fetoplacental circulation. In adult patients with acute decompensated heart failure, self-compensation of myocardium for heart failure occurred by increasing mature BNP secretion *via* accelerating proBNP processing and activating the BNP/cGMP cascade (Takahama et al., 2018). Further studies should be conducted to validate the pathophysiology and prognostic value of different ANP and BNP molecular forms in fetuses with heart failure.

Several studies have shown that there is no or little exchange of ANP, BNP, and NT-proBNP across the placenta (Castro et al., 1989; Bar-Oz et al., 2005; Lechner et al., 2009). Fetus and mother secrete ANP and BNP independently of each other, and high NP levels in the umbilical cord plasma of fetuses with CHD and/or arrhythmia are primarily derived from the fetal heart (Miyoshi et al., 2019b). Therefore, the plasma concentrations of ANP and BNP in the fetoplacental circulation are likely to be regulated by the balance between production by the fetal heart and metabolism and clearance in the placenta and umbilical vessels.

Origin of Amniotic Fluid NPs

The major source of NPs in the amniotic fluid has not yet been established (**Figure 1**). Fetal urine and lung fluid are contributors to amniotic fluid volume and NP concentrations (Merz et al., 2014; Carvajal et al., 2017). Amniotic membranes also produce and secrete NPs (Itoh et al., 1993; Carvajal et al., 2009). Gestational age should be considered in the assessment of amniotic fluid NT-proBNP concentrations. In normal fetuses with early gestation age, the amniotic membranes are the main source of NPs in the amniotic fluid. The reference values for amniotic fluid NT-proBNP in normal fetuses gradually decreased according to the progression of pregnancy, and it reaches a plateau after 34 weeks of gestation (Merz et al., 2014; Carvajal et al., 2017). A correlation between amniotic fluid and umbilical cord blood NT-proBNP concentrations was observed, even though the amniotic fluid had significantly lower NT-proBNP concentrations than the plasma (Miyoshi et al., 2018a). This correlation was similar to that between urinary and plasma NT-proBNP concentrations in adult patients with heart failure (Hammerer-Lercher et al., 2008). Taken together, at late preterm gestation or thereafter, amniotic fluid NT-proBNP is considered to be mainly derived from the fetal heart and can be used to assess fetal heart failure.

CONCLUSION

Plasma NP levels in the umbilical cord blood reflect the severity of heart failure in fetuses with CHD and/or arrhythmia. Elevated NP levels are mainly attributed to an increase in central venous pressure secondary to arrhythmia or atrioventricular valve regurgitation caused by CHD. The plasma concentrations of ANP, BNP, and NT-proBNP in the umbilical cord blood had similar correlation profiles with the severity of fetal heart failure. Meanwhile, NT-proBNP levels in the amniotic fluid and umbilical cord blood are strongly correlated and amniotic fluid NT-proBNP levels increase according to the severity of fetal heart failure. In contrast, the ANP and BNP concentrations in the amniotic fluid are extremely low and, thus, are not good markers for assessing fetal heart failure.

The fetus and mother produce and metabolize NPs independently of each other. Metabolism in the fetoplacental circulation is quite different between ANP and BNP. Fetal plasma ANP comprises the mature form, and the placenta and umbilical vessels may be the major sites of ANP metabolism. Fetal plasma BNP predominantly consists of the precursor form, which may reduce BNP metabolism in the fetoplacental circulation in addition to its lower affinity to NPR-C.

The features of ANP, BNP, and their related peptides in the umbilical cord blood and amniotic fluid provided a strong basis for their use as biomarkers that can complement the inadequate points of ultrasonography.

AUTHOR CONTRIBUTIONS

TM drafted the manuscript. HH and NM edited and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by JSPS KAKENHI Grants (19K08343) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. This work was also supported in part by grants from the National Cerebral and Cardiovascular Center (26-6-1, 27-1-5), the National Center for Child Health and Development (2020B-8), and the Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics. The funding sources were not involved in the study design, collection, analysis, and interpretation of data, writing of the manuscript, and decision to submit the article for publication.

ACKNOWLEDGMENTS

We would like to thank Enago (www.enago.jp) for English language review.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 21 May 2021
doi: 10.3389/fphys.2021.674254

Natriuretic Peptide Clearance Receptor (NPR-C) Pathway as a Novel Therapeutic Target in Obesity-Related Heart Failure With Preserved Ejection Fraction (HFpEF)

Emmanuel Eroume A. Egom[1,2]*

[1] Institut du Savoir Montfort, Hôpital Montfort, University of Ottawa, Ottawa, ON, Canada, [2] Laboratory of Endocrinology and Radioisotopes, Institute of Medical Research and Medicinal Plants Studies, Yaoundé, Cameroon

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Edited by:
Speranza Rubattu, Istituto Neurologico Mediterraneo Neuromed Scientific Institute for Research, Hospitalization and Healthcare (IRCCS), Italy

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Alexander E. Berezin, Zaporizhzhia State Medical University, Ukraine
Ines Falcão-Pires, Universidade do Porto, Portugal

***Correspondence:**
Emmanuel Eroume A. Egom
egomemmanuel@gmail.com

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *01 March 2021*
Accepted: *12 April 2021*
Published: *21 May 2021*

Citation:
Egom EEA (2021) Natriuretic Peptide Clearance Receptor (NPR-C) Pathway as a Novel Therapeutic Target in Obesity-Related Heart Failure With Preserved Ejection Fraction (HFpEF). Front. Physiol. 12:674254. doi: 10.3389/fphys.2021.674254

Heart failure (HF) with preserved ejection fraction (HFpEF) is a major public health problem with cases projected to double over the next two decades. There are currently no US Food and Drug Administration–approved therapies for the health-related outcomes of HFpEF. However, considering the high prevalence of this heterogeneous syndrome, a directed therapy for HFpEF is one the greatest unmet needs in cardiovascular medicine. Additionally, there is currently a lack of mechanistic understanding about the pathobiology of HFpEF. The phenotyping of HFpEF patients into pathobiological homogenous groups may not only be the first step in understanding the molecular mechanism but may also enable the development of novel targeted therapies. As obesity is one of the most common comorbidities found in HFpEF patients and is associated with many cardiovascular effects, it is a viable candidate for phenotyping. Large outcome trials and registries reveal that being obese is one of the strongest independent risk factors for developing HFpEF and that this excess risk may not be explained by traditional cardiovascular risk factors. Recently, there has been increased interest in the intertissue communication between adipose tissue and the heart. Evidence suggests that the natriuretic peptide clearance receptor (NPR-C) pathway may play a role in the development and pathobiology of obesity-related HFpEF. Therefore, therapeutic manipulations of the NPR-C pathway may represent a new pharmacological strategy in the context of underlying molecular mechanisms.

Keywords: obesity, heart failure with preserved ejection fraction, NPR-C, natriuretic peptide receptor C, adipose tissue, heart failure, co-morbiditie, natriuretic peptides

INTRODUCTION

Heart failure (HF) with preserved ejection fraction (HFpEF) is a major public health epidemic with an economic impact that is at least as great as that of HF with reduced ejection fraction (HFrEF) (Upadhya and Kitzman, 2017; Seferovic et al., 2019; Sweeney et al., 2020). HFpEF is common and is becoming increasingly more common (1% growth in cases per year) because of its association with aging and comorbidities (Owan et al., 2006; Sweeney et al., 2020). Despite the high prevalence,

there are currently no US Food and Drug Administration–approved therapies for the health-related outcomes of HFpEF, likely due to the marked heterogeneity of the HFpEF syndrome (Oh et al., 2019; Seferovic et al., 2019; Kirkman et al., 2020; Sweeney et al., 2020). Therefore, HFpEF management remains one of the greatest unmet needs in cardiovascular medicine.

The phenotyping of HFpEF patients into pathobiological homogenous groups has been recently suggested for the development of targeted therapies (Obokata et al., 2017; Kirkman et al., 2020). There are distinct phenotypes within this heterogeneous syndrome. Obesity has gained attention as one potential phenotype of HFpEF (Kirkman et al., 2020). Indeed, obesity is highly prevalent in patients with HFpEF as it is estimated that more than 80% of HFpEF patients are either overweight or obese (Shah et al., 2016; Obokata et al., 2017). Elevated body mass index (BMI) is one of the strongest independent risk factors for developing HFpEF, and this excess risk may not be explained by traditional cardiovascular risk factors (Ndumele et al., 2016; Obokata et al., 2017). Although obesity may be associated with other HFpEF comorbidities, recent data suggest that obesity-related HFpEF may represent not only a clinically relevant pathobiological mechanism but also a distinct phenotype within the broad spectrum of HFpEF (obesity-HFpEF phenotype) (Clerico et al., 2018). In addition, obesity may result in the induction of a systemic inflammatory state, which is believed to promote HFpEF progression (Ather et al., 2012; Paulus and Tschöpe, 2013).

There has recently been an increased interest in the intertissue communication between adipose tissue and the heart (Paulus and Tschöpe, 2013; Shah et al., 2016; Obokata et al., 2017; Clerico et al., 2018). Although volume overload may contribute to the development of HFpEF, recent data suggest that the metabolic, endocrine, and natriuretic peptide clearance receptor (NPR-C) signal transduction may play a crucial role in the pathobiology of the obesity-HFpEF phenotype (Matsukawa et al., 1999; Nakatsuji et al., 2010; Bordicchia et al., 2016; Oh et al., 2019). To support this hypothesis, several experimental studies have demonstrated a strong relationship between increased adiposity, NPR-C signaling, arterial hypertension, dyslipidemia, and inflammation and insulin resistance; which, in the long term, may result in impairment of endothelial, diastolic, systolic, arterial, and skeletal muscle functions (Paulus and Tschöpe, 2013; Shah et al., 2016; Obokata et al., 2017; Clerico et al., 2018). This review therefore summarizes the current understanding of the obesity-HFpEF phenotype, focusing on comorbidities and their impact on NPR-C signaling, as well as discussing how "crosstalk" exists between the heart and the adipose tissue within the context of NPR-C pathway.

SEARCH METHODS

For this narrative review, pertinent studies were retrieved from seven electronic databases (PubMed, Google Scholar, Cochrane Library, Web of Science, Science Citation Index, EMBASE, and Elsevier) using common keywords applied in the field of obesity, diabetes, metabolic syndrome, HFpEF, and NPR-C. The author also examined the complete list of relevant references in recent publications in English (both human and studies) on the topics investigated. Given the design of this article as a narrative review, no formal criteria for study selection or quality assessment were applied.

NATRIURETIC PEPTIDE CLEARANCE RECEPTOR

Evidence suggests that most of the physiological effects of natriuretic peptides (NPs), including the B-type (BNP), may be mediated through attachment to three distinct NP receptors A, B, and C (NPR-A, NPR-B, and NPR-C) (Egom, 2015; Egom et al., 2015a). The binding of NPs to NPR-A and NPR-B may result in the activation of the guanylyl cyclase (GC) enzyme and subsequent elevation of intracellular cyclic guanosine monophosphate (cGMP), which appears to mediate most of the physiological effects of NPs (Egom, 2015). NPR-C, which is bound by all NPs with similar affinity, does not contain a GC domain and was originally classified as a "clearance receptor" with no signal transduction function (Egom, 2019; Egom et al., 2019). Although the exact contribution of the NPR-C clearance pathway to the NP concentration in the plasma remains unclear, both the NPR-C and neprilysin pathways may contribute to the degradation of NPs (Potter, 2011). Although still commonly called a clearance receptor (Egom, 2015), evidence suggests that NPR-C may be coupled to a pertussis toxin–sensitive inhibitory G protein (G_i) and may reduce adenylyl cyclase activity and intracellular cAMP levels (**Figure 1**; Katada and Ui, 1982; Zhou and Murthy, 2003).

The relationship between the NP system and adipose tissue is well established. Circulating BNP levels appear to be lower in obese patients than in their normal-weight counterparts. This is even more evident in HFpEF, whereby obese HFpEF patients typically have lower circulating BNP levels than their normal-weight counterparts (Daniels et al., 2006; Obokata et al., 2017; Oh et al., 2019). This complex inverse relationship between circulating BNP and BMI is often termed the "natriuretic handicap" and has been observed in both healthy individuals and patients with HFpEF (Clerico et al., 2018; Nishimura et al., 2018). The molecular mechanisms of the "natriuretic handicap" may be partly related to the differential expression of NPR-C, leading to enhanced degradation of BNP in adipose tissue (Gentili et al., 2017).

Adipose tissue is the second largest expression site of NPR-C after the kidney, and the NPR-C signaling pathway within adipose tissue is the main target for endogenous agonists (Sarzani et al., 2017). Impaired activation of NPR-C signaling has been described in several pathological processes including obesity, inflammation, insulin resistance, fibrosis, ischemia, oxidative stress, remodeling, and arterial and pulmonary hypertension (Hobbs et al., 2004; Egom et al., 2017a,c, 2019; Sarzani et al., 2017; Egom, 2019). The human body may respond to these pathological processes through up-regulation of the NPR-C (a known protective pathway) gene expression as an adaptation mechanism to maintain homeostasis (Gower et al., 2006;

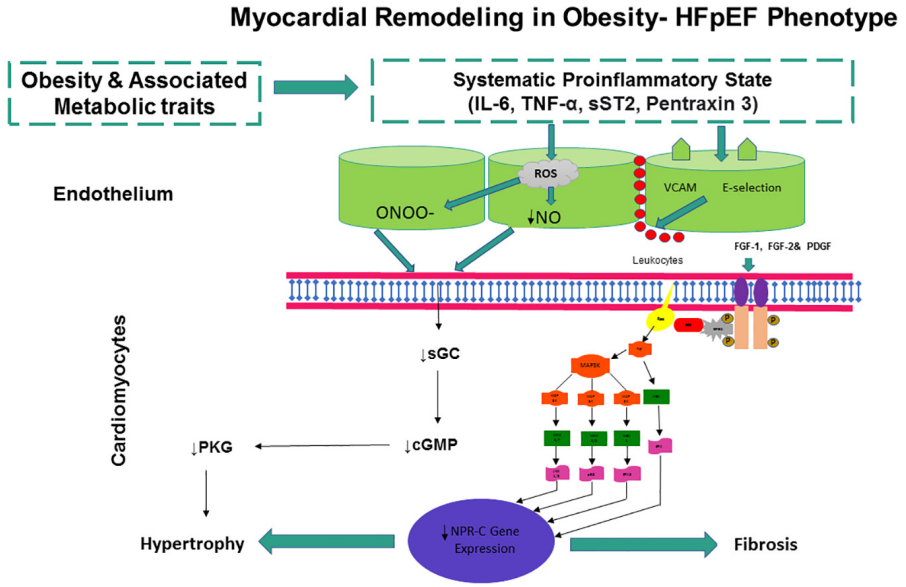

FIGURE 1 | The NPR-C pathway. NPs bind to NPR-C, which is also considered a clearance receptor responsible for receptor-mediated NP degradation. Recent evidence suggests that NPR-C may be coupled to inhibition of adenylyl cyclase (AC) through G_i protein or activation of phospholipase C (PLC) through G_i protein. Inhibition of PLC activity may result in decreased production of diacylglycerol (DAG) and inositol triphosphate (IP_3). NPR-C activation may also have some antiproliferative properties.

Egom et al., 2017a,c, 2019; Sarzani et al., 2017; Egom, 2019). Kuhn et al. (2004) have shown that the mRNA expression levels of the NPR-C receptor were increased in human failing hearts and that the reversal of cardiomyocyte hypertrophy during left ventricular assist device support was accompanied by normalization of the NPR-C mRNA levels. To that end, enhanced NPR-C mRNA levels in various cardiometabolic disorders may represent a compensatory response to low NPR-C activity with the goal of reestablishing cardiometabolic function.

OBESITY-HFPEF PHENOTYPE

The obesity-HFpEF phenotype is growing increasingly more common, with more than 1.8 million patients in the United States currently diagnosed with this clinical entity (Kitzman and Shah, 2016). The obesity-HFpEF phenotype may be considered as a disease of postmenopausal women (Tromp et al., 2019). Among postmenopausal women, HFpEF comprises nearly 90% of incident HF cases (Upadhya and Kitzman, 2017). These women are more likely to have hypertension with significant hypertrophy than age-matched men, as well as higher abdominal fat mass accumulation compared with premenopausal women (Agabiti-Rosei and Muiesan, 2002; Garaulet et al., 2002). The mechanisms that link hypertension, cardiac hypertrophy, obesity, and postmenopausal status have not fully been elucidated because of the lack of suitable experimental models (de Andrade et al., 2011). Experimental models are required tools to investigate the underlying molecular mechanisms linking the NPR-C pathway, obesity-associated cardiometabolic traits, and HFpEF and to explore the use of potential pharmacological agents in this specific phenotype (Valero-Muñoz et al., 2017; Oh et al., 2019). However, until recently, there were no experimental models that could accurately mimic the cardiometabolic changes typically seen in human obesity-related HFpEF (Valero-Muñoz et al., 2017; Oh et al., 2019). Thus, the unavailability of such models may have partly contributed to the current lack of understanding of the molecular mechanisms underlying obesity-HFpEF phenotype.

de Andrade et al. (2011) investigated the role of the NPR-C pathway in the development of obesity-related hypertension and cardiac hypertrophy in ovariectomized fat-fed experimental models. The authors found that the experimental obese postmenopausal models exhibit cardiac hypertrophy, increased mean blood pressure, and increased visceral fat mass, as well as increased NPR-C gene expression (de Andrade et al., 2011). Interestingly, whereas obesity or postmenopausal status alone did not induce the above alterations, a combination of the two conditions (i.e., obesity and postmenopause) was able to trigger some of the cardiovascular and renal alterations that are typically seen in human obesity-related HFpEF (de Andrade et al., 2011). The increased blood pressure and visceral fat mass were strongly correlated with the up-regulation of NPR-C gene expression (de Andrade et al., 2011). In a similar study, Belo et al. (2008) demonstrated that their overweight and estrogen-deficient experimental models had significantly higher renal and mesenteric adipocyte NPR-C gene expression than their wild-type counterparts. This effect was reversed by estrogen treatment, reinforcing the relationship between estrogen, fat deposit, and the NPR-C pathway (Belo et al., 2008).

Evidence suggests that NPR-C gene expression in human adipose tissue may be greater in obese hypertensive than in

obese normotensive individuals (Dessì-Fulgheri et al., 1997). In obese individuals, fasting-induced weight loss may result in a reduction of blood pressure, which is accompanied by a significant down-regulation of adipocyte NPR-C receptor (Dessì-Fulgheri et al., 1999). These observations suggest that the altered NPR-C pathway may play a role, at least in part, in a mechanism that reduces blood pressure in obese individuals.

In summary, an impaired NPR-C pathway may contribute to the susceptibility of postmenopausal women in developing hypertension and plays an essential role in the pathobiology of HFpEF in women. It would be interesting to investigate, at least in this patient cohort, the effects of long-term treatment with estrogen on the cardiovascular outcomes.

Cardiac Comorbidities Associated With Obesity-HFpEF Phenotype

Diastolic Dysfunction

Heart failure with preserved ejection fraction was initially labeled as "diastolic HF" based on hemodynamic studies that demonstrated elevated filling pressures in the absence of a significant increase in ventricular chamber size (Zile et al., 2004). As diastolic dysfunction may be observed in subjects with HFrEF, and patients with diastolic dysfunction may also have some degree of systolic dysfunction, the term "diastolic HF" was abandoned and replaced with HFpEF (Oh et al., 2019). Studies using experimental models have highlighted the relationships between the increased adiposity and metabolic alterations observed in obesity, diastolic dysfunction, and HFpEF (Oh et al., 2019). In order to investigate mechanisms linking NPR-C signaling, diastolic dysfunction, and HFpEF, we used an established model of hypertensive heart disease in which mice are chronically administered angiotensin II (Ang II) (3 mg/kg per day for 3 weeks by miniosmotic pump) (Frohlich et al., 1992; Houser et al., 2012; Mackasey et al., 2018). The administration of Ang II results in an initial compensated hypertrophy phase and HFpEF that ultimately transitions to HFrEF (Frohlich et al., 1992; Houser et al., 2012; Mackasey et al., 2018). Using this protocol, we have found that Ang II caused mice to become hypertensive and hypertrophic with diastolic dysfunction, as expected (Mackasey et al., 2018). The echocardiographic assessments (at 3 weeks) showed that wild-type mice developed ventricular hypertrophy but that systolic function was not yet impaired (no strain analysis by speckle-tracking echocardiography was performed) as ejection fraction (EF) and fractional shortening (FS) were not reduced compared to baseline (Mackasey et al., 2018). In contrast, we found that the same Ang II treatment in NPR-C$^{-/-}$ mice greatly expedited disease progression. Specifically, the mice lacking NPR-C that were treated with Ang II showed a much more severe decline in cardiac function and decompensation into overt HFrEF as indicated by ventricular dilatation and reductions in EF and FS (Mackasey et al., 2018).

Strikingly, the cotreatment of wild-type mice with Ang II and a selective NPR-C agonist (cANF) was able to largely prevent or slow the development of diastolic dysfunction, and cANF cotreatment did not prevent the increase in systolic blood pressure elicited by Ang II (Mackasey et al., 2018). Similarly, the increased fibrosis in NPR-C$^{-/-}$ mice is consistent with data demonstrating the strong antiproliferative and antifibrotic effects of NPs on cardiac fibroblasts (Tsuruda et al., 2002; Horio et al., 2003; Kapoun et al., 2004; Kawakami et al., 2004; Huntley et al., 2010), some of which involve NPR-C–dependent signaling (Huntley et al., 2006). In line with these experimental findings, NPR-C genetic alterations were independently associated with the development of diastolic dysfunction in humans, suggesting a potential critical role of NPR-C signaling in the pathobiology of HFpEF (Pereira et al., 2014).

The development of diastolic dysfunction appeared independent of age, gender, body weight, and systemic hypertension in subjects homozygous for the altered NPR3 genotype with an odds ratio of 1.9, which is similar to that of systemic hypertension, a major modifiable traditional cardiovascular risk factor for diastolic dysfunction (Pereira et al., 2014). The potential underlying mechanisms of the development of diastolic dysfunction in this cohort of individuals may be partially due to alterations in the NPR-C's cytoplasmic domain within cardiac myocytes contributing to cardiac fibrosis and diastolic dysfunction (Pereira et al., 2014; Egom et al., 2015b, 2017a,b).

Taken together, low NPR-C activity may remove the brake on cardiac hypertrophy, thereby inducing cardiac fibrosis and remodeling and leading to diastolic dysfunction, the major cardiac functional deficit in HFpEF.

Atherosclerotic Cardiovascular Disease

Susceptibility to Atherosclerotic Cardiovascular Disease

Coronary artery disease (CAD) is common and extensive in patients with HFpEF, with a prevalence approaching 40–50% (Shah, 2010; Hwang et al., 2014; Mohammed et al., 2015). In addition, the presence of CAD may predict incident HFpEF and cardiovascular death, especially sudden death (Shah, 2010; Hwang et al., 2014; Mohammed et al., 2015).

Evidence suggests that NPR-C may play a role in the pathogenesis of CAD. Hu et al. (2016) performed a multicenter genome-wide association study in 200 individuals from a Shandong cohort, a pathway-based candidate gene association from a Shanghai cohort (293 CAD/293 controls), and replication studies in additional 3,363 CAD patients and 3,148 controls. They identified new susceptibility loci of *NPR-C* that are specifically associated with CAD (Hu et al., 2016). Interestingly, multivariate logistical regression analyses revealed that the association between these single-nucleotide polymorphisms (SNPs) and CAD remained significant even after adjustment for the conventional atherosclerotic risk factors (age, gender, smoking, hypertension, diabetes, and dyslipidemia), suggesting that the NPR-C gene SNPs contribute to CAD susceptibility (Hu et al., 2016). The molecular mechanisms underlying the association between NPR-C gene polymorphism and CAD have not yet been fully elucidated.

Pathobiology of Atherosclerotic Cardiovascular Disease

Natriuretic peptide clearance receptor is expressed in the vasculature and may be involved in cellular proliferation, migration, and vascular remodeling. NPR-C is thus relevant

to the study of atherosclerotic cardiovascular disease because vascular cell proliferation and migration are central to the pathophysiology of this inflammatory-based condition. Casco et al. (2002) investigated the pattern of expression of NPR-C in human coronary arteries with various degrees of atherosclerotic lesions and found NPR-C in the intimal and the inner medial layers. Similarly, Naruko et al. (2005) immunohistochemically studied the expression of NPR-C during the post–percutaneous coronary intervention (PCI) healing process and demonstrated that NPR-C was strongly expressed in neointimal vascular smooth muscle cells from 1 to 9 months after PCI. Gene expression and histopathology analyses of coronary artery atheromatous lesions further demonstrated that NPR-C expression may be highest in the intima and inner media layers (Wei et al., 1994; Casco et al., 2002; Naruko et al., 2005). In these studies, as well as in other studies, the expression of NPR-C appeared to positively correlate with the severity of atherosclerotic cardiovascular disease (Furuya et al., 1995; Rollín et al., 2005; Scotland et al., 2005). Furthermore, Zayed et al. (2016) evaluated NPR-C expression by immunohistochemistry in carotid endarterectomy specimens isolated from 18 patients and found significant NPR-C expression in the intima of advanced carotid artery plaques, with an expression pattern correlating to the features of plaque vulnerability. These observations suggest that NPR-C may serve as a potential biomarker for plaque vulnerability and progression in patients with atherosclerotic cardiovascular disease (Zayed et al., 2016).

Periprocedural NPR-C Responses

The restoration of coronary blood flow during ischemic myocardium is important for limiting the injury caused by acute myocardial infarction and salvaging cardiac function. However, reperfusion may exert detrimental effects by extending myocardial necrosis and cardiac dysfunction beyond what was achieved by the ischemic insult itself [ischemia/reperfusion (I/R) injury] (Hobbs et al., 2004).

Evidence suggests that the activation of NPR-C signaling may contribute to the regulation of coronary blood flow. NPR-C signaling may also represent a protective mechanism against I/R injury by reducing infarct size and maintaining coronary perfusion pressure and left ventricular developed pressure at preischemic levels (Hobbs et al., 2004). In an experimental heart model, Hobbs et al. (2004) demonstrated that the selective NPR-C agonist $cANF^{4-23}$ elicits the potent relaxation of coronary arteries as well as offers a protective mechanism against I/R injury with suppression of both infarct size and myocardial dysfunction. Furthermore, the administration of $cANF^{4-23}$ during the reperfusion period alone may also protect against I/R injury, suggesting that the activation of NPR-C signaling may prove beneficial in patients presenting with an acute coronary ischemic event (Hobbs et al., 2004).

This NPR-C–induced protective effect may be enhanced in the setting of nitric oxide (NO) synthase inhibition (Hobbs et al., 2004). In fact, evidence suggests that endothelial NO synthase (eNOS) may regulate expression of NPR-C mRNA. Del Ry et al. (2020) investigated the NP system expression in whole blood obtained from normal-weight and obese adolescents and found that individuals with reduced endothelial function may have significantly higher expression level of NPR-C mRNAs. Using experimental models lacking eNOS, Yuan et al. (2010) investigated the effects of eNOS expression on the regulation of NPR-C gene expression. NPR-C mRNA levels were greater in the heart and kidney of the experimental models lacking eNOS compared to the wild-type counterpart (Yuan et al., 2010). Therefore, there may be complementary protective roles for the NPR-C and NO signaling pathways in the cardiovascular system, whereby the loss of one system may be compensated for with the up-regulation of the alternative signal transduction pathway. These complementary roles may be of particular significance for atherosclerotic cardiovascular disorders as they seem to be characterized by loss of the NO pathway (Hobbs et al., 2004). Under such circumstances, the influence of the NPR-C pathway may be heightened. Furthermore, pharmacological agents mimicking the biological activity of the NPR-C pathway may prove to be an important new strategy to treat these disorders.

Postprocedural NPR-C Responses

Maintaining the patency and integrity of the coronary arteries after successful restoration of coronary blood flow is of paramount importance. In patients undergoing PCI, reocclusion of the coronary artery often occurs within 6 months (Khambata et al., 2011; Ñato et al., 2018; Neumann et al., 2019a,b; Sousa-Uva et al., 2019; Modi et al., 2021). For these patients with reocclusion, their CAD is treated with a bare metal stent or drug-eluting stent (DES), which acts as a platform for new tissue growth (Khambata et al., 2011; Sousa-Uva et al., 2019). Current DESs (which are now the predominant implanted stents) release antiproliferative drugs that inhibit arterial smooth muscle cell proliferation, which is the most predominant cause of restenosis (Khambata et al., 2011; Ñato et al., 2018; Neumann et al., 2019a,b; Sousa-Uva et al., 2019; Modi et al., 2021). However, these agents may also inhibit endothelial cell proliferation (Matter et al., 2006; Khambata et al., 2011; Ñato et al., 2018; Neumann et al., 2019a,b; Sousa-Uva et al., 2019; Modi et al., 2021), which may increase the thrombogenicity of the stent surface. The antiproliferative effects of NPR-C signaling on vascular cells may therefore provide a promising treatment strategy of restenosis.

The beneficial effects of NPR-C agonists may also be applied to patients undergoing coronary artery bypass grafting (CABG). In patients undergoing CABG, the saphenous vein graft (SVG) is still commonly used as graft material; however, the 1-, 5-, and 10-year postoperative SVG patency rates may be 93, 74, and 41%, respectively (Gao et al., 2018). Furthermore, studies have shown that up to 12% of SVGs occlude within the first 6 months following CABG, with 3.4% occluding as early as 2–3 weeks principally due to thrombosis (Gao et al., 2018). Abnormal hyperplasia of the neointima severely affects a further 10% of grafts within 1 year (1–12 months after CABG) (Gaudino et al., 2017; McKavanagh et al., 2017). Here again, NPR-C signaling manipulation may be of therapeutic benefit. Indeed, NPR-C agonists were shown to be beneficial following balloon angioplasty and vein grafting in experimental models (Khambata et al., 2011).

Epicardial Fat

Compared to non-obese HFpEF patients, obese HFpEF patients may display increased epicardial fat thickness (Clerico et al., 2018). Clinical studies have shown that the amount of epicardial adipose tissue (EAT) may be associated with the presence, progression, or severity of CAD (Mahabadi et al., 2013; Nakanishi et al., 2014). Although the underlying pathophysiological mechanisms of EAT in CAD progression are not completely understood, recent evidence suggests that low levels of the EAT NPR-C expression may lead to dysregulation of the epicardial fat surrounding the myocardium, which in turn may contribute, at least in part, to the progression of CAD (Moreno-Santos et al., 2019). Moreno-Santos et al. (2019) investigated the relationship between the expression and signaling of EAT NPR-C and the progression of CAD in humans in a cohort of individuals with angiographically normal coronary arteries, stable CAD, and acute coronary syndrome (ACS). The authors showed that patients with ACS have lower expression of EAT NPR-C at both the protein and mRNA levels compared to patients with stable CAD or angiographically normal coronary arteries (Finck and Kelly, 2006; Díez, 2017; Moreno-Santos et al., 2019). Additionally, the authors showed that patients with ACS have reduced activation of p38 mitogen–activated protein kinase (p38 MAPK); lower expression of EAT uncoupling protein 1 (UCP1), which may in turn lead to reduced thermogenic capacity; and lower expression of peroxisome proliferator-activated receptor γ coactivator α, which may in turn lead to mitochondrial dysfunction.

Moreno-Santos et al. (2019) also found an inverse relationship between the mRNA levels of EAT NPR-C and the severity of CAD. Individuals with 3-vessel disease had lower EAT NPR-C mRNA levels compared with those with 1- or 2-vessel disease and no significant CAD (Moreno-Santos et al., 2019). Multivariate logistic regression models demonstrated significant associations of EAT NPR-C gene expression, EAT PGC1α mRNA levels, and the presence of ACS (Moreno-Santos et al., 2019).

Although it may be premature to make any final conclusions about cause and effect, some observations support the notion that low NPR-C levels may promote plaque vulnerability/instability and progression in patients with atherosclerotic cardiovascular disease (Hobbs et al., 2004; Zayed et al., 2016; Egom et al., 2019). One approach to determine a gene's function is to observe how a cell or a whole organism behaves when the gene of interest is absent or non-functional. As mentioned previously and as we will discuss further in subsequent sections, low NPR-C activity may remove the brake on cardiac hypertrophy, induce cardiac fibrosis and remodeling, and increase susceptibility to develop arrhythmias and pulmonary hypertension (Hobbs et al., 2004; Egom et al., 2015b, 2019; Jansen et al., 2018, 2019; Mackasey et al., 2018; Egom, 2019). On the other hand, enhanced NPR-C activity may protect against IR injury, reverse cardiac hypertrophy and fibrosis, and improve endothelial function (Hobbs et al., 2004; Egom et al., 2015b, 2019; Jansen et al., 2018, 2019; Mackasey et al., 2018; Egom, 2019).

Atrial Fibrillation

Atrial fibrillation (AF) is highly prevalent and commonly occurs in the setting of HFpEF and hypertension (Nattel, 2002; Dobrev and Nattel, 2010), conditions that are characterized by excessive activation of Ang II signaling (Mudd and Kass, 2008). HFpEF and AF may be inextricably linked, both to each other and to adverse cardiovascular outcomes (Chamberlain et al., 2011; Vermond et al., 2015; Upadhya and Kitzman, 2017). The interrelationships between AF and HFpEF remain complex and poorly understood, yet the number of patients with AF and HFpEF continues to increase worldwide (Al-Khatib et al., 2020). Thus, there is a need for experimental work that will provide insight into the mechanisms of the intersection between AF and HFpEF.

We have recently used a well-established model of hypertensive heart disease that leads to cardiac hypertrophy and HFpEF by chronically treating mice with Ang II (Mackasey et al., 2018). Consistent with prior studies (Wakisaka et al., 2007; Swaminathan et al., 2011; Fukui et al., 2013), we demonstrated that chronic Ang II may cause atrial enlargement, electrical and structural remodeling (fibrosis) of the atrial myocardium, and increased susceptibility to AF (Mackasey et al., 2018). In order to investigate the potential role of NPR-C signaling in the interrelationships between AF and HFpEF, we performed experiments in which we treated NPR-$C^{-/-}$ mice (and NPR-$C^{+/+}$ littermates) with saline or Ang II (Mackasey et al., 2018). As mentioned previously, echocardiography assessments demonstrated that NPR-$C^{-/-}$ mice responded much more severely to Ang II than wild-type mice. The NPR-$C^{-/-}$ mice displayed a rapid transition into HFrEF, as demonstrated by ventricular dilatation and reductions in EF and FS, whereas systolic function was not yet impaired in wild-type mice (no strain analysis by speckle tracking echocardiography was performed) (Mackasey et al., 2018). Furthermore, Ang II treatment resulted in significantly more atrial enlargement in NPR-$C^{-/-}$ mice compared to NPR-$C^{+/+}$ littermates (Mackasey et al., 2018). Intracardiac electrophysiology experiments demonstrated that Ang II treatment resulted in a greater susceptibility to AF in NPR-$C^{-/-}$ mice than in NPR-$C^{+/+}$ littermates (Mackasey et al., 2018). Strikingly, cotreatment of wild-type mice with Ang II and cANF prevented atrial electrical and structural dysfunction (Mackasey et al., 2018).

We also demonstrated that the increased susceptibility to AF in NPR-$C^{-/-}$ mice may be caused primarily by enhancing fibrosis in the atria, suggesting that NPs may act upon NPR-C in cardiac fibroblasts to regulate extracellular matrix deposition (Egom et al., 2015b). Consistently, the activation of the NPR-C pathway may have potent antifibrotic and antiproliferative effects on fibroblasts in the heart (Calvieri et al., 2012; Egom, 2019). To investigate the effects of Ang II on AF susceptibility and atrial function, we used *in vivo* electrophysiology, patch clamping, high-resolution optical mapping, and molecular biology on wild-type and NPR-$C^{-/-}$ mice (Jansen et al., 2019). While Ang II increased susceptibility to AF in wild-type mice, these effects were exacerbated in Ang II–treated NPR-$C^{-/-}$ mice (Jansen et al., 2019). Ang II also enhanced fibrosis in both atria in wild-type mice, whereas Ang II–treated NPR-$C^{-/-}$ mice exhibited substantially higher fibrosis burden throughout the atria. Cotreating wild-type mice with Ang II and cANF dose-dependently reduced AF susceptibility by preventing

most of the Ang II–induced atrial myocardial abnormalities (Jansen et al., 2019).

Taken together, these findings strongly implicate a potential signaling role for NPR-C in the pathobiology of the complex interrelations between AF and HFpEF.

Metabolic Traits Associated With Obesity-HFpEF Phenotype

Non-cardiac comorbidities, including but not limited to diabetes and arterial hypertension, are highly prevalent in patients with the obesity-HFpEF phenotype (Upadhya and Kitzman, 2017).

Our previous analysis of mice lacking NPR-C showed that these mice had a lean phenotype with a significantly reduced fat mass (Bordicchia et al., 2012; Egom et al., 2019). Mice lacking NPR-C appeared to have markedly smaller white and brown adipose tissue depots but higher expression of thermogenic genes (such as Ucp1) and other features of brown adipocytes (Bordicchia et al., 2012). The ability of humans to prevent body fat accumulation may be linked to their ability to expand the number and activity of brown adipocytes within white fat depots (Bordicchia et al., 2012). As the mice lacking NPR-C appear to have increased brown adipocytes in their white fat depots, they tend to resist diet-induced obesity and retain insulin sensitivity (Kovacova et al., 2016).

Sarzani et al. (2004) tested the association between the NPR-C A/C(-55)A polymorphism in 787 untreated male participants in the 1994–1995 follow-up examination of the Olivetti Heart Study in Naples. The authors found that individuals carrying the A/C(-55)A *NPRC* genotype had a significantly lower BMI and waist circumference, as well as a significantly lower rate of overweight and obesity at the 20-year follow-up observation (Sarzani et al., 2004). Interestingly, the authors did not find any association between either Blood pressure (BP) or fasting serum insulin concentration and the *NPRC* gene polymorphism (Sarzani et al., 2004).

Obese individuals have higher *NPR-C* gene expression in the subcutaneous abdominal adipose tissue than their lean counterparts (Kovacova et al., 2016). NPR-C is expressed most abundantly in white adipose tissue and more on visceral than subcutaneous adipose tissue (Nakatsuji et al., 2010). Del Ry et al. (2020) analyzed NPR-C expression in whole blood obtained from normal-weight and obese adolescents and found significant associations of circulating insulin, hemoglobin A_{1c} (HbA_{1c}) levels, and NPR-C expression, suggesting that hyperinsulinism and altered glucose metabolism may contribute, at least in part, to the elevated NPR-C expression in individuals with high BMI. There is a gradual and progressive increase of *NPR-C* transcripts in adipose tissue when an individual is transitioning from normal glucose tolerance to type 2 diabetes mellitus (T2DM) (Kovacova et al., 2016). Although individuals with normal glucose tolerance may have the same skeletal muscle NPR-C protein content regardless of their BMI, there may be an up-regulation of skeletal muscle NPR-C as glucose tolerance deteriorates in individuals with impaired glucose tolerance or T2DM (Coué et al., 2015).

In addition, hyperinsulinemic states, as found in patients with impaired glucose tolerance/insulin resistance due to metabolic syndrome or diabetes, may up-regulate subcutaneous NPR-C gene expression in a glucose-dependent manner (Nakatsuji et al., 2010). Kovacova et al. (2016) demonstrated that 12 weeks of treatment with pioglitazone significantly lowers levels of *NPRC* mRNA and improves insulin sensitivity in patients with T2DM or metabolic syndrome. Skeletal muscle NPR-C has also been shown to be positively related to fasting blood glucose, insulin, and HbA_{1c}, further suggesting that a functional NPR-C pathway is required for insulin sensitivity and blood glucose control (Coué et al., 2015). In a study by Christoffersen et al. (2006), diabetes and impaired glucose metabolism were demonstrated to confer increased NPR-C gene expression in the heart, suggesting a relationship between impaired NPR-C signaling, glucose dysmetabolism, and associated cardiac function.

Although it may be premature to make any final conclusions about cause and effect, the above findings suggest that the NPR-C pathway may be dysregulated in the context of metabolic disorders such as obesity, insulin resistance, and T2DM (Coué et al., 2015). The observed up-regulation of NPR-C mRNAs in these patients with these metabolic disorders may represent a compensatory mechanism to maintain or reestablish cardiometabolic health. The NPR-C pathway may thus represent a novel therapeutic target in cardiometabolic disorders, including but not limited to obesity and insulin resistance, in addition to HFpEF.

OBESITY PARADOX

Even though obesity is a strong risk factor for HF in the general population, the "obesity paradox" refers to the fact that obese patients with established HF tend to have better long-term prognosis than non-obese patients (Janovska et al., 2020). Patients with the obesity-HFpEF phenotype have better outcomes than their non-obese counterparts. In a cohort of 150 patients hospitalized with HFpEF, higher BMI values were associated with lower mortality (Stavrakis et al., 2013). Consistently, a U-shaped relationship between BMI and mortality has been reported for HFpEF (Kapoor and Heidenreich, 2010; Padwal et al., 2014; Ohori et al., 2021).

Although the association between obesity and HFpEF is well known, the pathophysiology of weight-related changes on the outcome of patients with HFpEF is still a matter of debate. Nishikido et al. (2019) investigated whether a change in BMI is associated with either prognosis or frequency of hospitalizations in patients who were hospitalized for decompensated HF. The authors found that a lowered BMI may be a significant predictive factor for the frequency of hospitalizations and increased mortality (Nishikido et al., 2019). Similarly, other studies have shown that increased body fat mass, but not appendicular skeletal muscle mass, corresponds to a lower risk of short-term cardiac events in HF patients (Thomas et al., 2019; Ohori et al., 2021). Every 5-unit increase in BMI corresponds to a 10% reduction in mortality (Fonarow et al., 2007).

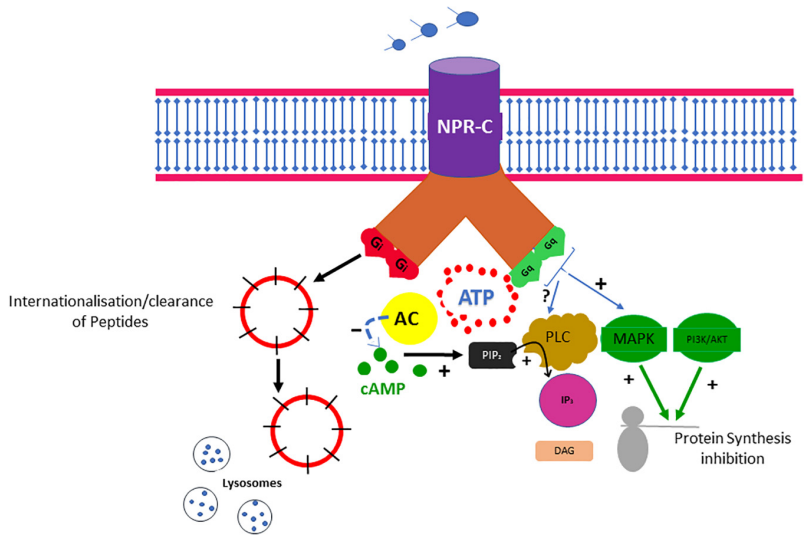

FIGURE 2 | Obesity and associated metabolic traits drive myocardial dysfunction and remodeling in obesity-HFPEF phenotype. Obesity and associated metabolic traits induce a systemic proinflammatory state characterized by high plasma levels of interleukin 6 (IL-6), soluble ST2 (sST2), tumor necrosis factor α (TNF-α), and pentraxin 3. Coronary microvascular endothelial cells produce vascular cell adhesion molecule (VCAM), E-selectin, and reactive oxygen species (ROS). The production of ROS results in reduced NO bioavailability and peroxynitrite ($ONOO^-$) production, both of which may lower soluble guanylate cyclase (sGC) activity in adjacent cardiomyocytes. Lower sGC activity results in decreased cGMP concentration and reduced protein kinase G (PKG) activity. Low PKG activity may trigger a cascade of events leading to cardiomyocyte hypertrophy. E-selectin and VCAM expression in endothelial cells may favor migration into the subendothelium of immune cells, which may release cytokines and growth factors including the fibroblast growth factors (FGF-1 and FGF-2) and platelet-derived growth factor (PDGF). FGF-1, FGF-2, and PDGF activate the membrane tyrosine kinase receptors, which then trigger a full range of intracellular Ras-Raf–mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase-MAPK signaling transduction pathways, leading to a down-regulation of NPR-C gene expression. Low NPR-C activity may remove the brake on cardiomyocyte hypertrophy, thereby inducing cardiac fibrosis and remodeling, leading to diastolic dysfunction, the major cardiac functional deficit in HFPEF.

Unfortunately, most obesity paradox studies used BMI cut points to classify subjects as normal, overweight, or obese (Egom et al., 2018). However, using BMI as a measure of true body fat content has been strongly criticized by some authors who argue that elevated BMI may overestimate the amount of body fat in subjects with greater muscle mass (and thus indicating a more favorable health status) or underestimate it in older individuals because of the loss of muscle mass related to aging (and thus indicating a worse health status) (Kragelund and Omland, 2005; Egom et al., 2018). The measurement of BMI in conjunction with other anthropometric indices such as percentage of waist circumference, body fat, and waist/hip ratio may thus not only be more accurate, but also provide additional prognostic value (Egom et al., 2018).

Although the potential underlying mechanisms of the obesity paradox have been extensively studied (Hamzeh et al., 2017; Thomas et al., 2019; Ohori et al., 2021), little attention has been given to the possible pathophysiological role of NPR-C. Crandall et al. (1989) studied the effects of weight reduction on NPR-C gene expression in an experimental obesity-associated HFpEF model. A cohort of patients who were subjected to a 15-week period of caloric restriction had, on average, a 40% reduction in body weight. These patients also had a significant decrease in NPR-C mRNA and ultimately had worse outcomes (Crandall et al., 1989). Consistently, Crandall et al. (1989) found that obesity-associated cardiac abnormalities persisted even after the normalization of body fat as the patients transitioned from an obese to lean condition, likely due to persistent low activity of NPR-C signaling.

Some evidence suggests that the prognostic impact of increased body fat mass may also be lost systematically as the disease progresses, and HF severity may overcome percent body fat in the prediction of short-term cardiovascular events (Ohori et al., 2021). Indeed, while fat mass may be associated with better survival in patients with advanced HF, cardiac cachexia (non-intentional weight loss) may be a stronger independent predictor for mortality (Janovska et al., 2020). Janovska et al. (2020) investigated the role of the NPR-C pathway in the development of cachexia in patient with advanced HF as well as its potential impact on the EAT–myocardium environment. High levels of mitochondrial phospholipid cardiolipin in various tissues have been causally linked to cachexia. A negative correlation was observed between NPR-C gene expression levels and the mitochondrial phospholipid cardiolipin levels in EAT, supporting the potential role of NPR-C in cardiolipin−induced changes of EAT metabolism in cachexia (Janovska et al., 2020). The authors also found that NPR-C gene expression was twofold to threefold lower in cachectic patients than in their body weight–stable counterparts (Janovska et al., 2020). Interestingly, NPR-C gene expression correlated positively with body weight change, BMI, and daily dose of both β−blockers and angiotensin−converting enzyme inhibitors or angiotensin receptor blockers (Janovska et al., 2020). These conventional HF agents represent the cornerstone of effective HF therapy and should be titrated to

the maximally tolerated doses. HF patients with a functional or enhanced NPR-C pathway may be more likely to tolerate higher doses of the neurohumoral inhibitors (Janovska et al., 2020).

The activation of the sympathetic nervous system is one of the compensatory mechanisms mounted by the body to maintain cardiac output in individuals with HF. Evidence suggests that an augmented activation of the sympathetic system may reduce the levels of NPR-C in a time- and dose-dependent manner by decreasing the transcriptional rate of the NPR-C gene (Kishimoto et al., 1994). The catecholamine-induced decrease in NPR-C density may be antagonized by carvedilol (Kishimoto et al., 1994). As obese patients with HF have higher NPR-C expression levels, they may have better tolerance for β-blockers at higher doses and should theoretically have improved outcomes (Litwin, 2008). The rational use of β-blockers in HF for improving the prognosis of the disease may also be supported by this observation (Farré et al., 2015).

The above findings suggest that enhancing the NPR-C pathway may represent an attractive therapeutic strategy to reduce body wasting, increase the ability to tolerate higher HF therapeutic doses, and improve HF outcomes.

CONCLUSION

Natriuretic peptide clearance receptor signaling may be central to the pathobiology of HFpEF in general and particularly to the obesity-HFpEF phenotype (as illustrated in **Figure 2**). Addressing the lack of effective available HFpEF therapies remains a priority given the rising demographics of obese, diabetic, hypertensive, and aging populations across the globe. Ultimately, HFpEF and the obesity-HFpEF phenotype are multisystem disorders, and pharmacological agents that alleviate both metabolic and cardiac dysfunction are most likely to provide the greatest clinical benefit. As NPR-C signal transduction may be a point of convergence for all upstream stimuli, from mechanical stretch to endocrine or paracrine mediators, targeting the NPR-C pathway would represent the most promising means of treating the obesity-HFpEF phenotype.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 25 May 2021
doi: 10.3389/fphys.2021.651246

Cardiac Natriuretic Peptide Profiles in Chronic Hypertension by Single or Sequentially Combined Renovascular and DOCA-Salt Treatments

Carolina S. Cerrudo[1†‡], Susana Cavallero[1†‡], Martín Rodríguez Fermepín[1‡], Germán E. González[2], Martín Donato[2], Nicolás M. Kouyoumdzian[3], Ricardo J. Gelpi[2], Cecilia M. Hertig[4], Marcelo R. Choi[1,3,5] and Belisario E. Fernández[1,5]*

[1] Facultad de Farmacia y Bioquímica, Cátedras de Fisiopatología y Anatomía e Histología, Universidad de Buenos Aires, Buenos Aires, Argentina, [2] Facultad de Medicina, CONICET, Instituto de Fisiopatología Cardiovascular, Universidad de Buenos Aires, Buenos Aires, Argentina, [3] Instituto Alberto C. Taquini de Investigaciones en Medicina Traslacional (IATIMET), Universidad de Buenos Aires, CONICET, Buenos Aires, Argentina, [4] Instituto de Ingeniería Genética y Biología Molecular (INGEBI), CONICET, Buenos Aires, Argentina, [5] Instituto Universitario de Ciencias de la Salud, Fundación H. A. Barceló, Buenos Aires, Argentina

The involvement of natriuretic peptides was studied during the hypertrophic remodeling transition mediated by sequential exposure to chronic hemodynamic overload. We induced hypertension in rats by pressure (renovascular) or volume overload (DOCA-salt) during 6 and 12 weeks of treatment. We also studied the consecutive combination of both models in inverse sequences: RV 6 weeks/DS 6 weeks and DS 6 weeks/RV 6 weeks. All treated groups developed hypertension. Cardiac hypertrophy and left ventricular ANP gene expression were more pronounced in single DS than in single RV groups. BNP gene expression was positively correlated with left ventricular hypertrophy only in RV groups, while ANP gene expression was positively correlated with left ventricular hypertrophy only in DS groups. Combined models exhibited intermediate values between those of single groups at 6 and 12 weeks. The latter stimulus associated to the second applied overload is less effective than the former to trigger cardiac hypertrophy and to increase ANP and BNP gene expression. In addition, we suggest a correlation of ANP synthesis with volume overload and of BNP synthesis with pressure overload-induced hypertrophy after a prolonged treatment. Volume and pressure overload may be two mechanisms, among others, involved in the differential regulation of ANP and BNP gene expression in hypertrophied left ventricles. Plasma ANP levels reflect a response to plasma volume increase and volume overload, while circulating BNP levels seem to be regulated by cardiac BNP synthesis and ventricular hypertrophy.

Keywords: natriuretic peptides system, cardiac hypertrophy, renovascular hypertension, DOCA-Salt hypertension, rat models, atrial natriuretic factor, B type natriuretic peptide

OPEN ACCESS

Edited by:
Massimo Volpe, Sapienza University of Rome, Italy

Reviewed by:
Alessandro Cataliotti, University of Oslo, Norway
Mihály Ruppert, Semmelweis University, Hungary
Valentina Cannone, University Hospital of Parma, Italy

****Correspondence:***
Susana Cavallero
ccavallero@mednet.ucla.edu

†Present address:
Susana Cavallero, Division of Cardiology, David Geffen School of Medicine at UCLA, University of California, Los Angeles and Veterans Affairs Healthcare System, Los Angeles, CA, United States
Carolina S. Cerrudo, Laboratorio de Ingeniería Genética y Biología Celular y Molecular, Departamento de Ciencia y Tecnología, Instituto de Microbiología Básica y Aplicada, Universidad Nacional de Quilmes, Buenos Aires, Argentina

‡These authors have contributed equally to this work

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *08 January 2021*
Accepted: *14 April 2021*
Published: *25 May 2021*

Citation:
Cerrudo CS, Cavallero S, Rodríguez Fermepín M, González GE, Donato M, Kouyoumdzian NM, Gelpi RJ, Hertig CM, Choi MR and Fernández BE (2021) Cardiac Natriuretic Peptide Profiles in Chronic Hypertension by Single or Sequentially Combined Renovascular and DOCA-Salt Treatments. Front. Physiol. 12:651246. doi: 10.3389/fphys.2021.651246

INTRODUCTION

Hemodynamic overload is a major determinant of the cardiac morphometric and functional response in cardiovascular diseases. Hypertension is a leading cause of congestive heart failure around the world. Elevation of the blood pressure results in left ventricular hypertrophy, an independent risk factor for cardiovascular mortality (González et al., 2018;

Polak-Iwaniuk et al., 2019; Yildiz et al., 2020). Remodeling of the heart can display a spectrum of geometric patterns as a result of complex interactions between pressure and volume overload; i.e., in aortic stenosis, concentric hypertrophy due to pressure overload evolves toward a dilated eccentric pattern and heart failure (Ganau et al., 1992; Opie et al., 2006). Several animal models of hypertension triggered mainly by pressure and/or volume stimuli have been used to address the role of hemodynamic overload in cardiac hypertrophy (Fenoy et al., 1997; Dobrzynski et al., 2000; Capuano et al., 2002), reviewed in Lerman et al., 2019); however, little is known about the mechanisms involved in the transition and the reversibility from one type of overload to another.

The natriuretic peptide (NP) family includes atrial (ANP) and B-type (BNP) natriuretic peptides that are secreted by the heart and are important regulators of cardiovascular homeostasis. They act on target organs through guanylyl cyclase- coupled receptors to induce diuresis, natriuresis and vasodilation, thus reducing cardiac preload and afterload in response to stress. They also modulate cardiac growth and display anti-inflammatory and anti-fibrotic properties (Nakagawa et al., 2019; Goetze et al., 2020). Gene expression of ANP and BNP is also induced in the adult heart as part of the fetal reprogramming associated to pathological hypertrophy (Sergeeva and Christoffels, 2013). C-type natriuretic peptide was traditionally considered the endothelial NP, but it was more recently implicated in the cardiac response to heart failure (Moyes et al., 2020). Circulating levels of ANP and BNP are used as clinical biomarkers with important diagnostic and therapeutic implications in hypertension and congestive heart failure (Burnett et al., 1986; Ruskoaho, 2003; Puyó et al., 2005; Rubattu et al., 2019). Elevated circulating levels are related to increased atrial wall stretch and ventricular synthesis due to left ventricular hypertrophy (Goetze et al., 2020); however, the response of ANP and BNP throughout transitions between pressure and volume overload is not well characterized.

Our lab previously described the combination of treatments to induce pressure and volume overload in defined times and sequences, in order to resemble the natural evolution of ventricular function in hypertensive disease and to evaluate the differential behavior of ANP and BNP (Cavallero et al., 2007, 2010). We used two established experimental models: renovascular hypertension (RV), and DOCA-salt hypertension (DS). RV is characterized by normal circulating renin levels (Seto et al., 1984) and an early transient increase of the plasmatic volume (Baker et al., 1990; Edmunds et al., 1991), followed by an increase of peripheral resistance that maintains elevated blood pressure (Brody et al., 1983; Fenoy et al., 1997; Lerman et al., 2019). In contrast, DS model is predominantly characterized by volume overload (Iyer et al., 2010; Ndisang and Jadhav, 2010; Lerman et al., 2019), low renin levels (Gavras et al., 1975; Makrides et al., 1988) and high circulating catecholamine, angiotensin II and endothelin-1 levels (Bianciotti and de Bold, 2000, 2001, 2002). As a consequence of volume expansion, cardiac stretch induces cardiomyocyte hypertrophy (Ogawa et al., 1999; Basting and Lazartigues, 2017). In our previous studies, we established models of cardiac hypertrophy by sequential combination of RV followed by DS, or the inverse sequence along a 4-week period. We reported that in a short-term setting, the lately applied overload stimulus determines the remodeling and cardiomyocyte hypertrophic pattern in the sequentially combined models, and moreover, that ANP gene expression but not plasma ANP correlates with volume overload in DS model (Cavallero et al., 2007, 2010).

We hypothesized that ANP and BNP could be used as biochemical markers for volume and pressure overload, respectively, and that a prolonged overload would be required to display differential profiles of ANP and BNP, and these would help to characterize the transition from one hypertrophic pattern to the other. Therefore, the aim of the present study was to evaluate the effect of a chronic hypertensive process in rats with RV and DS procedures along 6 to 12 weeks of treatment, as well as in rats subjected to combination of RV and DS in different sequences of induction. We measured the modifications of systolic blood pressure, cardiac hypertrophy indexes, cardiac function and the synthesis and secretion of ANP and BNP.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 180–220 g were obtained from the Animal Care Facility of the School of Pharmacy and Biochemistry at the University of Buenos Aires. The animals were housed in an environment with controlled temperature and humidity, 12-h light/dark cycle, and access to water and food *ad libitum* (Rodents Purina Chow, Cooperacion SRL, Argentina). The protocols were approved by the Institutional Review Board at the University of Buenos Aires.

Experimental Design

The experimental design is shown in **Figure 1**. Animals with renovascular (RV) or DOCA-salt (DS) hypertension were studied after 6 weeks (groups RV6 and DS6) or 12 weeks of treatment (RV12 and DS12). Groups receiving only RV or DS treatment are also referred to as "single treated groups." Animals subjected to combined treatment consisting of 6 weeks of either RV or DS and then switched to 6 weeks of the alternate treatment (DS or RV, respectively) were studied at 12 weeks. These groups were called RV6/DS6 and DS6/RV6 or "combined treatment groups."

Appropriate sham animals for each experimental group were included (see detailed description of procedures below). Sham animals for RV6 and DS6 groups did not show any statistical difference among them for several parameters (**Supplementary Table 1**) and were therefore pooled into one group, Sham 6 weeks (Sh6). Similarly, sham animals for single or combined procedures at 12 weeks did not show any statistical difference and were pooled into one group, Sham 12 weeks (Sh12). The number of animals (n) was 10–12 for experimental groups and 12–18 for sham groups.

Renovascular Hypertension

The surgical procedures were performed as previously described (Puyó et al., 2005). After being anesthetized with ketamine (80 mg/kg)/xylazine (2.5 mg/kg), the left kidney was removed,

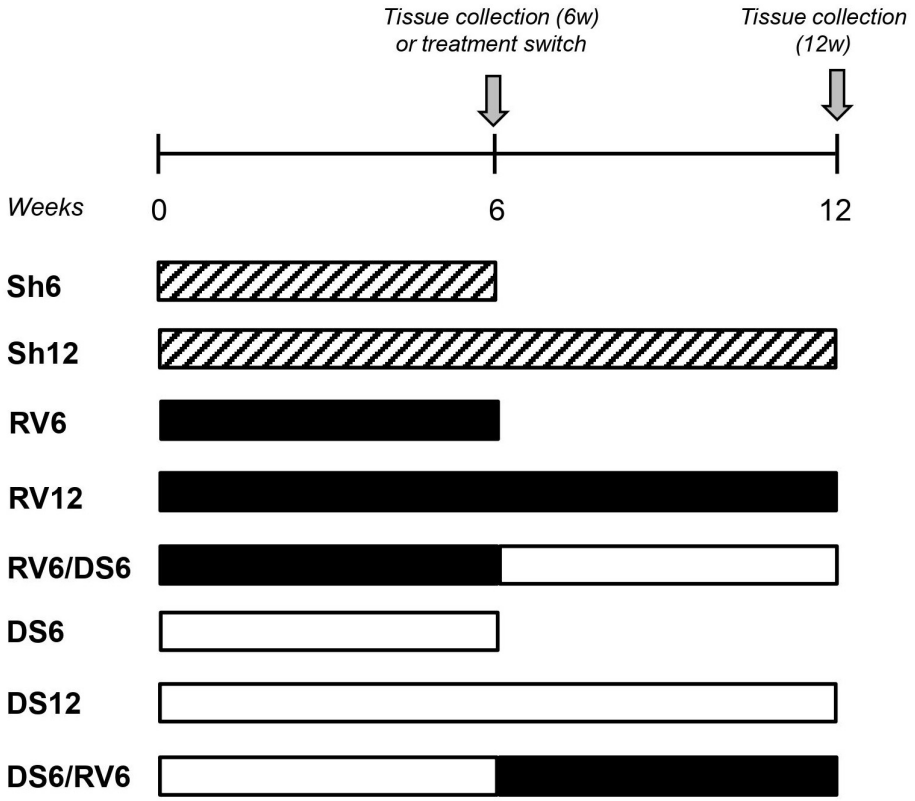

FIGURE 1 | Experimental design to study single and combined hypertensive models. Diagonally striped columns: sham-operated controls, Sham 6 weeks (Sh6) and Sham 12 weeks (Sh12). Solid black columns: renovascular hypertension 6 weeks (RV6) or 12 weeks (RV12). Solid white columns: DOCA-salt hypertension 6 weeks (DS6) or 12 weeks (DS12). Combined treatments: RV6/DS6 (black/white) and the inverse sequence DS6/RV6 (white/black). The switch occurs after 6 weeks from the beginning of treatment. Appropriate sham animals were studied for each group and pooled into two groups at 6 weeks (Sh6) and 12 weeks (Sh12).

and a silver clip with a 0.28 mm gap was placed on the right renal artery. For sham-operated rats, flank incisions were made to expose the kidneys and the renal artery without clipping the vessel. The animals were studied after 6 weeks (RV6) or 12 weeks (RV12). Animals in which the clip was observed outside the right renal artery at the time of sacrifice were excluded from the analysis.

DOCA-Salt Hypertension

Rats were anesthetized as described above and subjected to left nephrectomy (Cavallero et al., 2007; Iyer et al., 2010). They received weekly injections of deoxycorticosterone acetate (DOCA, 30 mg/kg; Sigma, St. Louis, MO) suspended in sesame oil and were supplied with 1% W/V NaCl in the drinking water. Sham animals were subjected to the same surgical procedure where the kidney was exposed but not removed, and received sesame oil injections and tap water to drink. The animals were studied after 6 weeks (DS6) or 12 weeks (DS12).

Combination of RV and DS Models

RV6/DS6

Six weeks after induction of RV treatment, some animals were randomly selected to undergo renal artery declipping. The clips were removed carefully by a surgery performed close to the initial incision. Upon recovery, rats received DOCA injections and 1% NaCl treatment during six additional weeks. Sham animals were subjected to simulated surgery, vehicle injections and drank tap water.

DS6/RV6

After 6 weeks of DOCA-salt treatment, a subgroup of rats was given no further DOCA injections or NaCl and underwent surgery for right renal artery constriction with 0.28 mm clip for six additional weeks. This group was named DS6/RV6. Sham animals discontinued vehicle injections and were subjected to simulated surgery.

Systolic Blood Pressure Measurement

Systolic blood pressure was measured in conscious animals using the standard tail-cuff method (Blood Pressure Analysis System, Hatteras Instruments, Cary, NC, United States). All measurements were performed between 9:00 am and 1:00 pm, after 3–5 days of training.

Plasma and Tissue Processing

At the end of the experimental period, blood samples were obtained from the abdominal cava vein under anesthesia and

immediately placed into ice-chilled plastic tubes containing 15% EDTA. Then, a solution of KCl 1 mol/L was injected to induce diastolic arrest. The hearts were immediately removed, washed in cold phosphate buffered saline, blotted and weighed. Heart samples were carefully dissected by the same operator and the four cardiac chambers were weighed individually. The interventricular septum was included with the left chamber. Samples were frozen in liquid nitrogen and kept at −80°C for analysis.

The ratio between heart weight and body weight (HW/BW) was calculated to evaluate cardiac hypertrophy. The ratios for each individual chamber weight to body weight were also calculated. LVW/BW and RVW/BW indicated left and right ventricular hypertrophy, and LAW/BW and RAW/BW indicated left and right atrial hypertrophy, respectively.

Plasma ANP and BNP Measurement

Atrial and BNP were extracted from plasma samples as previously described (Cavallero et al., 2007, 2010). Radioimmunoassay (RIA) was performed using commercially available kits to detect rat ANP and BNP-45 (Phoenix Pharmaceuticals, Burlingame, CA, United States).

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from left ventricular samples using Trizol (Invitrogen, Carlsbad, California, United States) and subjected to Northern Blot analysis as previously described (Cavallero et al., 2007). Scanning values for ANP and BNP mRNA were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA.

Transthoracic Echocardiography

Echocardiography was performed using an Acuson Sequoia C512 Ultrasound System with a 14-MHz linear transducer. Echocardiographic studies were performed under light anesthesia using ketamine (35 mg/kg) plus xylazine (5 mg/kg). The chest was shaved and the animal was positioned on a warm pad. Electrode needles were connected to each limb, and the electrocardiogram was simultaneously recorded. Rats were imaged in a shallow left lateral decubitus position. Two-dimensional parasternal short-axis imaging plane were used to obtain M-mode tracings at the level of the papillary muscles. LV internal dimensions and LV wall thickness were determined at systole and diastole. End-diastolic measurements were taken at the maximal LV diastolic dimension, and end systole was defined as the time of the most anterior systolic excursion of the posterior wall. Measurements were taken from three consecutive beats for each rat. Transmitral Doppler inflow waves were used to measure peak early diastolic filling velocity (E wave), peak filling velocity at atrial contraction (A wave), and the ratio between them (E/A) as well as isovolumetric relaxation time (ms), assessing diastolic function as previously described (Gao et al., 2011; González et al., 2016).

Systolic function was evaluated from LV dimensions by the cubed method as percentage of LV ejection fraction (LVEF): LVEF (%) = [(LVEDD3–LVESD3)/LVEDD3] × 100, where LVEDD and LVESD are LV end-diastolic diameter and LV end-systolic diameter, respectively. Diastolic and systolic wall stress was calculated by using hemodynamic and echocardiographic measurements according to Laplace's law, where Diastolic stress = LV End-diastolic pressure x LV-diameter in diastole/2 × diastolic posterior wall thickness, and Systolic stress = LV systolic pressure × LV-diameter in systole/2 × posterior wall thickness in systole.

Statistical Analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed across all eight groups by one-way ANOVA, using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, United States). For multiple comparisons, we performed Tukey-Kramer post-test comparing the mean of each group with the mean of every other group to identify significant differences. *P*-values less than 0.05 were considered statistically significant and are indicated in the figures and tables.

We created the correlation analysis plots using the mean values of each experimental group for the measured variables. The linear Pearson correlation was used for correlation studies. Fisher's *Z* test was used to compare two correlation coefficients (Marino et al., 2008). *P*-values of 0.05 or less were considered statistically significant.

RESULTS

Systolic Blood Pressure

All experimental animals regardless of the treatment became hypertensive after 6 and 12 weeks compared to sham animals (**Figure 2**). Single RV and DS treatments induced different temporal profile of SBP response. SBP increase in response to RV treatment was time-dependent, with higher levels at 12 weeks than at 6 weeks. In contrast, SBP increased in response to DS treatment at 6 weeks, but did not further increase after 12 weeks. We measured SBP measured after 2, 4, 6, and 12 weeks of single RV or DS treatment (**Supplementary Figure 1**). The time course study of SBP further evidences a continued steep increase in RV toward 12 weeks, in contrast to the plateau observed in DS from 6 to 12 weeks.

Comparing both treatments, DS6 and DS12 groups reached similar blood pressure values to RV12. The fact that only 6 weeks of DS treatment were equally effective as 12 weeks of RV suggests that DS treatment determines an earlier increase in SBP when compared with RV.

Both groups with combined RV and DS developed similar degree of hypertension regardless the sequence of treatment (mmHg: RV6/DS6: 184 ± 4; DS6/RV6: 181 ± 5; see shaded columns in **Figure 2**). Both combined groups presented lower SBP than single treated groups at 12 weeks (mmHg: RV12: 203 ± 4; DS12: 193 ± 4), which only reached statistical significance compared to RV12. In addition, in a subset of animals undergoing combined treatments we followed up the increase in SBP over time measured at 3, 6, 9, and 12 weeks of treatment (shown in **Supplementary Figure 2**). A transient reduction in SBP was observed at 9 weeks in both combined groups (3 weeks after withdrawal of the first treatment and after installation of

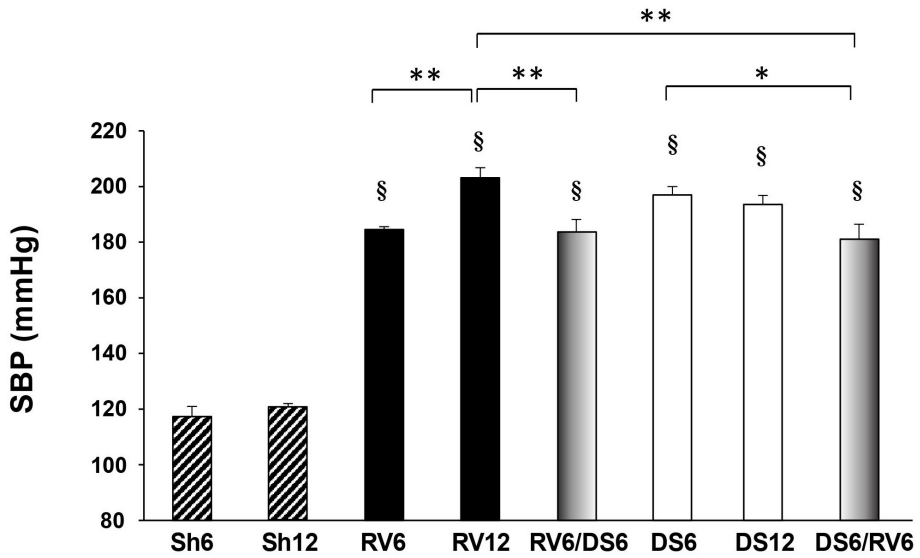

FIGURE 2 | Systolic blood pressure. The groups are indicated as described in **Figure 1**. Diagonally striped bars: Sham controls; black solid columns: RV treatment; white solid columns: DS treatment. Combined group RV6/DS6 is shaded black to white from left to right, and the inverse sequence DS6/RV6 is shaded white to black from left to right. Results are expressed as mean ± SEM, n = 10–18. Differences versus vs time- matched sham group (Sh6 or Sh12) are indicated on top of the column; differences among experimental groups are indicated within brackets: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\S}P < 0.001$.

the second treatment). The SBP continued to increase toward the 12-week end point, but it did not reach the SBP levels observed in the single treated groups RV12 and DS12. Taken together, these results suggest that a single type of stimulus persisting over time is more effective to induce hypertension that the sequential combination of two different types of hemodynamic stimuli, regardless the order of presentation.

We also verified if the withdrawal of DOCA-salt after 6 weeks of treatment is able to cause regression of hypertension and cardiac hypertrophy over the following 6-week period (**Supplementary Table 1**). At 12 weeks SBP was only partially normalized. However, cardiac hypertrophy reversed and was not different from the sham animals. On the other hand, it has been shown extensively in the literature that removal of the renal clip in the RV model reverses the SBP increase and the cardiovascular changes in both rats and mice (Edmunds et al., 1991; Kvist and Mulvany, 2003; Gao et al., 2005).

Cardiac Hypertrophy and Function

We calculated hypertrophy indexes and assessed functional parameters by echocardiography (**Table 1** and **Supplementary Figure 3**). All hypertensive groups developed cardiac hypertrophy at 6 and 12 weeks as evidenced by an increase in the heart-to-body weight ratio (HW/BW) and the LV posterior wall thickness in diastole (LVPWd). DS model showed a time-dependent increase in HW/BW ratio, with cardiac hypertrophy being more pronounced in DS12 compared to DS6 groups, while in RV we did not detect significant differences between 6 and 12 weeks. In addition, cardiac hypertrophy assessed by HW/BW was more pronounced in DS groups compared to RV groups at both time points (DS6 vs RV6 and DS12 vs RV12, $P < 0.05$ in both cases). Both combined treatments RV6/DS6 and DS6/RV6 had lower HW/BW compared to the single groups RV12 and DS12, respectively.

At the individual chamber level, RV as well as DS groups developed left ventricular hypertrophy when compared to sham animals, reaching similar LVW/BW ratios at 6 and 12-weeks of treatment. Single treated hypertensive groups developed right ventricular hypertrophy (RVW/BW) at 6 and 12 weeks. All hypertensive groups also developed left atrial hypertrophy, but only DS groups showed a time-dependent increase in LAW/BW (DS12 vs DS6, $P < 0.05$). Finally, right atrial hypertrophy (RAW/BW) was observed in DS groups at 6 weeks and was even more pronounced after 12 weeks. In contrast, right atrial hypertrophy developed in RV only after 12 weeks of treatment. Taken together, these results suggest that pronounced cardiac hypertrophy elicited by DS treatment may be sustained by an earlier development of hypertrophy in the right atria. Finally, both combined treatments RV6/DS6 and DS6/RV6 had less increase in RAW/BW compared to the single groups RV12 and DS12, respectively.

Functional data obtained by echocardiography are shown in **Table 1** and **Supplementary Figure 3**. All groups exhibited cardiovascular remodeling, with increased left ventricular wall thickness without dilation of the left ventricular chamber. The results of ejection fraction and shortening fraction show that left ventricular systolic function was preserved and none of the groups presented severe ventricular dysfunction. All hypertensive groups have compromised diastolic function as reflected by an increased isovolumic relaxation time (IVRT). At 6 weeks, DS group showed increased IVRT compared to RV6, however, at 12 weeks the IVRT did not differ between RV12 and DS12. Systolic parietal stress was elevated in DS at 6 weeks and more at 12 weeks, while in RV was increased only at 12 weeks.

TABLE 1 | Hypertrophic indexes and cardiac function assessed by echocardiography.

Group	HW/BW mg/g	LVW/BW mg/g	RVW/BW mg/g	LAW/BW mg/g	RAW/BW mg/g	LVEDD mm	LVPWd mm	Ejection fraction, %	Shortening fraction, %
Sh6	2.56 ± 0.05	1.95 ± 0.04	0.43 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	6.60 ± 0.11	1.43 ± 0.05	72.70 ± 0.81	36.53 ± 4.40
Sh12	2.46 ± 0.06	1.70 ± 0.04	0.52 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	6.63 ± 0.32	1.47 ± 0.03	68.03 ± 1.23	33.37 ± 0.89
RV6	3.30 ± 0.11†	2.48 ± 0.10*	0.59 ± 0.05*	0.17 ± 0.01*	0.12 ± 0.01	6.10 ± 0.20	1.71 ± 0.05*	77.60 ± 2.21	41.49 ± 2.03
RV12	3.61 ± 0.16‡	2.49 ± 0.20‡	0.65 ± 0.04*	0.17 ± 0.01‡	0.13 ± 0.01*	6.45 ± 0.25	1.85 ± 0.05*	77.01 ± 2.50	40.65 ± 2.35
RV6/DS6	3.19 ± 0.05‡	2.36 ± 0.06‡	0.59 ± 0.01	0.16 ± 0.01†	0.10 ± 0.01§	6.15 ± 0.15	1.77 ± 0.03*	76.40 ± 2.40	40.01 ± 2.10
DS6	3.89 ± 0.11$^{\ddagger\$}$	2.54 ± 0.13‡	0.59 ± 0.04*	0.17 ± 0.01*	0.14 ± 0.01*	6.47 ± 0.09	1.77 ± 0.07*	80.20 ± 3.10	43.83 ± 2.92
DS12	4.31 ± 0.16$^{\ddagger a\S}$	2.58 ± 0.26‡	0.73 ± 0.05†	0.21 ± 0.01$^{\ddagger a}$	0.17 ± 0.01$^{\ddagger a\S}$	7.00 ± 0.26	1.88 ± 0.07*	84.16 ± 2.90†	48.62 ± 3.26*
DS6/RV6	3.37 ± 0.08$^{\ddagger c}$	2.13 ± 0.11*	0.66 ± 0.03*	0.16 ± 0.01$^{\dagger b}$	0.12 ± 0.01*^{c}	6.93 ± 0.21	1.90 ± 0.16*	71.83 ± 3.44	36.58 ± 2.69
n	*10–18*	*5–9*	*5–9*	*10–18*	*10–18*	*3–8*	*3–8*	*3–8*	*3–8*

Ratios are calculated in mg tissue/g body weight. LVEDD and LVPWd are calculated in mm. Values are expressed as mean ± SEM. BW, body weight; HW, heart weight; LVW, left ventricle weight; RVW, right ventricle weight; LAW, left atria weight; RAW, right atria weight; LVEDD, LV end diastolic diameter; LVPWd, LV posterior wall thickness in diastole. The number of animals per group (n) is indicated for each parameter. The groups are nominated as described in ***Figure 1****. *$P < 0.05$, $^{\dagger}P < 0.01$ and $^{\ddagger}P < 0.001$ versus time-matched sham (Sh6 or Sh12), $^{\$}P < 0.01$ versus RV6; § $P < 0.05$ versus RV12; $^{a}P < 0.05$ versus DS6; $^{b}P < 0.05$, and $^{c}P < 0.001$ versus DS12.*

Furthermore, DS6/RV6 showed decreased systolic parietal stress compared to DS12. Taken together, these results indicate that DS treatment leads to an earlier compromise in both diastolic and systolic function compared to RV.

Plasma ANP and BNP Levels

We measured circulating levels of ANP and BNP as indicators of cardiac hormone secretion. Plasma ANP (**Figure 3A**) tended to increase in RV6 and RV12 compared to sham animals, although it did not reach statistical significance. In contrast, plasma ANP levels remarkably increased in both DS6 and DS12 compared to sham, and were statistically different from RV6 and RV12, respectively.

The RV6/DS6 combined model showed ANP concentrations that were not distinguishable from RV6 or RV12; however, the inverse sequence (DS6/RV6) showed significantly blunted ANP levels compared to DS6 and DS12.

Plasma BNP levels increased in both RV6 and RV12 groups (**Figure 3B**), and only in DS-treated animals after 12 weeks (DS12). Both combined models RV6/DS6 and DS6/RV6 had circulating BNP levels within the normal range when measured at 12 weeks.

Cardiac ANP and BNP Gene Expression

We evaluated ANP and BNP gene expression as an indicator of natriuretic peptide synthesis in cardiac tissues. Northern Blot analysis showed a time-dependent increase of ANP mRNA expression in the left ventricle for RV and DS, with higher values at 12 weeks in both treatments (**Figure 4A**). ANP gene expression was exacerbated in response to DS at 6 weeks compared to RV (4.5-fold-increase for DS6 versus 2-fold for RV6, $P < 0.001$), but only moderately at 12 weeks (6-fold-increase for DS12 versus 5-fold for RV12; the difference was not statistically different when comparing DS12 with RV12). Both combined treatments showed increased ANP mRNA levels compared to sham; RV6/DS6 showed significantly lower expression than RV12 or DS12.

Meanwhile, BNP mRNA did not significantly increase in RV6 and DS6, but increased 0.75-fold in RV12 and 1.35-fold in DS12 (**Figure 4B**). Only DS6/RV6 combined group had increased BNP mRNA compared to the opposite sequence RV6/DS6 and similar to DS12.

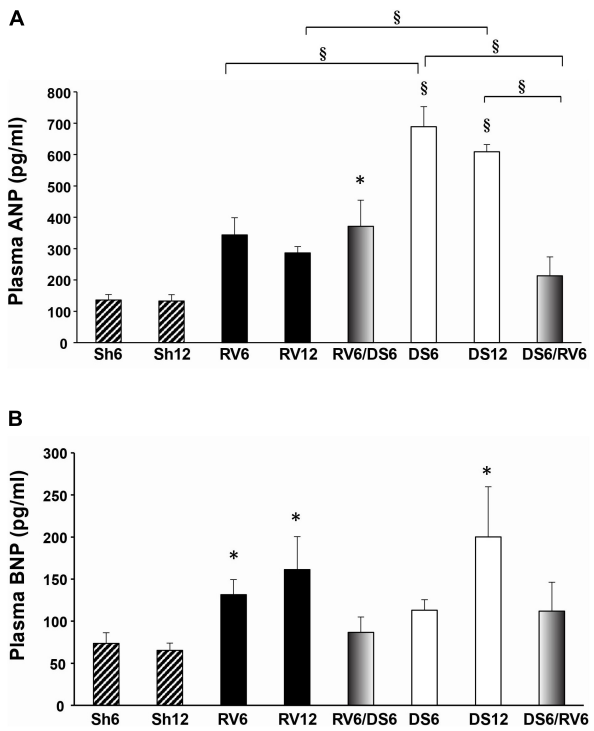

FIGURE 3 | Circulating natriuretic peptide levels. Plasma ANP **(A)** and BNP **(B)**. Results are expressed as mean ± SEM. The groups are nominated as described in **Figure 1**, and the columns are color coded as in **Figure 2**. Differences versus vs time- matched sham group (Sh6 or Sh12) are indicated on top of the column (no brackets); differences among experimental groups are indicated within brackets: *$P < 0.05$, $^{\S}P < 0.001$.

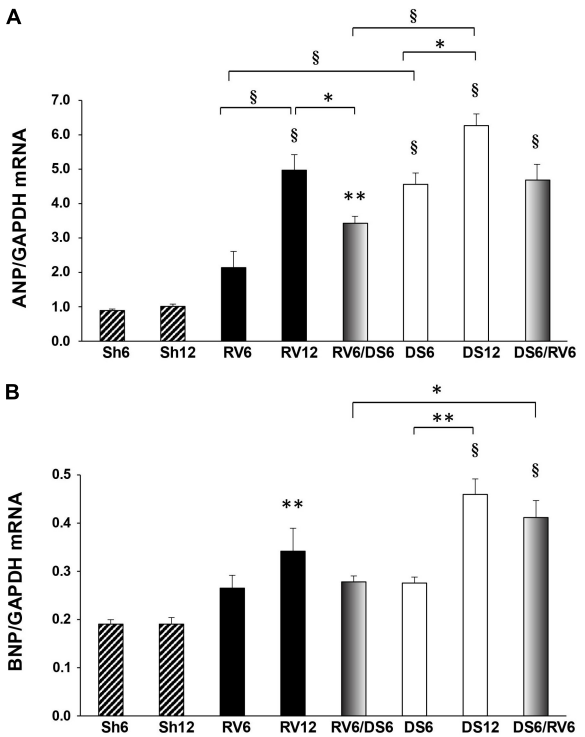

FIGURE 4 | Left ventricular gene expression of ANP and BNP. ANP mRNA **(A)** and BNP mRNA **(B)** in the left ventricle. Results are expressed as mean ± SEM, n = 3–5. The groups are nominated as described in **Figure 1**, and the columns are color coded as in **Figure 2**. Differences versus vs time-matched sham group (Sh6 or Sh12) are indicated on top of the column (no brackets); differences among experimental groups are indicated within brackets: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\S}P < 0.001$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

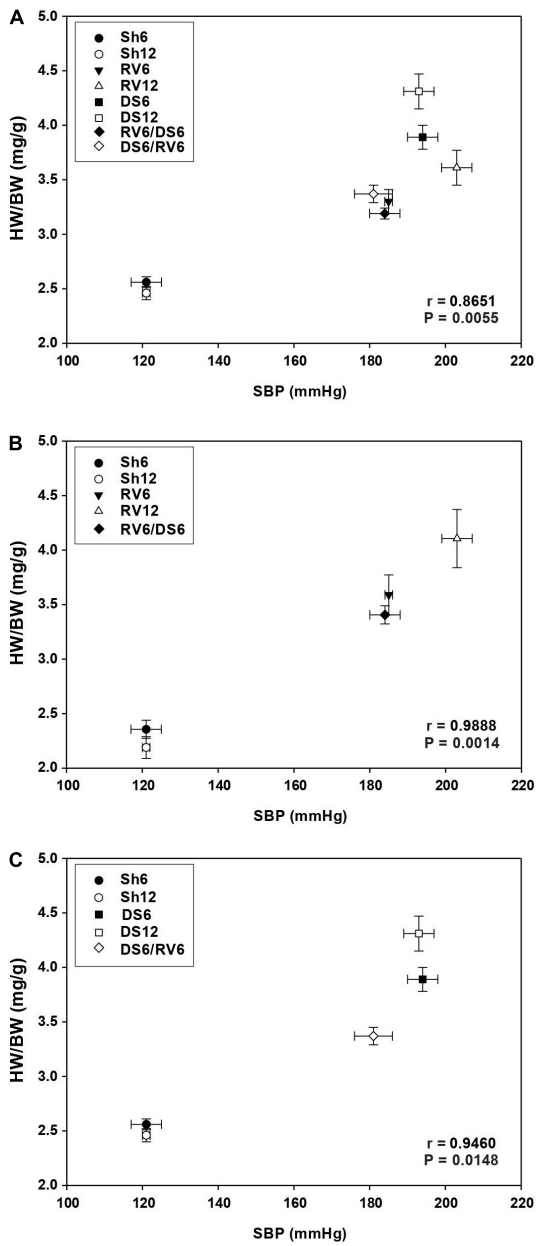

FIGURE 5 | Relationship between the evolution of cardiac hypertrophy (HW/BW) and systolic blood pressure (SBP) levels. Panel **(A)** analysis including all RV and DS groups; panel **(B)** analysis including only RV groups; panel **(C)** analysis including only DS groups. Each point on the graph represents the mean ± SE for each group. The groups are nominated as described in **Figure 1**.

Correlation Analysis

Hypertension Versus Cardiac Hypertrophy

We analyzed the relationship between hypertension (SBP value) and cardiac hypertrophy (HW/BW ratio). There was a positive correlation between SBP and HW/BW when all the groups were taken together (**Figure 5A**) and also when the RV (**Figure 5B**) and DS (**Figure 5C**) groups are analyzed separately. The correlation was more significant in RV than in DS (Fisher's z test between correlation coefficients for RV vs. DS: −4.7411, P: 0.0002). This result can be explained, at least in part, by the time dependent increase in SBP in response to RV treatment between 6 and 12 weeks, which is not observed in DS treatment (see **Figure 2** and **Supplementary Figure 1**).

ANP and BNP Circulating Levels Versus Ventricular Gene Expression

We analyzed the relationship between plasma levels of ANP and BNP peptides and their ventricular gene expression (graphs not shown). We did not find any correlation between circulating ANP and ANP gene expression neither in DS (r: 0.7717, P: 0.1263) nor RV groups (r: 0.6718, P: 0.2143). In contrast, the concentration of circulating BNP correlated with BNP gene expression in RV groups (r: 0.8816; P: 0.0480), but not in DS groups (r: 0.8750; P: 0.0525).

Cardiac Hypertrophy Versus Natriuretic Peptide Ventricular Expression

Figures 6A,B show the relationship for all experimental groups between left ventricular ANP and BNP gene expression level and cardiac hypertrophy, respectively. ANP as well as

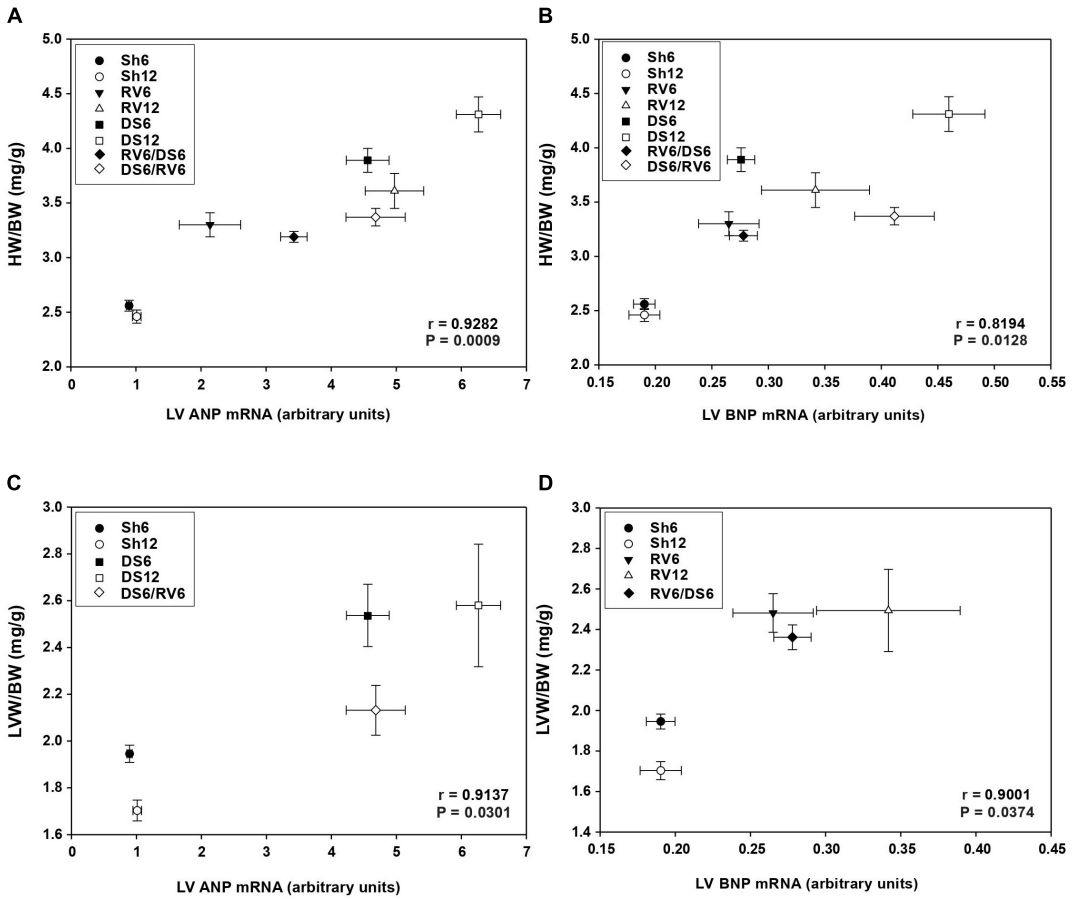

FIGURE 6 | Relationship between cardiac hypertrophy (HW/BW) and left ventricular NPs mRNA expression. Panels **(A,B)** analysis for ANP and BNP mRNA, respectively, including all RV and DS groups. Panel **(C)** analysis for ANP mRNA including only DS groups; panel **(D)** analysis for BNP mRNA including only RV groups. Each point on the graph represents mean ± SEM for each group. The groups are as nominated as described in **Figure 1**.

BNP mRNA were positively correlated to cardiac hypertrophy expressed as HW/BW ratio.

In addition, ANP mRNA showed positive correlation with the degree of left ventricular hypertrophy (LVW/BW ratio) when all groups were analyzed together (*r*: 0.7415, *P*: 0.0355; graph not shown). Moreover, a stronger positive correlation was exhibited when the DS groups were evaluated separately (**Figure 6C**; Fisher's *z* test between correlation coefficients for RV vs. DS: −2.2126, *P*: 0.0135). For the RV groups evaluated separately, the correlation was not statistically significant (*r:* 0.6353, *P*: 0.1750, graph not shown).

Finally, left ventricular BNP mRNA expression showed positive correlation with left ventricular hypertrophy only in the RV groups (**Figure 6D**), but not in DS groups (*r:* 0.6880, *P*: 0.1991; graph not shown).

DISCUSSION

Remodeling of the heart in response to persistent hemodynamic overload can lead to a spectrum of geometric, functional and hormonal patterns. We aimed to identify differential profiles of cardiac natriuretic hormones ANP and BNP in rats undergoing sequential combination of 6 weeks of renovascular followed by 6 weeks of DOCA-salt treatment or vice versa, and compared to the single treatments applied for 6 or 12 weeks. Our results show that in the models combining 6 weeks of each treatment, regardless of the sequence of presentation the overload stimulus associated to the second treatment is less effective than the first stimulus to sustain cardiac hypertrophy and to increase ANP and BNP expression. Moreover, in a chronic setting ANP correlates with volume overload while BNP correlates with pressure overload- induced cardiac hypertrophy. Thus, the combination of overload stimuli in different sequences provide an experimental tool to gain understanding about the events involved in the transition of hemodynamic states that occur along the evolution of cardiac disease.

Systolic Blood Pressure and Cardiac Hypertrophy

The development of hypertension in response to RV was gradual and time-dependent, in agreement with previous studies in this model (Gao et al., 2005) while in DS the blood pressure reached

a plateau at 6 weeks, with higher levels than RV, and persisted elevated with no further increase up to 12 weeks. Consistently, a study by Abrams et al. (2010) described distinct phases in the development of DOCA-salt hypertension: an initiation phase of rapid increase in blood pressure, followed by a development phase with a slower rise and a maintenance phase.

Renovascular model developed cardiac hypertrophy at 6 and 12 weeks, consistent with previous studies (de Simone et al., 1992; Sharifi et al., 2004; Gao et al., 2005). In DS treatment the hypertrophic process was even more pronounced than in RV, despite the lack of difference in SBP levels between 6 and 12 weeks. These results suggest that mechanisms other than SBP contribute to cardiac hypertrophy in DS. In this regard, Yokota et al. (1995) suggested that hypertension is not the initial cause for cardiac hypertrophy in DS model, given that 1 week of DS treatment was able to trigger hypertrophy even before the development of hypertension. The analysis of individual cardiac chamber hypertrophy indexes showed that right and left atrial hypertrophy in DS12 rats contributed to the difference observed in whole heart hypertrophy compared with RV.

All groups exhibited cardiovascular remodeling, with increased left ventricular wall thickness without dilation of the left ventricular chamber. The results of ejection fraction and shortening fraction show that left ventricular systolic function was preserved and none of the groups presented severe ventricular dysfunction. In addition, DS treatment leads to an earlier compromise in both diastolic and systolic function compared to RV. However, RV and DS did not show distinctive features of defined concentric or eccentric hypertrophy. Hypertensive disease per definition involves both pressure and volume overload components, therefore RV and DS are not 100% pure models for pressure and volume, respectively. Both models rather have a predominant component of pressure or volume, which leads to geometric patterns that are not completely concentric or eccentric, as it would happen in hypertrophic versus dilated cardiomyopathies of genetic origin.

Both combined models (RV6/DS6 and DS6/RV6) had lower SBP levels at 12 weeks than the single overloaded models RV12 and DS12, respectively. Cardiac hypertrophy, as well as the hypertrophy of the four chambers at 12 weeks was less pronounced in the combined treated groups than in the single treated models. A similar behavior was observed in our previous study, where we evaluated combined models at 4 weeks (Cavallero et al., 2007). These results confirm that suppression of one type of overload and substitution by another type is less effective than a single overload maintained throughout time, and that switch between treatments results in a slowdown of the hypertensive and hypertrophic process.

Circulating ANP and BNP

The circulating ANP and BNP levels are the result of a balance between gene regulation, peptide release and clearance by NPR-C receptors and the metallopeptidase neprilysin (Rubattu et al., 2020). In addition, the levels of biologically active NPs are also regulated by cardiac and renal transmembrane proteases (corin and furin) that cleave the inactive precursors proANP and proBNP into active smaller fragments (Goetze et al., 2020). Plasma ANP and BNP levels were measured as an index of NPs cardiac secretion. Plasma ANP was increased in both RV and DS treatments after 6 and 12 weeks. In our previous studies (Cavallero et al., 2007, 2010) we reported that both treatments induced a modest increase of similar magnitude in plasma ANP of 2 and 4 weeks of treatment, and that only the ventricular gene expression of ANP, but not the plasma ANP, was indicative of volume overload at 4 weeks. In this study, plasma ANP is much more increased in response to DS than RV treatment in the chronic setting at both 6 and 12 weeks. Interestingly, when volume overload is suppressed and replaced by RV treatment (group DS6/RV6) the increased ANP levels were reverted to the level of the sham animals. Therefore, plasma ANP could be considered a marker of volume overload in the DS model in a chronic setting.

The main source of ANP in plasma and the main stimulus for the release of ANP in DS is volume overload that leads to stretching of the right atrium. In contrast, the RV model has predominantly pressure overload that mainly involves stretching of the left ventricular myocardium. These facts may explain why plasma ANP increases much more in response to DS than to RV treatment. Furthermore, these results are also in agreement with Yokota et al. who have reported a marked increase in cardiac ANP production and its secretion into the circulation in DS model (Yokota et al., 1995).

However, in the RV model when pressure overload is suppressed and replaced by DS treatment (group RV6/DS6) the ANP levels did not further increase, and showed similar levels to RV6 and RV12. A possible explanation to this observation is that after the interruption of RV model there is a partial regression of the hypertensive process (**Supplementary Figure 2**). Moreover, DS model is based on pharmacological treatment with DOCA injections and salt intake, which in a heart that is already hypertrophied may not generate such a pronounced response as it would happen in a normal heart. We speculate that there is a longer latency period for the RV/DS transition than for the opposite sequence DS/RV (in which the effect of the constrictor clip takes place immediately after the surgery). Further investigations will be needed to provide a definitive explanation to this lack of ANP response in the RV/DS model.

We previously showed that plasma BNP levels increased only after 4 weeks of RV treatment (Cavallero et al., 2007) and in the present study we observed elevated BNP in RV at both 6 and 12 weeks. In contrast, plasma BNP did not increase at 6 weeks and only increased after 12 weeks of DS treatment. These results suggest that BNP responds to volume overload only after a sustained exposure, and therefore we conclude that BNP plasma level is an earlier indicator for pressure overload in RV.

ANP and BNP Gene Expression

We evaluated ANP and BNP gene expression as a more reliable index of natriuretic peptide synthesis than the tissue protein level, since the amount of tissue protein may be influenced by several processes like synthesis, storage, intragranular cleavage, clearance, and intracrine/autocrine release or endocrine secretion (Yokota et al., 1995). More recently, epigenetic mechanisms have

been shown to modulate several components of the NP system (Rubattu et al., 2020).

The results obtained in the present study allow us to suggest that hypertrophic growth and NPs gene induction are processes that develop in parallel in the single-overloaded models. Our results show that DS treatment elicits an earlier induction of ANP gene expression compared to RV, since ANP mRNA was increased at 6 weeks in DS treated animals but not in RV. ANP mRNA expression markedly increased in RV and DS treatment from 6 to 12 weeks, and at 12 weeks there was no difference between RV and DS treatment. In contrast, BNP mRNA did not increase at 6 weeks in RV and DS but increased in both treatments at 12 weeks. These data are in agreement with previous reports (Yokota et al., 1990; Liu and Yoshimi, 1995; Bianciotti and de Bold, 2000, 2001; Wolf et al., 2001) and our previous studies (Cavallero et al., 2007, 2010). Moreover, mRNA expression of ANP and BNP positively correlate with ventricular weight (Kohno et al., 1992).

In RV model, the increase in gene expression was not sufficient to increase plasma levels on ANP at 12 weeks. A recent study also showed that the accumulation and release of ANP from myocyte granules depends on the pathogenesis of hypertension, and described a higher number of ANP secretory granules in a salt loading model compared to two-kidney 1-clip hypertension, providing a possible explanation for the lack of increase in plasma ANP in our RV model (Bugrova and Galkina, 2020). Another possible explanation is a differential regulation of the tissue cleavage enzymes and clearance receptors, which we have not addressed in this study.

In the combined models, left ventricular ANP mRNA expression showed intermediate levels between those observed in both single treated RV12 and DS12 and their corresponding sham groups, supporting the concept that combined models display an intermediate behavior between those observed in single models.

Hormonal and Hypertrophic Profiles

BNP gene expression in the left ventricle was more positively correlated with left ventricular hypertrophy index only in RV groups, while ANP gene expression was positively correlated with left ventricular hypertrophy index only in DS groups. These results strongly suggest that throughout the evolution of left ventricular hypertrophy, ventricular re-expression of ANP is mainly induced in volume-overloaded DS groups and BNP in pressure-overloaded RV model. Both combined groups had a similar increase of left ventricular hypertrophy. However, left ventricular BNP expression was higher in DS6/RV6. Then, the induction of both ventricular hypertrophy and the natriuretic peptide expression in the combined treated groups would be two independent processes.

Plasma ANP did not correlate with ANP gene expression in left ventricle neither in DS nor RV groups, while plasma BNP correlated with left ventricular BNP mRNA in RV groups, but not in DS groups. These results suggest that plasma ANP levels not only are influenced by cardiac ANP secretion, but also by other tissue sources. On the other hand, circulating BNP levels in RV model seems to be regulated by cardiac BNP synthesis, which depends, among other factors, of ventricular hypertrophy. In agreement, Yokota et al. (1995) reported that while an increase in circulating levels of both ANP and BNP reflects ventricular hypertrophy, increases in plasma ANP without a concomitant increase in plasma BNP indicates atrial hemodynamic overload independently of ventricular hypertrophy.

Combined Models

The switch RV-to-DS treatment as well as the opposite sequence DS-to-RV in chronic combined models of hypertension slowed down the progression of hypertension, the cardiac hypertrophy, and the increase of ANP and BNP expression, although the cardiac overload continued for 12 weeks. This behavior differs from the acute combined models, where we reported that the second stimulus of mechanical overload determines the evolution of hypertrophy and the synthesis and secretion of NPs (Cavallero et al., 2007, 2010). In the acute setting, hypertension and cardiac hypertrophy elicited in response to the first treatment are rapidly reverted when the treatment is withdrawn- and eventually would completely regress after 2 weeks without treatment-, thus allowing the second stimulus to rapidly predominate over the first one, and as a result the heart displays the characteristics of the second mechanical overload. However, in a chronic setting where the hypertrophic process is well established after 6 weeks of the first type of overload, the effects of the second overload may not be independent of the effects of the previous overload. As a consequence, the "new" type of hypertrophic process that takes place after the switch is slower and the heart maintains a pattern of response more similar to the first than to the last stimulus. Further experimentation will be needed in order to study the biological and molecular sequence of events developed in response to the switch pressure-volume and volume-pressure in the combined overloaded hearts.

Study Limitations

As mentioned above, RV and DS are not pure models and have a predominant component of pressure or volume overload, respectively. We chose these models because alternative surgical procedures for pure hemodynamic overload in rats such as aortic constriction (pressure) and aortocaval shunt (volume) involve complex surgical interventions that are difficult to revert; therefore they are not amenable to perform regression and combination studies (Wu et al., 2015). In addition, in the present study we evaluated the effect of the treatments only at the end points (6 and 12 weeks). The goal of future studies will be to investigate the complex events that happen during the critical process of transitioning between models, including the behavior of the RAAS system, and this will carry critical implications to understand the progression of disease in humans.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Review Board at the University of Buenos Aires.

AUTHOR CONTRIBUTIONS

CC, SC, and BF conceived and designed the study. CC, SC, and MRF performed the experiments and statistical analysis. MD, GG, and RG performed echocardiography analysis and wrote sections of the manuscript. CC and CH performed Northern Blot analysis. CC, SC, MRF, GG, NK, MC, and BF wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

This study was supported by grants from the University of Buenos Aires (UBACyT B014 and B018 to BF), the National Council of Scientific and Technological Research (CONICET PIP 6161 to BF), the National Agency for Science and Technology (ANPCyT PICT 05-13775 and 05-22037 to BF, PICT 2018-01105 to MC), and the "Hector A. Barceló" Foundation (BA-MED-039-2017 to BF).

ACKNOWLEDGMENTS

The authors wish to thank Federico Matorra for contribution with echocardiography analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.651246/full#supplementary-material

Supplementary Figure 1 | Systolic blood pressure (SBP) in RV (upper panel) and DOCA-salt hypertension (lower panel) single treatments compared to their sham at 2, 4, 6, and 12 weeks of treatment. Results are expressed as mean ± SEM. $^{*}P < 0.01$ and $^{***}P < 0.001$ versus corresponding sham; $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.001$ versus RV2; $^{\S}P < 0.01$ versus RV6: $^{\S}P < 0.05$ vs. DS2; $^{\#}P < 0.05$ vs. DS4.

Supplementary Figure 2 | Systolic blood pressure (SBP) in single and combined RV and DS treatments measured at 3, 6, 9, and 12 weeks of treatment. Results are expressed as mean ± SEM.

Supplementary Figure 3 | Ventricular function parameters. Differences versus vs time- matched sham group (Sh6 or Sh12) are indicated on top of the column; differences between experimental groups are indicated within brackets: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{\S}P < 0.001$.

Supplementary Table 1 | Systolic blood pressure (SBP) and hypertrophic indexes in sham animals for different groups. Ratios are calculated in mg tissue/g body weight. Values are expressed as mean ± SEM. BW, body weight; HW, heart weight; LVW, left ventricle weight; RVW, right ventricle weight; LAW, left atria weight; RAW, right atria weight. The number of animals (n) per group is indicated for each parameter. Sh-RV6 and Sh-RV12 are the sham animals for renovascular groups; Sh-DS6 and Sh-DS12 are the sham animals for DOCA-salt groups; Sh-RV6/DS6 and Sh-DS6/RV6 are the sham animals for combined groups. Animals were subjected to simulated surgeries and vehicle injections at the same time points as the experimental groups as indicated in the Methods section.

Supplementary Table 2 | Regression of hypertension and cardiac hypertrophy at 12 weeks after withdrawal of DOCA-salt treatment for 6 weeks. A group of animals were subjected to left nephrectomy and DOCA/salt administration as described in the Methods section; after 6 weeks the treatment was discontinued and the parameters were evaluated at 12 weeks. Values are expressed as mean ± SEM. Number of animals (*n*) is indicated between brackets for each group. $^{\S}P < 0.001$ vs corresponding sham; $^{@}P < 0.001$ vs DS6; $^{\$}P < 0.01$ and $^{\#}P < 0.001$ vs DS12; $^{*}P < 0.05$ vs DS6/RV6.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

MINI REVIEW
published: 28 May 2021
doi: 10.3389/fphys.2021.680213

Protective Renal Effects of Atrial Natriuretic Peptide: Where Are We Now?

Marcelo Roberto Choi[1,2,3]* *and Belisario Enrique Fernández*[3]

[1]*Instituto Alberto C. Taquini de Investigaciones en Medicina Traslacional (IATIMET), CONICET - Universidad de Buenos Aires, Buenos Aires, Argentina,* [2]*Departamento de Ciencias Biológicas, Facultad de Farmacia y Bioquímica, Cátedra de Anatomía e Histología, Universidad de Buenos Aires, Buenos Aires, Argentina,* [3]*Instituto Universitario de Ciencias de la Salud, Fundación H.A. Barceló, Buenos Aires, Argentina*

Atrial natriuretic peptide belongs to the family of natriuretic peptides, a system with natriuretic, diuretic, and vasodilator effects that opposes to renin-angiotensin system. In addition to its classic actions, atrial natriuretic peptide exerts a nephroprotective effect given its antioxidant and anti-inflammatory properties, turning it as a beneficial agent against acute and chronic kidney diseases. This minireview describes the most relevant aspects of atrial natriuretic peptide in the kidney, including its renal synthesis, physiological actions through specific receptors, the importance of its metabolism, and its potential use in different pathological scenarios.

Keywords: atrial natriuretic peptide, nephroprotection, kidney, natriuresis, antioxidant

OPEN ACCESS

Edited by:
Rubattu Speranza,
Istituto Neurologico Mediterraneo Neuromed (IRCCS), Italy

Reviewed by:
Luis Miguel Ruilope,
University Hospital October 12, Spain
Riccardo Sarzani,
Marche Polytechnic University, Italy
Thomas Langenickel,
Ethris GmbH, Germany

***Correspondence:**
Marcelo Roberto Choi
marcelinkchoi@yahoo.com.ar

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *13 March 2021*
Accepted: *29 April 2021*
Published: *28 May 2021*

Citation:
Choi MR and Fernández BE (2021) Protective Renal Effects of Atrial Natriuretic Peptide: Where Are We Now? Front. Physiol. 12:680213. doi: 10.3389/fphys.2021.680213

INTRODUCTION

The natriuretic peptide system involves the family of natriuretic peptides ANP, BNP, CNP, DNP, and urodilatin; the receptors NPR-A, NPR-B, and NPR-C; and the enzymes involved in their synthesis (furin, corin, and PCSK6) and degradation [neutral endopeptidase (NEP)] (Rukavina Mikusic et al., 2018b; Rubattu et al., 2019). Its main functions are to regulate hydro-saline homeostasis and blood pressure through renal actions combined with vascular effects. This system opposes the renin-angiotensin system (RAS), thus representing a protective mechanism against hypertension and their associated pathologies, including kidney damage (Rubattu et al., 2019). ANP was discovered in 1981 by de Bold et al. (1981) as a 28-amino acid peptide that is synthesized and stored in atrial and ventricular myocytes and released in response to various stimuli, such as stretching of the cardiac wall, endothelin, diverse cytokines, or adrenergic agents (Rukavina Mikusic et al., 2018b). Subsequently, it has been described that the kidney has all the biosynthetic machinery to produce ANP, its receptors, and the catabolic enzymes to degrade it (Wu et al., 2009; Choi et al., 2014). Given its actions in the kidney, it has been proposed that ANP could be involved in the pathogenesis of chronic kidney disease (CKD).

Synthesis, Receptors, and Degradation of ANP

Corin is a type II transmembrane serine protease responsible for the cleavage of inactive pro-atrial natriuretic peptide (pro-ANP) into active ANP (Ricciardi et al., 2016). Synthesized as a zymogen, corin needs to be cleaved by a proprotein convertase subtilisin/kexin-6 (PCSK6, also called PACE4)

in order to be activated (Chen et al., 2018). The kidney is the second organ with the highest amount of corin after the heart (Xue et al., 2020). Corin expression has been detected in the proximal tubule, the distal tubule (largely in the thick ascending medullary loop), and the collecting duct (especially in the internal medullary collector duct; Theilig and Wu, 2015). Several facts reveal the importance of corin or PCSK6 in the metabolic pathway of ANP and its effects on sodium homeostasis and blood pressure: (1) mutations in corin gene that affect its activation are associated with hypertension and preeclampsia (Cui et al., 2012; Dong et al., 2013), (2) in CKD patients, as well as proteinuric rats, it has been described reduced levels of corin in the kidney (Polzin et al., 2010; Fang et al., 2013), and (3) PCSK6 KO mice develop salt-sensitive hypertension and exhibit undetectable levels of corin and pro-auricular ANP activity (Chen et al., 2015).

To date, three types of natriuretic peptide receptors have been described: NPR-A, NPR-B, and NPR-C. ANP binds preferentially to NPR-A and NPR-C receptors (Rukavina Mikusic et al., 2018b). The NPR-A receptor is a transmembrane receptor encoded by the Npr1 gene, with its mRNA expressed in different organs, including the kidney. In the kidney, NPR-A is located at the renal vessels, podocytes, mesangial cells, proximal tubule, thin and thick ascending loop of Henle, collecting duct, and juxtaglomerular cells (Ogawa et al., 2012; Staffel et al., 2017). The guanylyl cyclase activity of NPR-A catalyzes the production of the second messenger cyclic guanosine monophosphate (cGMP), which triggers downstream activation of cGMP-dependent protein kinases (PKG) I and II, cyclic nucleotide-activated ion channels, and cyclic nucleotide phosphodiesterase (Beavo and Brunton, 2002). NPR-A displays a high affinity for ANP, BNP, and urodilatin, and its activation not only mediates vasodilator and natriuretic effects but also activates cellular mechanisms that regulate cell growth, apoptosis, proliferation, and inflammation (Rukavina Mikusic et al., 2018b). Several factors can regulate the expression of NPR-A, such as angiotensin II (Ang II), vitamin D, endothelin, endothelial NO synthase (eNOS), p38 MAPK, and osmotic stimuli. Also, the same ANP can negatively regulate its own Npr1 gene expression through cGMP response element-binding protein (Theilig and Wu, 2015). Regarding NPR-C, two subtypes with different molecular weights have been identified. The 77 kDa NPR-C lacks the guanylyl cyclase activity since it has a short intracellular domain, acting more as a clearance receptor through the internalization and degradation of natriuretic peptides (Theilig and Wu, 2015; Zhao and Pei, 2020). On the other hand, the 67 kDa NPR-C acts as an inhibitor of the adenylyl cyclase activity by coupling to an inhibitory Gi protein and activation of phospholipase C to exert multiple effects at the vascular, cardiac, metabolic, and bone (Rubattu et al., 2019). Recently, it has been demonstrated that both NPR-A and NPR-C mediate the renal effects of ANP by enhancing $Ca2^{+}$/calmodulin-dependent NOS activity. NPR-A-induced NO production was shown to be cGMP dependent, while NPR-C-dependent NO release is partially mediated by Gi protein (Theilig and Wu, 2015).

ANP can be catabolized by two different pathways: (1) ANP binding to NPR-C receptor leads to ANP-NPR-C complex, which is internalized, and then, ANP is cleaved by lysosomal actions and (2) circulating ANP can be degraded by the NEP, neprilysin (Yandle et al., 1989; Nussenzveig et al., 1990). Neprilysin is a zinc-dependent metallopeptidase expressed in different tissues, including the kidney. This catabolic pathway takes relevance in scenarios with high levels of ANP, such as in heart failure (Potter, 2011). Although the main substrate for neprilysin is ANP, given its high complementary affinity, it also degrades other substrates, and thereby, its inhibition is not only associated with increased levels of ANP but also with other substances with vasodilator and natriuretic properties, as bradykinin, adrenomedullin, and angiotensin 1-7 (Volpe et al., 2019). In contrast, NEP inhibition can increase Ang II and endothelin-1 levels, blocking the beneficial effects of ANP through their vasoconstrictor and pro-fibrotic effects (Ferro et al., 1998). Recently, it has been reported that dipeptidyl peptidase-4 (DPP-4) would also degrade ANP, since inhibitors of DPP-4 exhibit antioxidant and anti-inflammatory effects, partially due to rising ANP levels and eNOS activity (Kamel et al., 2019).

Renal Physiological Actions and Its Role as a Nephroprotector Hormone

In the kidney, ANP stimulates diuresis and natriuresis by different mechanisms (**Figure 1**): (1) At glomerular level: ANP vasodilates the afferent arteriole and contracts the efferent arteriole, thus increasing glomerular capillary pressure and therefore improving glomerular filtration rate (GFR) and fractional filtration (Dunn et al., 1986). This hemodynamic effect on the glomerulus is a consequence of ANP actions on the mesangial and smooth muscle cells of the renal arterioles. On podocytes, ANP exerts a protective effect, as it was demonstrated in mice with specific KO of NPR-A in podocytes and exposed to deoxycorticosterone acetate and high salt intake, where markedly increased levels of albuminuria and glomerular damage were observed related to a decrease in podocin, nephrin, and synaptopodin expression in the slit diaphragm and a greater influx of calcium ATP due to increased expression of canonical channel 6 (TRPC6; Ogawa et al., 2012; Staffel et al., 2017).

(2) At tubular level: ANP inhibits sodium reabsorption throughout the nephron (Choi et al., 2014). In the proximal tubule, ANP inhibits different Na^{+} transporters, as the Na^{+}/H^{+} exchanger, Na/Pi type IIa cotransporter, and the Na^{+}, K^{+}-ATPase, and also counteracts angiotensin-stimulated sodium reabsorption (Theilig and Wu, 2015). Additionally, ANP can regulate other transporters as organic cation transporters (OCTs) and chloride channels (Darvish et al., 1995; Kouyoumdzian et al., 2016). In Henle's thick ascending loop, ANP inhibits Cl^{-} transport and the activity and expression of the $Na^{+}/K^{+}/2Cl^{-}$ cotransporter, reducing the ability to concentrate urine and increasing urine formation (Theilig and Wu, 2015). In the cortical collecting duct, ANP decreases aquaporin-2 phosphorylation, thus reducing vasopressin-dependent water permeability (Dillingham and Anderson, 1986; Klokkers et al., 2009). In the medullary collecting duct, acting on main cells, ANP reduces epithelial sodium channel (ENaC) opening as

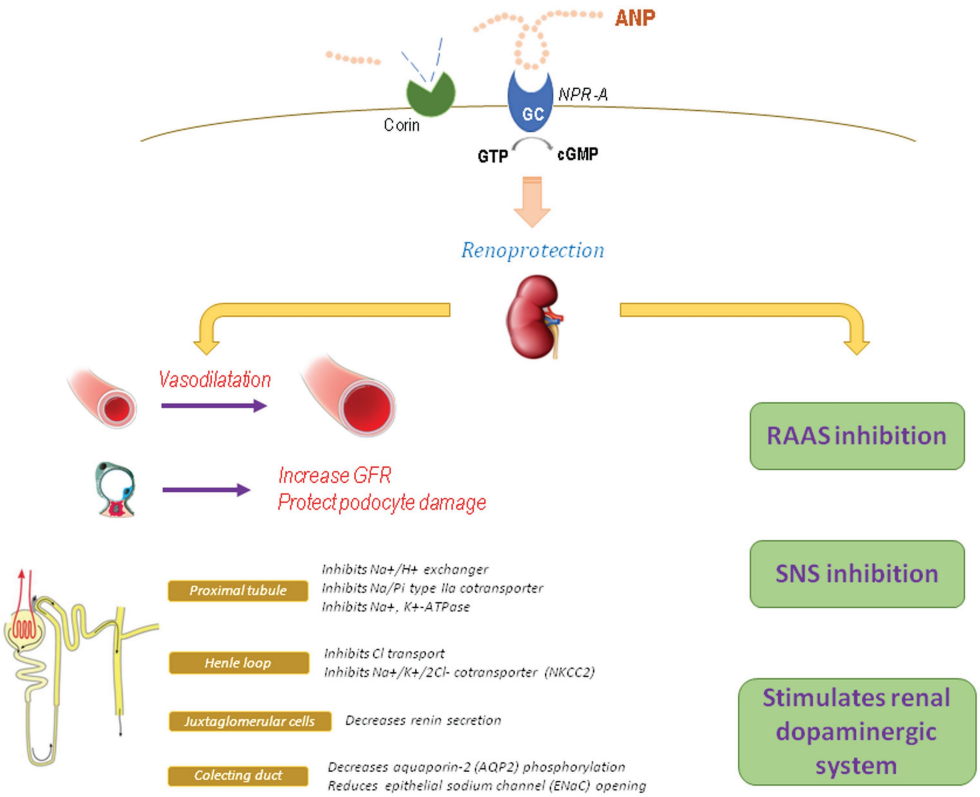

FIGURE 1 | Physiological actions of ANP. GFR, glomerular filtration rate; RAAS, renin-angiotensin-aldosterone system; SNS, sympathetic nervous system.

well as other sodium channels as cyclic nucleotide-regulated cation channels (CNG; Light et al., 1990, Guo et al., 2013). The ENaC channel is a key target for ANP, since corin knockout mice display an increase in the β subunit of medullary ENaC with enhanced reabsorption of sodium and water and development of salt-sensitive hypertension (Polzin et al., 2010; Wang et al., 2012).

(3) At hormonal level: ANP has antagonic effects on the RAS, which contributes to its nephroprotective actions, since ANP decreases renin secretion from juxtaglomerular cells, reduces Ang II and vasopressin effects, and blocks aldosterone synthesis and release (Kurtz et al., 1986; Brenner et al., 1990; Volpe et al., 2019). Aldosterone is a determining factor for CKD progression. As a physiological antagonist, ANP can block aldosterone-induced MAPK phosphorylation in podocytes and also avoid aldosterone-induced nuclear translocation of the mineralocorticoid receptor *via* PKG I (Kato et al., 2017). Additionally, hypertension and glomerular injury (mesangial expansion, segmental sclerosis, severe podocyte injury, and increased oxidative stress) induced by chronic administration of aldosterone and high salt diet are deeply impaired and accelerated in mice lacking of NPR-A receptor, even when blood pressure is controlled with hydralazine, suggesting that these effects are independent of hypertension (Ogawa et al., 2012). All these alterations can be reduced by the AT1 receptor blocker olmesartan or with the antioxidant tempol, suggesting that the nephroprotective properties of ANP are related to its antagonic action against the RAS as well as its antioxidant effects (Baradaran et al., 2014; Rukavina Mikusic et al., 2014). It has also been reported that ANP is capable to regulate another local natriuretic system, the renal dopaminergic system. In this way, the diuretic and natriuretic effects of ANP are partially exerted by favoring the recruitment of D1R receptors and by enhancing the inhibitory effect of dopamine on Na^+/H^+ exchanger in the proximal tubules (Choi et al., 2014). Furthermore, ANP stimulates tubular dopamine uptake *via* NPR-A, cGMP, and PKG, increases the activity of dopa decarboxylase, and reduces the activity of COMT, thus favoring the tubular bioavailability of dopamine (Fernández et al., 2005; Correa et al., 2007, 2008). These effects were reproduced in an experimental *in vivo* model, in which the infusion of ANP at low doses increased the urinary excretion of dopamine by stimulating the activity of OCTs with subsequent over-inhibition of the Na^+, K^+-ATPase (Kouyoumdzian et al., 2016). Additionally, Ang II opposes to ANP by downregulating the renal dopaminergic system (Rukavina Mikusic et al., 2018a; Kouyoumdzian et al., 2021). This synergic interaction between ANP and renal dopamine not only favors the maintenance of hydro-electrolyte balance, blood pressure, and a stable redox state, but also its alteration would be implicated in the pathophysiology of arterial hypertension and inflammatory renal damage (Armando et al., 2011; Choi et al., 2014).

In addition to its hemodynamic and renal effects, it has been described that ANP exhibits antioxidant and anti-inflammatory properties that justify its nephroprotective effects (Rukavina Mikusic et al., 2014). Oxidative stress and inflammation are determining factors for the development of kidney damage (Harrison et al., 2011; **Figure 2**). It has been shown that ANP is capable of attenuate ROS levels in different models of kidney injury such as animals fed with chronic high sodium diet, in which the hypoxia generated increases the levels of HIF-1α, an important stimulus for ANP synthesis and release (Chun et al., 2003; Della Penna et al., 2014). In addition, it has been demonstrated that ANP exhibits anti-inflammatory effects by inhibiting the activation of the nuclear factor NF-kB (by inducing the expression of the inhibitor IκB of NF-κB), iNOS, RANTES, cyclooxygenase-2, and TNF-α, thus decreasing the production of peroxynitrites, cytokines, and chemokines (Kiemer and Vollmar, 1998; Kiemer et al., 2002a,b). Additionally, ANP exerts an indirect effect by facilitating the action of the renal dopaminergic system, which also exhibits an antioxidant effect by inhibiting NADPH oxidase and stimulating the antioxidant enzymes superoxide dismutase, glutathione peroxidase, glutamyl cysteine transferase, Parkinson's protein 7 (PARK7 or DJ-1), paraoxonase 2 and heme oxygenases 1 and 2 (George et al., 2012; Yang et al., 2012; Rukavina Mikusic et al., 2014).

ANP as a Renal Therapeutic Agent

Several experimental and clinical studies have demonstrated the benefit of ANP administration in the prevention or reversion of renal damage. Given its vasodilator and renal hemodynamic effects, the ANP analog, anaritide, has been shown to improve diuresis in patients with oliguric acute renal failure and reduce the need for dialysis (Allgren et al., 1997). In the renal ischemia-reperfusion injury model, the infusion of ANP (0.2 mg/kg/min) prevented metabolic acidosis, increased plasma creatinine and lactate, reduced tubular injury, increased activity of eNOS, and attenuated the expression of TNF-a in the kidney (Chujo et al., 2010; Tulafu et al., 2014). On the other hand, the acute kidney injury developed during surgery is associated with a greater risk of adverse events and death; therefore, strategies that protect the kidney are of interest to prevent or reduce them. In this context, it has been reported that the administration of ANP, especially in low doses, exerts beneficial and protective effects against acute kidney injury after cardiac surgery by preserving renal function, increasing intraoperative diuresis, and improving the degree of acute renal failure (Mitaka et al., 2011; Moriyama et al., 2017). In addition, the infusion of ANP or its analog, carperitide, in CKD patients undergoing cardiac surgery reduces postoperative serum creatinine concentration both in the acute postoperative stage and even up to 1 year after surgery with a lower rate of postoperative cardiac events and need of dialysis (Sezai et al., 2011; Mori et al., 2014). In contrast, a prospective, multicenter, randomized, double-blind, and placebo-controlled clinical study, carried out in 77 patients with acute kidney injury associated with cardiac surgery, demonstrated that a low-dose ANP infusion of 0.02 μg/kg/min significantly increased urine output but failed to improve kidney function. The authors postulate that this lack of response observed with ANP could be due to the fact that in the pathophysiological process of acute injury associated with cardiac surgery, renal blood flow decreases due to several mechanisms such as ischemia and reperfusion injury, neurohormonal activation, pro-inflammatory mediators and

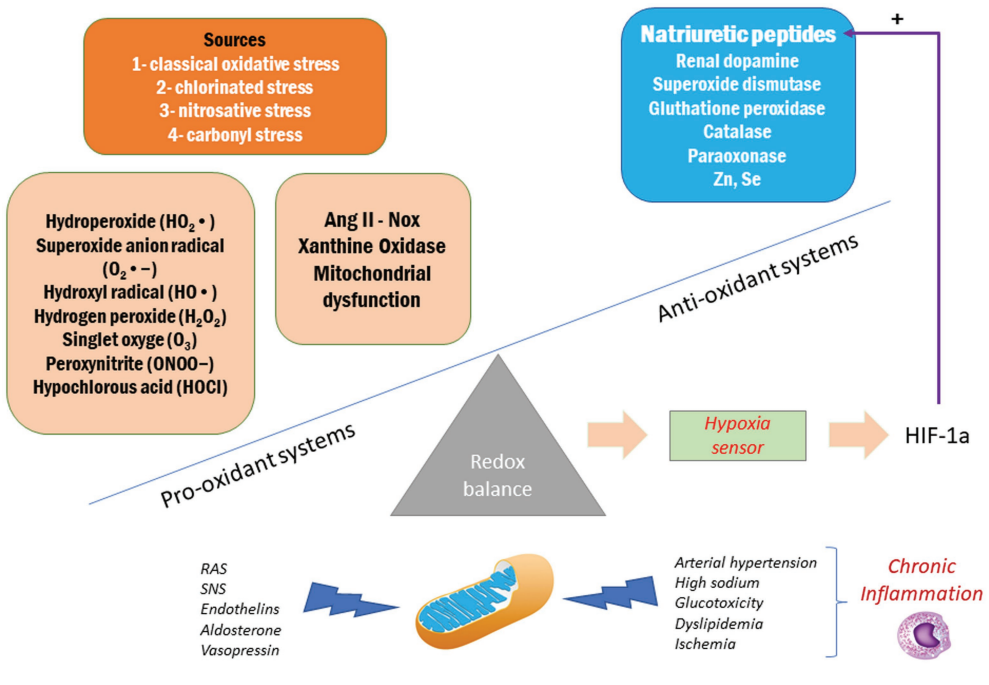

FIGURE 2 | Oxidative stress in the progression of chronic kidney disease.

vasoconstrictor agents, oxidative stress, and the presence of exogenous and endogenous toxins (Mitaka et al., 2017).

The renal benefits of ANP have also been demonstrated in the initial oliguric phase of endotoxemia and in renal failure induced by nephrotoxic agents. In this way, the addition of carperitide (a recombinant ANP) at a dose of 1.8 μg/kg/h to fluid resuscitation therapy significantly improved both the glomerular filtration and the tubular flow rates in rats exposed to lipopolysaccharides (Kitamura et al., 2018). Additionally, subcutaneous infusion of ANP in rats at 1.5 μg/kg/min was shown to significantly reduce the cisplatin-induced increase in creatinine and urea nitrogen, the urine albumin/creatinine ratio, tubular necrosis, and renal expression of inflammatory markers, such as IL-1β, IL-6, intercellular adhesion molecule 1, and monocyte chemoattractant protein 1 mRNA (Nojiri et al., 2015).

The Imbalance Between ANP and Ang II as a Potential Nephroprotective Strategy in Chronic Kidney Disease

Chronic kidney disease is associated with an imbalance of vasoactive substances in the kidney, with an increase in vasoconstrictor agents, such as Ang II and endothelin-1, and a reduction in vasodilator agents, such as nitric oxide, bradykinin, and ANP, which causes renal hemodynamics alterations and intraglomerular capillary hypertension (Benigni et al., 2004). Considering the beneficial effects of ANP in the kidney and the deleterious effects of the RAS, the use of a combined inhibition that favors the protective actions of ANP and blocks the harmful effects of Ang II represents an interesting approach to evaluate. In this way, the dual inhibition of the angiotensin receptor AT1 and neprilysin (called ARNI) is a novel therapy that combines a neprilysin inhibitor (sacubitril) that enhances the action of ANP, and a selective antagonist of the AT1 receptor (valsartan) that counteracts the increase in Ang II induced by sacubitril while avoiding the incidence of cough and angioedema caused by ACE inhibitors (Uijl et al., 2020). ARNIs are currently indicated for patients with heart failure (HF) with reduced ejection fraction, where they have shown benefits in terms of morbidity and mortality (McMurray et al., 2014). Experimentally, in rats undergoing 5/6 nephrectomy, chronic use of the dual inhibitor sacubitril-valsartan for 8 weeks was associated with cardiorenal benefit by reducing cardiac hypertrophy, aortic fibrosis, and improvement of renal function (Suematsu et al., 2018). Another study demonstrated that treatment with ARNI was associated with increased ANP levels and significant protection against kidney damage in SHRSP rats compared to valsartan alone (Rubattu et al., 2018). Other studies that used an experimental model of angiotensin II-dependent hypertensive diabetic rats [TGR (mREN2) 27 rats] demonstrated that chronic treatment with sacubitril/valsartan was associated with increased in urinary ANP levels, reduced albuminuria, and less development of segmental glomerulosclerosis compared to valsartan monotherapy (Roksnoer, 2016; Uijl et al., 2020). This renoprotective effect of ANP would be independent of its antihypertensive efficacy and could be related to a reduction in renal inflammation. The benefits of inhibiting neprilysin are not limited only to glomerular protection but also extend to the tubulointerstitial fibrosis. It was demonstrated in renomedullary interstitial cells from neprilysin knockout mice exposed to a hyperglycemic environment that ANP reduced cell proliferation and extracellular matrix synthesis induced by Ang II, being this effect more pronounced in the presence of a selective antagonist of the AT1 receptor (Maric et al., 2006). Finally, a recent systematic review and meta-analysis that included 10 randomized controlled trials (n = 16,456 patients) evaluated the renal outcome of ARNI against RAS inhibitors (ACE inhibitors or AT1 receptor blockers) in patients with or without HF (Spannella et al., 2020). Compared with RAS inhibitors alone, ARNI treatment resulted in a lower risk of renal dysfunction, especially with a strong association in older patients or HF patients with preserved ejection fraction. However, no significant association was found in patients without HF (Spannella et al., 2020).

Although, in experimental models of kidney damage, beneficial and superior effects of ARNI were observed compared to RAS inhibitors, the real impact of these agents in CKD patients is still unknown. In this sense, the United Kingdom Heart and Renal Protection-III trial was designed to evaluate if ARNI therapy can slow the rate of kidney decline better than current standard treatment with RAS inhibitor irbesartan in patient with CKD without HF (Haynes et al., 2018). This double-blind, randomized trial included 414 patients with moderate to severe CKD (estimated GFR 20 to 60 ml/min/1.73 m^2) who were randomly assigned to sacubitril/valsartan 97/103 mg twice daily (n = 207) or irbesartan 300 mg once daily (n = 207). There was no difference in the primary outcome (GFR at 12 months) between both treatment arms urinary neither in the albumin:creatinine ratio. There was only a decrease in systolic and diastolic blood pressure and some cardiac biomarkers (troponin I and NT-proBNP) in the ARNI group against irbesartan, suggesting a possible role in the cardiovascular risk reduction in advanced CKD patients (Haynes et al., 2018). Therefore, conversely to what was reported experimentally, this clinical study indicates that ARNI therapy in patient with moderate to severe CKD has similar effects on kidney function and albuminuria than current standard treatment with RAS inhibitors. However, it is necessary to emphasize that this lack of response could be due to some limitations of this study, like short time of study, number of patients, and type and stage of CKD. Therefore, the evidence on the clinical benefits of ARNI in CKD regardless the presence of HF is still scarce, but enough to demonstrate its safety at the renal level.

CONCLUSION

The expression of all the components of this natriuretic system in the kidney is relevant to maintain renal function and to exert a nephroprotective effect, and its alteration can predispose to kidney damage with increased levels of pro-inflammatory cytokines and oxidative stress, albuminuria, renal tubular injury,

and interstitial fibrosis. Although most of the evidence supports a beneficial protecting renal effect of ANP or its analogs, some clinical trials reported no benefit in terms of kidney function improvement during cardiovascular surgery. Since ANP also antagonizes the renin-angiotensin-aldosterone system, its potential benefit could be extent to those situations in which exist an imbalance in favor of Ang II and in detriment of ANP such as CKD. Given the fact that CKD is a constantly growing entity and can progress over time to a greater renal and cardiovascular deterioration even under renoprotection with RAS inhibitors, the search for new treatments to improve renal protection is imperative. The beneficial effects of pharmacological use of dual angiotensin receptor AT1-neprilysin inhibitors are currently limited to HF patients, raising the urgent need to investigate the renal outcome of ARNI therapy outside the HF setting, such as type 2 diabetes mellitus and CKD, scenarios in which the evidence is still scarce and of low quality.

AUTHOR CONTRIBUTIONS

MC and BF wrote, designed, and revised the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by UBACYT 20020170100621BA, PICT 2018-01105, Fundación H.A. Barcelo 2018-2020.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 08 July 2021
doi: 10.3389/fphys.2021.691407

Atrial Natriuretic Peptide$_{31-67}$: A Novel Therapeutic Factor for Cardiovascular Diseases

*Gustavo Jose Justo da Silva[1], Raffaele Altara[1,2], George W. Booz[3] and Alessandro Cataliotti[1]**

[1] Institute for Experimental Medical Research, Oslo University Hospital and University of Oslo, Oslo, Norway, [2] Department of Pathology, School of Medicine, University of Mississippi Medical Center Jackson, Jackson, MS, United States, [3] Department of Pharmacology and Toxicology, School of Medicine, The University of Mississippi Medical Center, Jackson, MS, United States

The characterization of the cardiac hormone atrial natriuretic peptide (ANP$_{99-126}$), synthesized and secreted predominantly by atrial myocytes under stimulation by mechanical stretch, has established the heart as an endocrine organ with potent natriuretic, diuretic, and vasodilating actions. Three additional distinct polypeptides resulting from proteolytic cleavage of proANP have been identified in the circulation in humans. The mid-sequence proANP fragment 31–67 (also known as proANP$_{31-67}$) has unique potent and prolonged diuretic and natriuretic properties. In this review, we report the main effects of this circulating hormone in different tissues and organs, and its mechanisms of actions. We further highlight recent evidence on the cardiorenal protective actions of chronic supplementation of synthetic proANP$_{31-67}$ in preclinical models of cardiorenal disease. Finally, we evaluate the use of proANP$_{31-67}$ as a new therapeutic strategy to repair end-organ damage secondary to hypertension, diabetes mellitus, renal diseases, obesity, heart failure, and other morbidities that can lead to impaired cardiac function and structure.

Keywords: natriuretic peptides, atrial natriuretic peptide, proANP$_{31-67}$, cardiovascular disease, heart Failure

OPEN ACCESS

Edited by:
Speranza Rubattu, Istituto Neurologico Mediterraneo Neuromed (IRCCS), Italy

Reviewed by:
Valentina Cannone, University Hospital of Parma, Italy
Aldo Clerico, Sant'Anna School of Advanced Studies, Italy

***Correspondence:**
Alessandro Cataliotti
alessandro.cataliotti@medisin.uio.no

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *06 April 2021*
Accepted: *14 June 2021*
Published: *08 July 2021*

Citation:
da Silva GJJ, Altara R, Booz GW and Cataliotti A (2021) Atrial Natriuretic Peptide$_{31-67}$: A Novel Therapeutic Factor for Cardiovascular Diseases. Front. Physiol. 12:691407. doi: 10.3389/fphys.2021.691407

INTRODUCTION

Natriuretic Peptides

The human natriuretic peptides (NPs) consist of a family of three known peptides encoded in the human genome, with each being a distinct gene product with similar structure (**Figure 1**). The atrial natriuretic peptide (ANP$_{99-126}$), a hormone synthesized and secreted predominantly by cardiac cells, was the first member of the NP family to be discovered in de Bold et al. (1981), and established the heart as an endocrine organ. Wall stretch, due to increased intravascular volume and/or cardiac transmural pressure, is the major stimulus for cardiac ANP release (Goetze et al., 2020). ANP is encoded by the NPPA gene located on chromosome 1 in the human genome, and is primarily expressed by atrial myocytes. The NPPA gene translates a 151-amino acid polypeptide known as preproANP. A post-translational modification process cleaves the 25 amino acid signal sequence to produce proANP, a 126 amino acid peptide that is stored in intracellular granules of atrial myocytes (Yan et al., 2000). Under stimulation, atrial cells release proANP that is rapidly converted to the 28-amino-acid C-terminal mature ANP by the transmembrane serine protease corin (Yan et al., 2000), a transmembrane cardiac serine protease, to form the biologically active carboxyl-terminal

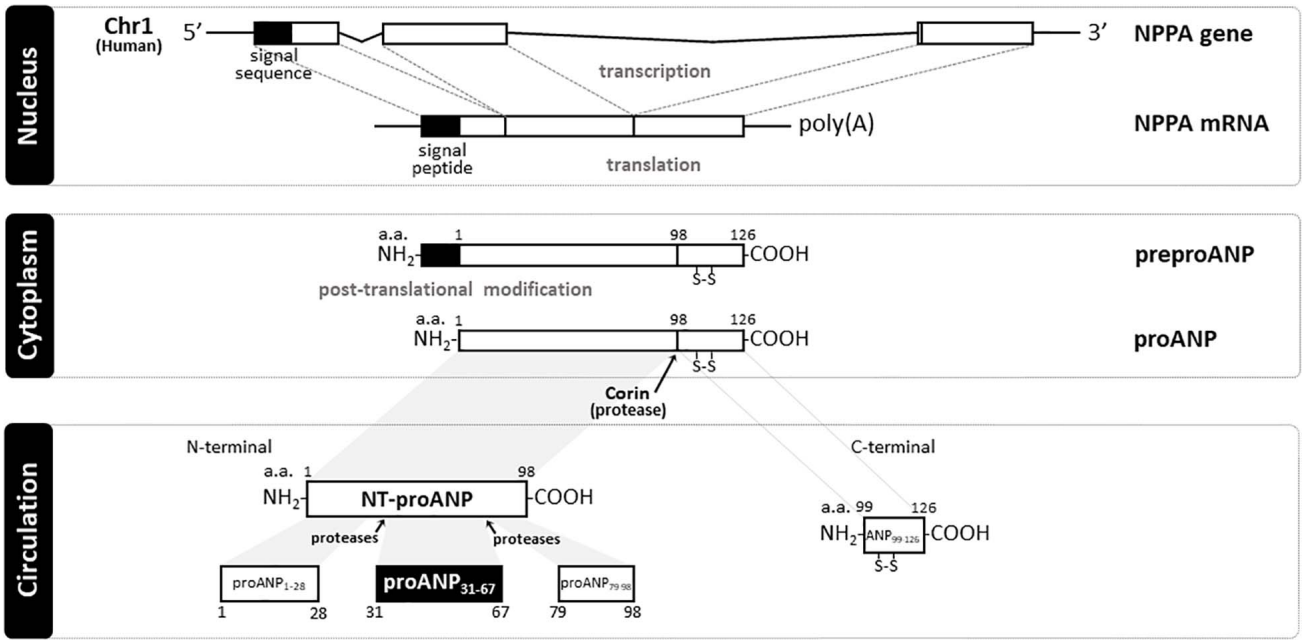

FIGURE 1 | Schematic representation of natriuretic peptide gene product processing and generated peptide fragments. In humans, atrial natriuretic peptide (ANP) is encoded by NPPA gene (Chr1:11,845,709-11,848,345:-) that translates a 151 a.a. polypeptide (preproANP). Post-translational modification process cleavages the 25 a.a. signal sequence to produce proANP, a 126 a.a. peptide that is stored in intracellular granules of atrial myocytes. Under stimulation, atrial cells release proANP that is rapidly converted to the 28-a.a. C-terminal mature ANP by the transmembrane serine protease Corin, and a 98 a.a. N-terminal mature ANP (NT-proANP or ANP$_{1-98}$). Further cleavage by proteases of NT-proANP generates different fragments, i.e., amino acids 1–28 (proANP$_{1-28}$), amino acids 31–67 (proANP$_{31-67}$), and amino acids 79–98 (proANP$_{78-98}$). NH2, free amine group located at the N-terminal end of a polypeptide; COOH, free carboxyl group located at the C-terminal end of a polypeptide; poly(A), multiple adenosine monophosphates mRNA tail; a.a., amino acid; Chr 1, Chromosome 1; NPPA, human atrial natriuretic peptide gene; ANP, Atrial natriuretic peptide; NT-proANP, N-terminal proatrial natriuretic peptide; preproANP, precursors to prohormone of atrial natriuretic peptide; proANP, prohormone of atrial natriuretic peptide.

28-amino-acid peptide called ANP$_{99-126}$ (Forssmann et al., 1998). The 28-amino acid peptide contains a 17-amino acid ring in the center of the molecule (**Figure 1**), formed by a disulfide bond between two cysteine residues at positions 7 and 23. The highly biologically active ANP$_{99-126}$ is formed at equimolar amounts as the biologically inactive amino-terminal portion (98 amino acid) of proANP (termed NT-proANP, or proANP$_{1-98}$) (Yan et al., 2000). However, as ANP$_{99-126}$ has a very short half-life (less than 5 min) compared with NT-proANP (60–120 min), NT-proANP is considered a more reliable biomarker than ANP$_{99-126}$ (Buckley et al., 1999). Originally, the ring structure was thought to be essential for the ANP$_{99-126}$ biological actions (Currie et al., 1984), but linear forms of the N-terminal ANP prohormone containing internal sequences believed to account for activity without the ring structure were shown also to have biological activity, albeit significantly reduced (Brenner et al., 1990; Vesely, 2007). The B-type natriuretic peptide (BNP), also known as brain natriuretic peptide, is a hormone secreted primarily by cardiomyocytes in the heart atria and ventricles (Sudoh et al., 1988) in response to stretching caused by increased ventricular blood volume and increased filling pressure (de Lemos et al., 2003). While the main source of BNP in normal conditions is the atrium, the production of BNP from the ventricles increases under pathological conditions such as cardiac remodeling (Luchner et al., 1998). Under stimulation, a 32-amino acid polypeptide is secreted attached to a 76-amino acid N-terminal fragment in the prohormone called NT-proBNP. A specific convertase (furin or corin) subsequently cleaves proBNP between arginine-102 and serine-103 into NT-proBNP and the biologically active 32-amino acid polypeptide BNP 1–32. Last, the C-type natriuretic peptide (CNP), encoded by the gene NPPC located on human chromosome 2, is synthesized and secreted from the central nervous system (e.g., cerebellum, hypothalamus, and anterior pituitary), kidney, and vascular endothelial cells (Mukoyama et al., 1991; Heublein et al., 1992; Stingo et al., 1992; Suga et al., 1992), and by the heart (Vollmar et al., 1993; Del Ry et al., 2011; Sangaralingham et al., 2020), in response to shear stress and certain proinflammatory cytokines. CNP is structurally related to ANP and BNP molecules, but has less intensive natriuretic and diuretic effects (Goetze et al., 2020). A recent study revealed that CNP regulates distal arteriolar and capillary blood flow via NPR-B-induced cGMP signaling in microvascular smooth muscle cells and pericytes (Spiranec et al., 2018), controlling microvascular resistance and blood pressure through vasodilating actions (Sangaralingham and Burnett, 2018). Other NPs have been identified in nature. The *Dendroaspis* natriuretic peptide (DNP), is structurally similar to ANP, BNP, and CNP, and possesses comparable biologic properties to other NPs. Additionally, the NP urodilatin (URO or CDD/ANP 95–126), known as renal ANP among the NPs,

is secreted by cells of the distal tubule and collecting duct in the kidney in response to increased blood pressure and blood volume. Urodilatin is transcribed by the NPPA gene, but differentially processed in the kidney, and only detected in urine (Meyer et al., 1998). Both DNP and Urodilatin bind to NPR-A resulting in a cGMP-dependent signal transduction (Pandey, 2014).

Biological actions of NPs are mediated by membrane-bound guanylyl cyclase receptors, which are expressed in a variety of cells. Three NP receptors are known: NPR-A (or NPR1), NPR-B (or NPR2), and NPR-C (or NPR3). ANP and BNP bind primarily to NPR-A and CNP binds to NPR-B (Charles et al., 1996; Kuhn, 2004). When activated, NPR-A and NPR-B receptors generate the second messenger cyclic guanosine monophosphate (cGMP), while the activation of NPR-C does not generate cGMP. In cardiac myocytes, cGMP-mediated signaling is regulated in a spatial and temporal manner by specific phosphodiesterases, which act to localize and temper levels of this signaling second messenger (Dunkerly-Eyring and Kass, 2020). All three NPs are ligands with similar affinity (Anand-Srivastava and Trachte, 1993) to the receptor NPR-C, which is not a guanylyl cyclase-linked receptor (Kuhn, 2004). It is known that NPR-C couples to inhibitory G proteins (G_i) and causes inhibition of adenylyl cyclase and activation of phospholipase-C (Anand-Srivastava, 2005).

Physiological Actions of the Linear Fragment ANP$_{31-67}$

A comprehensive biological understanding of NPs emerged following studies in cultured cells, rodent models of altered NPs production or receptor function, and integrative physiologic studies in disease models and in humans. The biological properties of the NPs, which include natriuresis, vasodilatation, inhibition of the renin-angiotensin-aldosterone system (RAAS), positive lusitropy, and inhibition of fibrosis, have led to the unique concept of cardiorenal protection by activation of cGMP (de Bold et al., 1981; Burnett et al., 1984; Wada et al., 1994; Kishimoto et al., 1996; Stevens et al., 1996; Wright et al., 1996; Lainchbury et al., 2000). An accumulating body of evidence demonstrated the tissue-specific distribution of NPs (Saito et al., 1989; Kojima et al., 1990; Ogawa et al., 1990; Mukoyama et al., 1991) and their receptors (Fuller et al., 1988; Martin et al., 1989; Schulz et al., 1989). Additionally, proANP$_{1-98}$ can break down into multiple peptides (**Figure 1**), i.e., amino acids 1–28 (proANP$_{1-28}$), amino acids 31–67 (proANP$_{31-67}$), and amino acids 79–98 (proANP$_{78-98}$), which also have potent vasodilatory properties (Vesely et al., 1987). Interestingly, these proANP forms have been identified in the circulation in humans (Vesely, 1995). For instance, by using high performance-gel permeation chromatography (HPGPC) and radioimmunoassay (RAI), Vesely (1995) was able to demonstrate that proANP$_{1-98}$ is further cleaved by proteases to generate these proANP fragments in the circulation. Among these forms, proANP$_{31-67}$ has unique potent and prolonged diuretic and natriuretic properties (Gunning et al., 1992) and will be the main form described in the current review.

Here, we review in detail, the actions of the linear fragment proANP$_{31-67}$, in particular on the heart, kidneys, and metabolism, which are independent of cGMP production. Originally, Gower et al. (1994) demonstrated the presence of different circulating molecular forms of the N-terminal and the C-terminal ANP prohormone peptides in plasma and their metabolites excreted in urine. These authors subjected plasma and urine samples from humans to high performance gel permeation chromatography (HP-GPC), followed by radioimmunoassay assessment of all ANP fragments, to reveal that proANP$_{31-67}$ and ANP circulate as distinct peptides (Gower et al., 1994). Interestingly, the proANP$_{31-67}$ levels in the circulation were found to be 10–20-fold higher than ANP$_{99-126}$ in normal humans (Winters et al., 1989; Hartter et al., 2000) and dogs (Habibullah et al., 1995). This is explained by differences in the clearance rates of both peptides, i.e., it takes 45 min for proANP$_{31-67}$ to be removed from the body compared to a half-life of 3–5 min for ANP (Greenwald et al., 1992). Additionally, proANP$_{31-67}$ appears to be resistant to degradation by endopeptidases, such as neutral endopeptidase (NEP), being excreted in the urine largely intact (less terminal 2–3 a.a.) (Gower et al., 1994; Hartter et al., 2000). This unique characteristic of this polypeptide, contributes to the prolonged renal actions, and the potential therapeutic effects of proANP$_{31-67}$.

It has been shown that the upstream and C-terminus fragments of the ANP prohormone are released by central hypervolemia induced by head-out water immersion (Vesely et al., 1989) or cardiac pacing or tachycardia (Ngo et al., 1989). These ANP fragments have physiologic actions similar to the ring structured ANP form, producing vasodilation (Vesely, 1995), natriuresis (Martin et al., 1990; Villarreal et al., 1999b), diuresis (Martin et al., 1990), and affecting metabolic phenotypes (Moro, 2013). In rodents, intravenous infusion of proANP$_{31-67}$ (at doses of 0, 10, 30, and 100 ng/kg/min) in anesthetized normotensive and spontaneously hypertensive rats elicited natriuresis and diuresis (Villarreal et al., 1999b). Moreover, an increase in sodium excretion was also observed in intravenously infused anesthetized Munich-Wistar (Martin et al., 1990) and Sprague-Dawley (Dietz et al., 1994) rats. Both ANP and proANP$_{3l-67}$ also inhibit sodium transport in suspensions of inner medullary collecting duct cells (Zeidel et al., 1986; Gunning et al., 1992). Because both peptides inhibit sodium transport in the collecting duct, it is possible that they act in an additive fashion on these cells in the intact animal. In conscious non-human primates (*Macaca fascicularis*), Benjamin and Peterson showed that infusion of proANP$_{31-67}$ (15 pmol. kg^{-l}. min^{-l} i.v.) increases renal sodium excretion, due to tubular and hemodynamic components (Benjamin and Peterson, 1995). Similarly, Vesely et al. (1994) demonstrated that intravenous infusion of proANP$_{31-67}$ (100 ng/kg body weight/min, for 60 min) produced blood pressure-lowering, and diuretic and natriuretic properties in healthy individuals. Interestingly, these authors additionally showed that proANP$_{31-67}$ has natriuretic properties that are significantly prolonged compared with ANP (Vesely et al., 1994).

NPs play also a key role in human metabolism (Cannone et al., 2019), thus connecting the heart with insulin−sensitive organs like adipose tissue, skeletal muscle, and liver. In fact,

accumulation of NPs is associated with protein energy wasting and activation of browning in white adipose tissue (Luce et al., 2020). Importantly, ANP increases mitochondrial uncoupling and thermogenic gene expression in human adipocytes, induces thermogenic programs in brown (BAT) and white (WAT) adipose tissue and so increases energy expenditure (Bordicchia et al., 2012). These actions are considered favorable effects. However, in pathological conditions, these favorable actions are blunted or abolished. Accumulating evidence suggests that impaired cardiac endocrine function contributes to the development of obesity, type 2 diabetes, and other cardiometabolic complications (Verboven et al., 2017). The ring-structured form of ANP was reported to induce lipid mobilization and oxidation and to enhance insulin sensitivity (Coue and Moro, 2016). ANP infusion in humans increases energy expenditure and leads to lipolysis, with an increase in plasma levels of glycerol and non-esterified fatty acids regardless of body mass index (Birkenfeld et al., 2005, 2008). Also, intravenous administration of ANP increases plasma levels of adiponectin (Tsukamoto et al., 2009), an adipocyte-derived cytokine, which protects against atherosclerosis and insulin resistance or diabetes. However, the potential clinical utility of ANP might be limited by its inherent, sustained blood pressure lowering effects that can cause hypotension. Regarding the mid-sequence of the ANP, it has been shown that proANP$_{31-67}$ might play an important role in the acute and chronic physiological responses to physical exercise. For instance, Cappellin et al. (2004) showed that measured proANP$_{31-67}$ levels before and at the end of dynamic exercise in 28 trained cyclists and found that a single bout of exercise induce an increase in the urinary proANP$_{31-67}$ levels. This could be, at least in part, explained by the increase in the venous return to the heart, and perhaps the higher heart rate levels, during a single exercise session. Interestingly, Freund et al. (1988) have demonstrated that the increase in the ANP levels occurs in a dose- and time-dependent manner. Additionally, proANP$_{31-67}$ plasma concentration was also found higher in endurance trained athletes than in sedentary subjects (De Palo et al., 2000). Although protection of the vasculature, heart, and kidneys are favorable effects in the setting of metabolic diseases, the role of proANP$_{31-67}$ in metabolism is unknown.

Novel Therapeutic Strategies to Target ANP$_{31-67}$

One of the hallmarks of heart failure (HF) is the marked increase in plasma levels of NPs (Burnett et al., 1986; Sugawara et al., 1988; Yamamoto et al., 1996; Cataliotti et al., 2001; Maisel et al., 2003; Richards and Troughton, 2004). It is established that elevated cardiac filling pressure is accompanied by increased circulating levels of ANP, and that congestive HF is not characterized by a deficiency in ANP, but with its elevation (Burnett et al., 1986). The increased circulating NP levels during HF are a compensatory response to volume overload and to hyperactivation of the adrenergic system and renin-angiotensin-aldosterone system (RAAS). However, not all HF patients seem to increase the circulating levels of NPs. In a recent clinical study, approximately 26% of acutely decompensated heart failure patients presented a lack of increase of circulating levels of ANP (Reginauld et al., 2019), which might suggest the existence of a relative state of ANP deficiency in a subgroup of patients, possibly due to reduced production, altered release, or enhanced enzymatic degradation by neprilysin. It should also be underscored, however, that the role of potential confounders responsible for this apparent ANP deficiency status remains yet to be fully elucidated (Richards and Januzzi, 2019). Nevertheless, as discussed herein, the increased cardiac production and circulation of NPs can be differently processed in the periphery in chronic HF patients, resulting in inactive forms with no efficient benefit, thus supporting the rational for using NPs or their analogs as anti-HF therapy (Belluardo et al., 2006; Macheret et al., 2012). Others and we have previously demonstrated the existence of a deficiency state of the endogenous biologically active NPs system in HF patients starting with the early stage of HF (Hawkridge et al., 2005; Belluardo et al., 2006; Niederkofler et al., 2008; Macheret et al., 2012). Additionally, a blunted natriuretic response has been observed after treatment with different pharmacological agents (e.g., angiotensin-converting enzyme inhibitors, angiotensin-II blockers, β-blockers, and spironolactone) in experimental models and in patients with chronic heart failure, suggesting a resistance to the biological effects of NPs (Cody et al., 1986; Saito et al., 1987; Komeichi et al., 1995; Charloux et al., 2003). This resistance to biological effects of ANP is probably mainly due to up-regulation of clearance receptors in patients with chronic heart failure (Andreassi et al., 2001; Clerico et al., 2006).

Winters et al. (1989) have evaluated the N-terminus and C-terminus ANP fragments in the circulation of thirty patients with varying severity degrees of congestive HF using high-pressure liquid chromatography. Compared to the other ANP peptides, proANP$_{31-67}$ was the only one that accurately discriminated the severity of congestive HF (Winters et al., 1989). In light of these findings, the impaired production and release of mature forms of the NPs and of their linear precursors seems to play a fundamental role in the evolution and progression of HF, and thus the exogenous supplementation of such cardiac hormones may prove to be of therapeutic importance in HF. In fact, the biologic properties of the NPs have supported the development of as therapeutic agents for cardiovascular diseases (Marcus et al., 1996; Yamamoto et al., 1997; Colucci et al., 2000; Hobbs et al., 2001; Boerrigter and Burnett, 2004; Rubattu et al., 2019; Rubattu and Volpe, 2019). Here we will further discuss the development of novel therapeutic strategies based on exogenous supplementation of a linear fragment of the ANP, the proANP$_{31-67}$, in HF.

The effort to develop novel therapeutic strategies to prevent the progression of cardiovascular disease is also focused on restoring the impaired NP system, for instance, by augmenting the circulating levels of NPs through exogenous supplementation. For instance, the NP drugs carperitide and nesiritide have been approved for use in patients in Japan and United States, respectively, as intravenous agents for the treatment of acute decompensated HF. These forms stimulate the production of cGMP, and frequently result in inadequate cardioprotective

effects due to significant reductions in blood pressure levels, leading to reduced renal perfusion and further deterioration of kidney function.

ProANP$_{31-67}$ was shown to enhance renal function acutely in persons with congestive heart failure (Vesely et al., 2001) and to protect against ischemia-induced acute tubular necrosis and renal failure in a rat model of ischemic non-oliguric acute renal failure (Clark et al., 2000). Overall, proANP$_{31-67}$ induced renal vasodilation and diuresis with enhanced sodium excretion, but with no associated increase in oxygen consumption. Of note, as mentioned above, the biological actions of proANP$_{31-67}$ were independent of cGMP activation and therefore, are characterized by a less intense vasodilatory effect (Vesely et al., 1987).

Potential clinical indications for proANP$_{31-67}$ include reducing symptoms in patients with worsening HF or those diagnosed with stable congestive HF and compromised renal function, or cardiorenal syndrome. Clinical trials showing safety and efficacy of synthetic proANP$_{31-67}$ peptide were conducted on stable congestive HF and renal impairment patients (ACTRN12612000576820 and ACTRN12611000806965), and on acute decompensated congestive HF patients (ACTRN12609000998246). Intravenous and subcutaneous delivery of proANP$_{31-67}$ was shown to preserve renal function in both chronic and acute heart failure with reduced ejection fraction (HFrEF) (Delacroix et al., 2016). Additionally, the infusion of proANP$_{31-67}$ (100 ng/kg/min, i.v. for 1 h) has been shown to possess several cardiac enhancing effects in congestive HF patients (NYHA III), including augmenting cardiac output, cardiac index, and stroke volume index, while reducing pulmonary capillary wedge pressure (Vesely et al., 1998). Of note, proANP$_{31-67}$ has similar effects to those observed with the ring forms of the NPs, which are currently in use for the treatment of acute decompensated overt HF, but has shown a less intense blood pressure lowering effect.

Based on these observations and on the known unique renal protective effects of proANP$_{31-67}$, we investigated the therapeutic value of proANP$_{31-67}$ for maladaptive cardiac and renal remodeling in a rat experimental model of salt-induced hypertension (Altara et al., 2020). This is a preclinical model for heart failure with preserved ejection fraction (HFpEF), as evidenced by concentric remodeling/hypertrophy and diastolic dysfunction, i.e., increased cardiac stiffness. We also sought to extend current knowledge on the protective actions of chronic exogenous supplementation of proANP$_{31-67}$ on the kidney, knowing that it stimulates natriuresis and diuresis, but has moderate effect on blood pressure. With hypertension in this preclinical model, we observed that proANP$_{31-67}$ increased urine output, natriuresis, and glomerular filtration rate (GFR), while preventing detrimental perivascular collagen deposition in the renal cortex. Remarkably, proANP$_{31-67}$ was shown to be beneficial to the heart. Characteristic signs of adverse cardiac remodeling and function that manifested as diastolic dysfunction were attenuated with chronic administration of proANP$_{31-67}$. These beneficial actions on the heart, included attenuated cardiac hypertrophy, as indicated by decreased heart weight to body weight ratio and left atrial diameter, as well as reduced fibrosis (both interstitial and perivascular left ventricular fibrosis) and normalized ratio of the diastolic mitral inflow E wave to A wave, a measure of cardiac stiffness (Altara et al., 2020). Of note, the beneficial effects on the heart were retained absent of a marked lowering of blood pressure and when animals were treated with a renal sub-therapeutic dose of proANP$_{31-67}$, suggesting a unique mode of action directly on the heart, beyond its renal actions, which warrants further investigation.

MECHANISMS OF ACTION OF ANP$_{31-67}$

The mechanisms of action of proANP$_{31-67}$ associated with the cardiorenal protective and diuretic effects were not attributed to any effects on blood pressure. Competitive binding experiments revealed that proANP$_{31-67}$ does not activate the canonical NP receptors (e.g., NPR-A and NPR-B), resulting in the activation of the cGMP pathway but rather has its own separate and distinct receptor (Vesely et al., 1990, 1992). However, the nature of the proANP$_{31-67}$ receptor is still unknown.

Renal Mechanisms

With regard to its mechanism of action in the kidney, several studies have shown that proANP$_{31-67}$ endogenously (i.e., paracrine like) induces prostaglandin E_2 (PGE_2) formation (Gunning et al., 1992; Vesely et al., 2000). PGE_2, a product of the cyclooxygenase 2 (COX-2) pathway (**Figure 2**), is an important homeostatic regulator of nephropathy, as well as hypertension, adipogenesis, dyslipidemia, diabetes, neuropathy, atherogenesis and retinopathy, contributing to global cardiovascular risk (Nasrallah et al., 2016). PGE_2 was also reported to modulate growth, fibrosis, and apoptosis phenotypes by influencing inflammatory, immune, and oxidative stress responses (Makino et al., 2002; Zahner et al., 2009; Nasrallah et al., 2015). Felder et al. (2017) reported that proANP$_{31-67}$ directly stimulated PGE_2 release in the renal medullary tissue, more precisely by cells located in the collecting tubules/ducts at the cortical-medullary interface. Through PGE_2, proANP$_{31-67}$ is also a potent inhibitor of the Na^+-K^+-ATPase (or sodium-potassium pump) of inner medullary collecting duct cells, resulting in Na^+ transport inhibition and natriuretic actions. Furthermore, proANP$_{31-67}$ was shown to increase GFR, both in preclinical models as well as in humans with congestive HF, and to attenuate tubular necrosis in a rat model of acute renal failure (Afsar et al., 2017). Intrarenal administration of proANP$_{31-67}$ also increases creatinine clearance and inhibits renin secretion in a Na^+ depleted canine model of renin system activation induced by unilateral nephrectomy (Villarreal et al., 1999a). These data suggest that inhibition of renin secretion is, at least in part, in response to a proANP$_{31-67}$-induced increase in the sodium load delivered to the macula densa.

In mammals, PGE_2 exerts its signals through four G protein-coupled receptors, designated EP1, EP2, EP3, and EP4 (**Figure 2**; Sugimoto and Narumiya, 2007). Although highly conserved among mammals, the PGE_2 receptors have distinct signal transduction pathways, and tissue and cellular distribution, reflecting their diverse properties (Nasrallah et al., 2016). In the kidney, EP1 and EP4 receptors

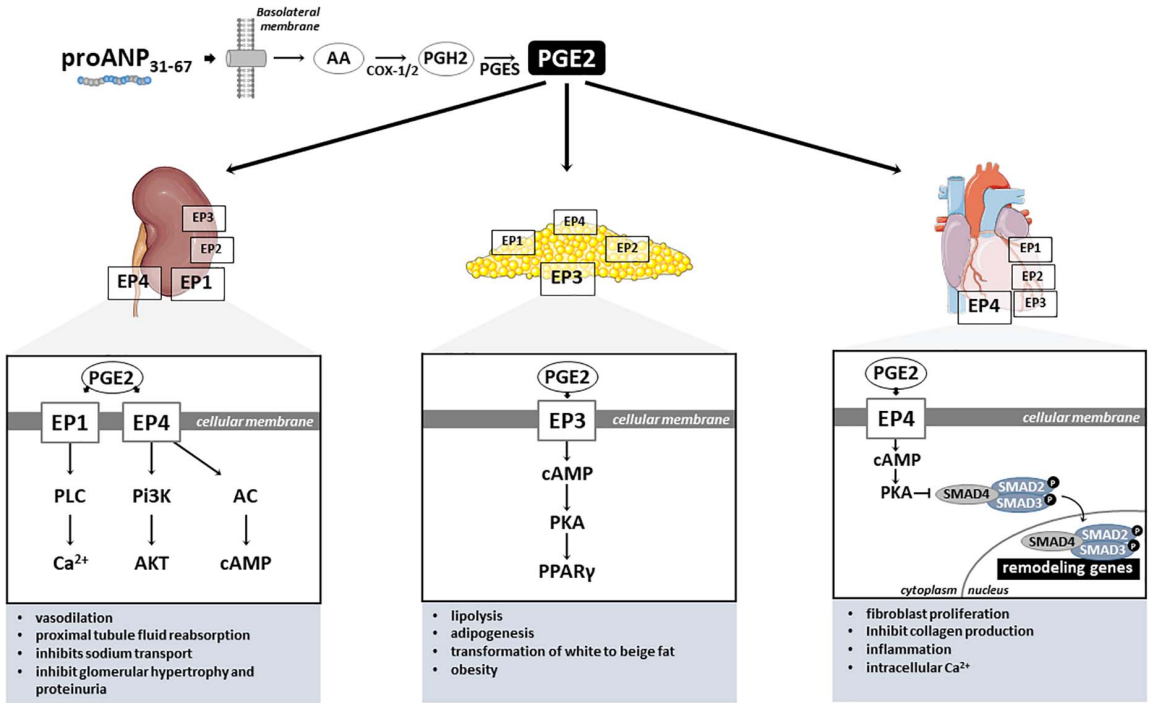

FIGURE 2 | PGE_2-mediated proANP$_{31-67}$ cardiac, renal and metabolic mechanisms of action. PGE_2, Prostaglandin E2; EP1–4, PGE_2 receptors (coupled to G_q); AA, Arachidonic acid; COX-1/2, Cyclooxygenase-1 and –2; PGH2, Prostaglandin H2; PGES, Prostaglandin E synthase; PLC, Phospholipase C; PI3K, Phosphoinositide 3-kinases; AC, Adenylate cyclase; Ca^{2+}, Calcium ion; AKT, protein kinase B; cAMP, Adenosine 3′,5′-cyclic monophosphate; PKA, protein kinase A; SMAD2–4, SMAD (Mothers against decapentaplegic) family member 2, 3, and 4; PPARγ, Peroxisome proliferator-activated receptor gamma.

seem to mediate PGE_2 microcirculation actions (**Figure 2**). Purdy and Arendshorst (2000) identified by RT-PCR the expression of EP1 (*Ptger1*) and EP4 (*Ptger4*) receptors in freshly isolated preglomerular arterioles of Sprague-Dawley rats. These authors also demonstrate that the EP4 receptor is the major receptor located in preglomerular vascular smooth muscle cells, mediating PGE_2-induced vasodilation through cAMP formation and reduction of cytosolic Ca^{2+} levels (Purdy and Arendshorst, 2000). Curiously, the renal vascular tone response induced by PGE_2 stimulation seems to vary depending on the type of the receptor (Schweda et al., 2004). For instance, $EP2^{-/-}$ and $EP4^{-/-}$ mice presented an augmented vasoconstriction in response to higher PGE_2 concentrations, contrasting with the markedly blunted response observed in EP1 and EP3 knockout mice (Schweda et al., 2004). Furthermore, EP1 and EP4 were detected in transformed murine proximal tubular cells (MCTs), mediating PGE_2-induce fluid reabsorption (Nasrallah et al., 2015). Loss-of-function *in vivo* experiments in mice have shown that PGE_2 stimulates the renin-angiotensin-aldosterone system by activation of EP4 receptor (Poschke et al., 2012). Similarly, Schweda et al. (2004) demonstrated that PGE_2 stimulates renin release in juxtaglomerular cells via activation of both EP2 and EP4 receptors. Interestingly, the EP1 receptor attenuates vasopressin-dependent water reabsorption and inhibits sodium transport in the collecting duct (Nasrallah et al., 2018). Activation of PGE_2-EP4 signaling with proANP$_{31-67}$ also can exert multiple biochemical effects on the kidney and other organs, suggesting the potential wide-ranging use of EP4 in both cardiovascular and metabolic disorders. For instance, by inhibiting Na^+ transport in the inner medullary collecting duct cells, proANP$_{31-67}$ is known to reduce renal oxygen consumption (Gunning et al., 1992).

Under different pathological conditions, the PGE_2 receptors seem to be involved in the development of renal disease. For instance, the oral administration of PGE_2 receptor EP1-selective antagonist prevented the progression of nephropathy, evidenced by improved glomerular hypertrophy, decreased mesangial expansion, and suppression of proteinuria in streptozotocin-induced diabetic rats (Makino et al., 2002). Mechanistically, the authors demonstrated that mesangial cells cultured under high-glucose conditions and treated with this selective agonist for EP1 receptor exhibit inhibited transforming growth factor-beta (TGF-β) and fibronectin upregulation, key regulators of the extracellular matrix. Similarly, an EP4-specific agonist significantly attenuated the development of tubulointerstitial fibrosis induced by unilateral ureteral obstruction in mice by suppressing inflammatory responses (Nakagawa et al., 2012). On the other hand, knockout mice for EP4 showed exacerbated tubulointerstitial fibrosis response after ureteral obstruction. Additionally, cultured renal fibroblasts treated with EP4 agonist significantly inhibited the platelet-derived growth factor (PDGF)-induced proliferation and profibrotic connective tissue growth factor production (Nakagawa et al., 2012). Hence, these data indicate that both PGE_2 receptors EP1 and EP4 play critical roles in the development of renal injury (**Figure 2**), and

might explain the renal protective benefits of proANP$_{31-67}$ observed by our group in hypertensive rats. For instance, chronic administration of proANP$_{31-67}$ prevented perivascular collagen deposition in the rat experimental model of salt-induced hypertension, accompanied by improvements in renal function (Altara et al., 2020).

Myocardial Mechanisms

Cardiac phenotypes are equally affected by the different PGE_2 receptors (**Figure 2**). Although all four PGE_2 receptors are detected in the cardiac tissue, EP4 is highly expressed in the heart (Muraoka et al., 2019), and seems to have protective effects against adverse remodeling. In fact, EP4 agonist administration to mice subjected to pressure overload (Wang et al., 2017) and cardiac injury (Hishikari et al., 2009; Pang et al., 2016) exhibited antifibrotic effects and prevented the progression to systolic dysfunction. In a mouse model of cardiac hypertrophy generated by transverse aortic constriction (TAC) surgical procedure, EP4 agonist ONO-0260164 treatment significantly prevented myocardial fibrosis and progression of systolic dysfunction 5 weeks after pressure overload (Wang et al., 2017). Hishikari et al. (2009) used another EP4 selective agonist (EP4RAG) to treat rats submitted to myocardial ischemia-reperfusion injury and demonstrated that EP4RAG significantly reduced ischemic myocardium, attenuated interstitial fibrosis, and ameliorated cardiac contractility and dilatation compared with vehicle.

The generation of genetically engineered animals has contributed with the understanding of the role of PGE_2 receptors in the cardiac tissue. Qian et al. (2008) generated cardiac specific EP4 deficiency, using site-specific recombination by the Cre recombinase method (Cre-loxP) to inactivate EP4 only in cardiomyocytes (CM- EP4 knockout [KO]), and showed that CM-EP4 KO mice are defective in their ability to activate Stat-3, presenting a worsening of systolic function after myocardial infarction injury. These studies are interpreted as indicating that EP4 plays both protective and damaging roles in the heart with the protective effects of EP4 due at least in part to its ability to suppress inflammation.

We cannot exclude, however, the role of PGE_2 stimulation of its receptors in cells other than cardiomyocytes, for instance cardiac fibroblasts, endothelial cells, and smooth muscle cells. In fact, it has been demonstrated recently that EP4 signal also regulates fibrotic phenotypes in cardiac fibroblasts. In this regards, Umemura et al. (2019) showed that cardiac fibroblasts isolated from adult rats treated with EP4 agonist (ONO-AE1-437) decreased the expression of transforming growth factor-β (TGF-β), connective tissue growth factor (CTGF) and ACTA2 (a-smooth muscle actin) mRNA, suggesting that that EP4 signaling suppresses fibroblasts to myofibroblast transdifferentiation. Consistently, Wang et al. (2017) demonstrated in cultured neonatal rat cardiac fibroblasts that treatment with EP4 agonist ONO-0260164 inhibited the TGF-β1 induced upregulation of collagen type 1 (*Col1a1*) and type 3 (*Col3a1*) gene expression.

Mechanistically, EP4 is a G protein-coupled receptor with seven transmembrane domains that when bound to PGE_2 or another agonists, mobilizes G proteins containing the Gs alpha subunit (i.e., Gαs) and G beta-gamma (i.e., Gβγ) (Tuteja, 2009). In particular, Gsα stimulates adenylyl cyclase to raise the production of cyclic adenosine monophosphate (cAMP) (Yokoyama et al., 2013), that subsequently activates protein kinase A (PKA), which in turn phosphorylates downstream proteins, such as the transcription factor cAMP response element binding protein (CREB). Of note, CREB regulates the expression of genes that control cellular proliferation, cellular differentiation, cellular survival, and angiogenesis. The activated CREB(p) binds to specific sites and regulates the expression of genes, such as B-cell lymphoma 2 and tumor necrosis factor α (TNFα), which are involved in development of ischemic heart disease (Ichiki, 2006). EP4 activation of G proteins also triggers PI3K/AKT/mTOR, ERK, and p38 MAPK pathways (Xu et al., 2018). Regarding the other PGE_2 receptors expressed in the cardiac tissue, it is known that PGE_2 stimulates cardiac fibroblast proliferation via both EP1 and EP3, p42/44 MAPK and Akt-regulation of cyclin D3, possibly modulating cardiac fibrosis (Harding and LaPointe, 2011).

As mentioned, in addition to its renoprotective effects, proANP$_{31-67}$ inhibited cardiac hypertrophy and early onset of diastolic dysfunction in our salt-induced hypertension model of HFpEF, as indicated by reduced cardiac fibrosis (Altara et al., 2020). The cardioprotective actions of proANP$_{31-67}$ may have resulted from a local increase in PGE_2 and activation of the EP4, which recently has been demonstrated to have antifibrotic and antihypertrophic actions in the heart (Yamagami et al., 2015; Harada et al., 2017; Wang et al., 2017; Bryson et al., 2018; Lai et al., 2018; Zhu et al., 2019; Jin et al., 2020). ProANP$_{31-67}$ seems to activate the PGE_2-EP4-SMAD signaling pathway, reducing the phosphorylation of SMAD2 (Altara et al., 2020), possibly inhibiting the activation in TGF-β1 mediated collagen deposition. Evidence indicates that EP4 attenuates cardiac fibrosis by inhibiting SMAD signaling through activation of protein kinase A (PKA) (Harada et al., 2017; Wang et al., 2017). In our study, urine levels of PGE_2 were elevated by proANP$_{31-67}$, although we did not observe a significant increase in plasma PGE_2. However, local PGE_2 production in the heart, where levels tended to be increased by treatment with proANP$_{31-67}$, may have been responsible for the inhibition of cardiac remodeling process observed in our study (Altara et al., 2020), Therefore, the cardioprotective actions of proANP$_{31-67}$ observed in our study (e.g., improved diastolic function, attenuated cardiac fibrosis and hypertrophy, and anti-remodeling effect on cardiomyocytes) (Altara et al., 2020) may have resulted from the activation of the EP4.

However, this response might be dependent on the cardiac cell type involved. In fact, proANP$_{31-67}$ may have direct effects on the ultrastructure of cardiomyocytes. We have demonstrated that chronic administration of proANP$_{31-67}$ reduced t-tubule density in our rat model of hypertensive heart disease and renal damage (Altara et al., 2020). Normal ultrastructure of cardiac t-tubules is important in electrical-mechanical coupling and Ca^{2+} handling in cardiomyocytes as any abnormalities may predispose toward heart failure (Manfra et al., 2017). In HF patients, we have demonstrated etiology-dependent differences in mechanisms for diastolic dysfunction (Frisk et al., 2021). For instance, myocardial biopsies from HFrEF hearts under high

ventricular wall stress were linked to disruption of t-tubules, local collagen deposition and of systolic calcium homeostasis impairment. In contrast, maintained wall stress in HFpEF patients was associated with compensatory t-tubule proliferation and largely maintained calcium release (Frisk et al., 2021). In keeping with this, we observed that proANP$_{31-67}$ treatment protects t-tubular structure and density, and also preserves intracellular distances between t-tubules and the sarcolemmal membrane. In our study, we did not examine the effect of proANP$_{31-67}$ on Ca^{2+} dynamics in cardiomyocytes, which remains an area for future investigation.

Taken together, our study previously discussed supports the hypothesis that proANP$_{31-67}$ cardioprotective benefits might directly affect both cardiac fibroblasts and cardiomyocytes, via the activation of PGE_2/EP4 signaling. We cannot exclude the possibility that proANP$_{31-67}$ mediates the secretion of growth factors by myofibroblasts indirectly induces hypertrophy of cardiomyocytes via a paracrine like-manner, which is a landmark of heart failure. Therefore, the detailed investigation of the proANP$_{31-67}$ molecular mechanisms involving anti-fibrotic signaling pathways and cellular processes, including inflammation, signaling kinases, apoptosis, fibroblast-to-myofibroblast differentiation, cardiomyocytes ultrastructure is absolutely crucial to understand the cardiorenal protective actions of this compound in a heart failure scenario.

Mechanisms Associated With Metabolic Phenotypes

With regard to the role of PGE_2 mediating metabolic phenotypes, these effects seem to be mainly mediated by EP3 and EP4 receptors in the adipose tissue. Of those, the EP3 is the most widely abundant receptor in adipose tissue (Tang et al., 2015; Xu et al., 2016), and is involved in various pathophysiological processes (Cai et al., 2015). Accordingly, PGE_2 receptor EP3 seems to regulate both lipolysis and adipogenesis in white adipose tissue (Strong et al., 1992; Fain et al., 2000; Xu et al., 2016), as well as adipocyte transformation of white to beige fat, protecting against obesity and metabolic disease (Garcia-Alonso et al., 2013). In fact, it has been shown that loss-of-function of EP3 in mice resulted in obese and insulin resistant phenotypes (Sanchez-Alavez et al., 2007; Ceddia et al., 2016). Mechanistically, using both pharmacological blockade and genetic disruption, Xu et al. (2016) elegantly showed that PGE_2 EP3 receptor inhibits adipogenesis via the cAMP/PKA/PPARγ pathway, and blocks lipolysis mainly through the cAMP/PKA/HSL pathway in white adipose tissue. These data demonstrates that PGE_2/EP3 axis is critical for lipid and glucose metabolism.

Activation of PGE_2-EP4 signaling seems also to exert important role in adipose tissue and metabolic disorders. Loss-of-function mice model for EP4 submitted to high fat diet exhibited reduced body weight gain and adiposity, and shorter life span when compared with wild type (Cai et al., 2015). Additionally, EP4 deficiency induced disruption in lipid metabolism due to impaired triglyceride clearance (Cai et al., 2015). Nevertheless, it is still unknown any direct or indirect metabolic properties of proANP$_{31-67}$ and future investigation is fundamental. However, there are some evidence of the possible connection of proANP$_{31-67}$ and metabolic phenotypes. For instance, in inner medullary collecting duct (IMCD) cells it has been previous shown that proANP$_{31-67}$ reduces O_2 not by direct inhibition of mitochondrial O_2 consumption, but by reducing the demand for metabolic energy of the Na^+-K^+-ATPase (Gunning et al., 1992). Accumulation of NPs is, in fact, associated with protein energy wasting and activation of browning in white adipose tissue (Luce et al., 2020). The incubation of primary adipose cells exposed to ANP led to a significant increase of uncoupling protein 1 content. Therefore, it is reasonable to believe that proANP$_{31-67}$ might also has potential in metabolic disorders associated or not with cardiovascular diseases.

POTENTIAL USEFUL COMBINATION OF proANP$_{31-67}$ WITH CURRENT MEDICATIONS

Notwithstanding the substantial advance achieved in treatment, the incidence of heart failure has not been reduced and remains the major cause of morbidity and mortality in developing and developed countries (Roger, 2013). Nevertheless, current medical procedures aim to increase survival of cardiac tissue and limit cardiac damage, whereas an effective treatment to improve and/or protect renal function, which often deteriorates after cardiac injury, is still lacking and urgently needed. More recently, the combination of NEP inhibitor (Sacubitril) and angiotensin II receptor blocker (Valsartan) (sold as Entresto) became a first-choice treatment for HFrEF patients (Volpe et al., 2015; Seferovic et al., 2019; Volpe et al., 2019), based on its superior benefits to reduce cardiovascular death, and HF symptoms and hospitalizations compared to angiotensin-converting enzyme inhibitor (ACEi) (McMurray et al., 2014). Inhibition of NEP, by decreasing NPs degradation (Zile et al., 2016), elicits hypotensive actions. Of note, Entresto presented higher proportions of hypotension and non-serious angioedema cases, but lower proportions of patients with renal impairment, hyperkalemia, and cough than ACEi (Enalapril). In this regards, proANP$_{31-67}$ may provide an ideal complementary therapeutic strategy by directly targeting end-organ remodeling in the setting of HFpEF. Given the fact that proANP$_{31-67}$ does not appreciably lower blood pressure, this peptide may be especially efficacious as an add-on-therapy to target end organ damage, and could be tested in other heart disease settings, including coronary heart disease, as well as aortic valve stenosis, cardiac dysfunction in the presence of metabolic disease, along with both forms of HF (e.g., HFpEF or HFrEF), and cardiorenal syndrome, for which it has been first developed. An additional beneficial action of proANP$_{31-67}$ is increased urinary excretion of potassium, suggesting that this peptide might be combined with potassium sparing drugs or medications frequently used in the treatment of HF (Altara et al., 2020). An interesting area of future investigation would be the impact of proANP$_{31-67}$ on the metabolic syndrome, a collection of conditions that contribute to the development of heart disease, diabetes and stroke. We anticipate a synergistic

beneficial action of proANP$_{31-67}$ in preventing end organ damage and attenuating metabolic dysfunction when used in combination with current therapies to ameliorate lipid and glucose metabolism. Of note, proANP$_{31-67}$ pharmacokinetics and high stability also render it an interesting candidate as therapeutic agent for chronic administration. Equally important, no adverse effects, gross or microscopic pathology changes were observed when proANP$_{31-67}$ was tested in various pre-clinical models (Benjamin and Peterson, 1995; Villarreal et al., 1999b; Clark et al., 2000; Vesely, 2007; Altara et al., 2020), ranging from mice to non-human primates (monkeys or Macaca fascicularis). With respect to the administration to patients, proANP$_{31-67}$ proved to be well tolerated at all doses via both intravenous (IV) and subcutaneous (SQ) infusions, without the profound vasodilatory and hypotensive complications evident with CT ring agents (blood pressure was maintained with no adverse hemodynamic effects noted) (Vesely et al., 1994, 1998, 2000). Additionally, pharmacokinetic analysis with pharmacodynamic parameters showed no adverse effects on any parameters measured, including cardiac and renal performance.

CONCLUSION

Considering that unique mechanism of action, its intrinsic resistance to enzymatic degradation, and its complementary actions to other members of the NPs system, make it compelling to evaluate the effects of proANP$_{31-67}$, as single therapy as well as in combination with current medications, in the treatment of cardiac diseases and metabolic syndrome. The long half-life, and its safe pharmacological profile, make proANP$_{31-67}$ a promising therapeutic option for currently difficult to treat clinical conditions. Therefore, further studies aimed to demonstrate its protective effects and exploit its clinical potential are clearly warranted.

AUTHOR CONTRIBUTIONS

AC was the lead author and conceived the concept of the manuscript. All authors contributed to the literature review and writing of the manuscript and approved the final version.

FUNDING

GS was supported by the South-Eastern Norway Regional Health Authority (HSØ-RHF, Project No. 25674). RA was supported by the Olav Raagholt og Gerd Meidel Raagholts, and internal grant funding. AC was funded by the Nasjonalforeningen for folkehelsen (Project No. 13173) and the HSØ-RHF (Project Nos. 2016089 and 2019009).

ACKNOWLEDGMENTS

We would like to thank Dr. Lawrence Mahan for his valuable comments in the preparation of this article.

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Conflict of Interest: AC serves as consultant for the advisory board of Madeleine Pharmaceuticals. RA and AC have applied for a patent related to the cardiac protective properties of proANP$_{31-67}$ (UK # 2000945.2).

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 16 July 2021
doi: 10.3389/fphys.2021.643721

A Contemporary View of Natriuretic Peptides in the SARS-CoV-2 Era

Speranza Rubattu[1,2], Giovanna Gallo[1] and Massimo Volpe[1]*

[1] Cardiology Unit, Department of Clinical and Molecular Medicine, Sapienza University of Rome, Sant'Andrea Hospital, Rome, Italy, [2] IRCCS Neuromed, Pozzilli, Italy

The heart releases natriuretic peptides (NPs) which represent an important hormonal axis with cardiorenal protective effects. In view of their properties, NPs have pathophysiologic, diagnostic and prognostic implications in several cardiovascular diseases (CVDs). Severe pulmonary inflammation, as induced by the SARS-COV2, may increase pulmonary pressure with potential influence on NPs release, whereby normal cardiovascular integrity becomes impaired. Moreover, pre-existing CVDs are strong negative prognostic factors since they exacerbate the effects of the viral infection and lead to worse outcomes. In this context, it may be expected that NPs exert a key protective role toward the virus infection whereas an impairment of NPs release contributes to the virus deleterious effects. In this review article we explore the potential involvement of NPs in the COVID-19 disease. To this aim, we will first focus on the interactions between NPs and the Ang II/ATIR arm of the renin-angiotensin-aldosterone system (RAAS) as well as with the protective ACE2/Ang (1-7) arm of the RAAS. Subsequently, we will review evidence that strongly supports the role of increased NT-proBNP level as a marker of cardiac damage and of worse prognosis in the COVID-19 affected patients. Finally, we will discuss the potential therapeutic benefits of these protective hormones toward the viral infection through their endothelial protective function, anti-inflammatory and anti-thrombotic effects. In conclusion, the potential implications of NPs in the SARS-CoV-2 infection, as discussed in our article, represent an important issue that deserves to be fully investigated.

Keywords: natriuretic peptides, RAAS, ACE2, Ang (1-7), COVID-19, ARNi

OPEN ACCESS

Edited by:
Gianluigi Pironti, Karolinska Institutet (KI), Sweden

Reviewed by:
Sang-Bing Ong, The Chinese University of Hong Kong, China
Aldo Clerico, Sant'Anna School of Advanced Studies, Italy

****Correspondence:***
Speranza Rubattu
speranzadonatella.rubattu@uniroma1.it

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *18 December 2020*
Accepted: *16 June 2021*
Published: *16 July 2021*

Citation:
Rubattu S, Gallo G and Volpe M (2021) A Contemporary View of Natriuretic Peptides in the SARS-CoV-2 Era. Front. Physiol. 12:643721. doi: 10.3389/fphys.2021.643721

INTRODUCTION

The heart releases natriuretic peptides (NPs), including atrial and brain natriuretic peptides (ANP and BNP) (Woodard and Rosado, 2008; Volpe et al., 2014; Forte et al., 2019; Rubattu and Volpe, 2019), which play important functional effects within the heart, blood vessels and kidneys in order to maintain sodium electrolytes and blood pressure homeostasis (Potter et al., 2009). Within the heart, the myocytes stress is the main stimulus to release ANP from the atria and BNP from the ventricles. The endocrine role of the heart is associated to a local synthesis of NPs in several tissues, including the myocardium and the endothelium, and in few organs, including the brain and the kidneys, with the final goal to achieve a fine control of the mechanisms implicated in the cardiovascular functions (Atlas et al., 1986; Baron et al., 1989; Barbato et al., 2011; Arcari et al., 2020).

Of note, the COVID-19 disease selectively impacts the lungs with potential influence on the right ventricle due to potential increase in pulmonary pressure. As a consequence of the increased atrial distension, secondary to the increased pulmonary pressure, the release of ANP might also be affected.

Noteworthy, NPs play tight interactions with other hormonal systems within the cardiovascular apparatus. The most important is the renin-angiotensin-aldosterone system (RAAS), which has been implicated in the SARS-CoV-2 infection. In fact, ACE2 plays a role as the virus receptor (Gheblawi et al., 2020). We know that NPs inhibit renin and aldosterone synthesis in the kidneys and adrenal glands (Atlas et al., 1986; Cody et al., 1986). Furthermore, NPs counteract the salt-retaining, vasoconstrictive, pro-inflammatory, pro-hypertrophic and pro-fibrotic functions of the angiotensin converting enzyme 1 (ACE1)-Ang II-Ang II/type 1 receptor (AT1R) arm of the RAAS (Nishikimi et al., 2006). On the other hand, it is not surprising to observe that the protective arm of the RAAS, represented by ACE2, Angiotensin (1-7) (Ang 1-7) and Mas receptor, has a tight functional connection with NPs (Shah et al., 2010).

Based on the above-mentioned functional properties within the cardiovascular system and on their tight interactions with the RAAS, NPs may be involved in the SARS-CoV-2 infection.

The potential link between NPs and Covid19 will be the topic of the present review article. In particular, we will discuss all available evidence supporting the implications of NPs in the SARS-CoV-2 disease, involving their potential pathogenetic, prognostic and therapeutic roles.

INTERACTION BETWEEN NPS AND ACE1-Ang II-AT1R

ANP is the NPs family component that has been mostly investigated in this regard since its discovery. It has been observed that ANP acts in different ways to counteract the actions of the classical RAAS, driven by ACE1-Ang II-AT1R, in order to maintain blood pressure and electrolytes homeostasis (Atlas et al., 1986; Cody et al., 1986). In fact, through the interaction with NPRA-cGMP, it exerts vasodilation, that is most pronounced in Ang II-preconstricted blood vessels. It inhibits both renin secretion by the iuxtaglomerular cells within the kidney and ang II-induced aldosterone secretion by the adrenal cortex. Furthermore, ANP opposes the sodium-retaining action of aldosterone through its natriuretic effects (Atlas et al., 1986; Cody et al., 1986) (**Figure 1**). Thus, the two hormonal axes play a complementary hemodynamic role in the regulation of sodium-volume and of blood pressure. NPs and RAAS also exert opposing actions at the local tissue/cellular level to control the cardiovascular remodeling process. Herein, NPs promote beneficial effects with anti-proliferative, anti-inflammatory, anti-fibrotic and anti-hypertrophic actions (Volpe et al., 2014). The opposite holds true for the Ang II-AT1R arm which acts through the signaling pathway of the calcium/inositol triphosphate (Li et al., 2006). Therefore, the cardiovascular remodeling results from the effects exerted by the two opposing systems (**Figure 1**).

The complementary regulation of cardiovascular functions by NPs and RAAS becomes more evident in pathological conditions at both cardiac and vascular level. It is likely that it may play a relevant role in conditions where an excess of Ang II stimulates vasoconstriction, inflammation, oxidative stress, blood clotting such as the infection by SARS-CoV-2.

INTERACTION BETWEEN ACE2-Ang (1-7) AND NPs

The protective arm of the RAAS is driven by ACE2-Ang (1-7)-Mas receptor and it plays complementary functions to the Ang II-AT1R arm (Santos et al., 2003). Whereas AT1R signals through the calcium inositol triphosphate pathway to produce its effects, the Ang (1-7) acts through the Mas receptor to mediate vasodilation, protect endothelial function, and to oppose hypertrophy, fibrosis and thrombosis (**Figure 1**) (Santos et al., 2003; Wang et al., 2016). Activation of the ACE2-Ang (1-7)-Mas receptor reduces inflammation and fibrogenesis in several diseases (Zhuo et al., 2013). Interestingly, from the beginning of the SARS-CoV-2 pandemic and of its related COVID-19 outbreak, ACE2 has attracted increasing attention since it has been shown as the cell receptor through which the virus enters into the cells (Korber et al., 2020). However, it has been largely demonstrated that ACE2 counterbalances the detrimental effects of Ang II, reducing inflammation, fibrosis, thrombosis, vasoconstriction and increased vascular permeability and, as a consequence, the progression of lung and systemic damage during COVID-19 disease (Battistoni and Volpe, 2020). In addition, it has been reported that the pharmacological blockade of the RAAS, that influences the ACE2 level, have no impact on the clinical course and outcome of the SARS-CoV-2 infection (Battistoni and Volpe, 2020; Danser et al., 2020; Vaduganathan et al., 2020; Volpe et al., 2020).

Of note, a bidirectional interaction between NPs and ACE2 has been described in several previous studies. *In vitro*, ANP reduced Ang II induced hypertrophy of cardiac myocytes through the inhibition of the mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) pathway. Moreover, ANP, through the stimulation of NPRA and the production of cGMP, which upregulates the MAP phosphatase MKP1, prevented the reduction of ACE2 mRNA mediated by Ang II or endothelin-1 and preserved cellular growth (Gallagher et al., 2008).

On the other hand, Ang (1-7), the product of ACE2, has been demonstrated to stimulate ANP secretion through the Mas receptor/phosphatidylinositol 3-kinase/protein kinase B (Mas/PI3K/Akt) pathway. These data were confirmed by performing *ex vivo* high atrial pacing (Shah et al., 2010) and by using a rat model of cardiac hypertrophy, with evidence of Ang (1-7)-mediated increase of ANP release. In the presence of inhibitors of Mas, PI3K, Akt, NOS, sodium-hydrogen antiporter 1 (NHE-1) and Ca 2 + /calmodulin-dependent protein kinase II (CAMKII), Ang (1-7) was unable to induce ANP secretion, thus confirming the involvement of these molecular pathways in its ability to regulate ANP (Shah et al., 2010).

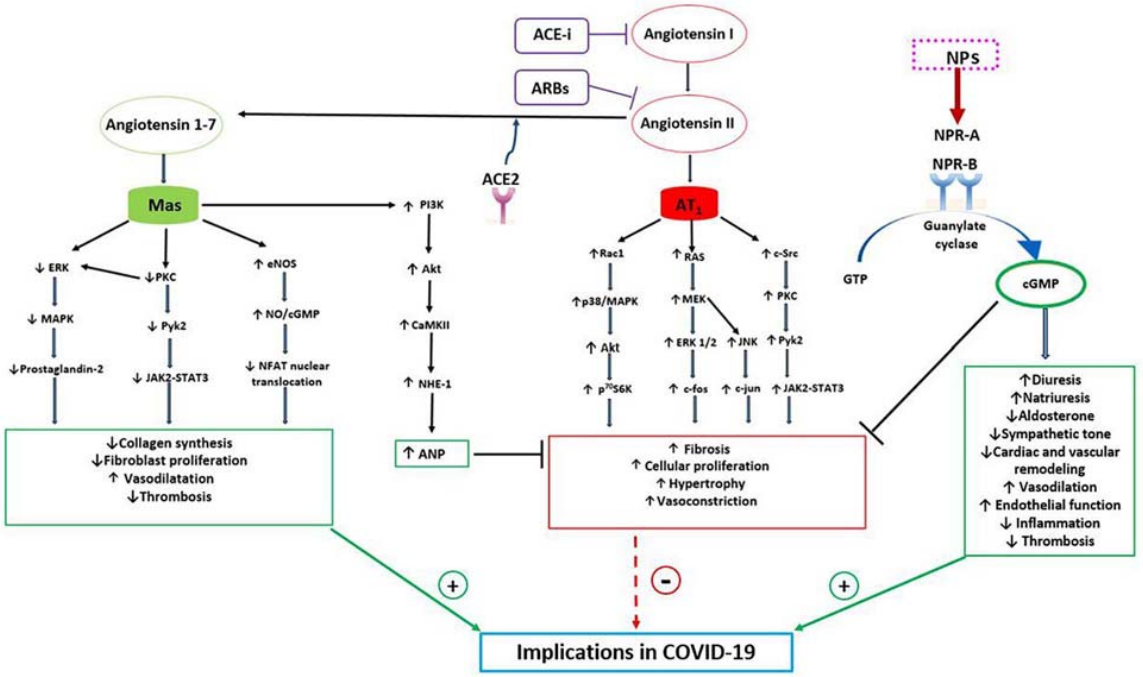

FIGURE 1 | Bidirectional interaction between NPs and RAAS with potential implications in COVID-19. The RAAS may exert both protective and detrimental effects. Angiotensin II, through its receptor AT1, activates signaling pathways involved in fibrosis, cellular proliferation, hypertrophy and vasoconstriction. Indeed, AT1 stimulates the RAC1/p38/MAPK pathway which is involved in the activation of Akt, mTOR and its downstream target p^{70}S6K. Moreover, AT1 activates both Ras/MEK/ERK pathway, which leads to an increased expression of c-fos mRNA, and JNK, which increases cJUN activity. AT1 also induces c-Src which, through PKC and PyK2, a non-receptor tyrosine kinase, stimulates JAK2-STAT3 signaling pathway. On the other hand, the protective arm of the RAAS is driven by ACE2-Ang (1-7)-Mas receptor, which plays complementary functions to the Angiotensin II-AT1 receptor. Mas receptor, indeed, reduces the activation of the MAPK, ERK and JAK2-STAT3 signaling pathways and increases the eNOS/NO pathway, down-regulating the NFAT transcription factor activity. As a consequence, Mas receptor activation reduces collagen synthesis, fibroblast activation and thrombosis. Moreover, Mas receptor activates PI3K/AKT and consequently CAMKII and NHE-1, increasing ANP gene expression, which further contributes to the protective role of ACE2-Ang (1-7). NPs indeed counteract the salt-retaining, vasoconstrictive, pro-inflammatory, pro-hypertrophic and pro-fibrotic functions of AT1. Through the stimulation of NPR-A and NPR-B and the production of cGMP, NPs increase diuresis, natriuresis, endothelial function and vasodilatation and reduce aldosterone production, sympathetic tone, inflammation and cardiac remodeling. As a consequence of their properties, NPs and Ang (1-7) may play a protective role during COVID-19 infection, reducing systemic inflammation, thrombosis, myocardial, lung and renal injury and improving prognosis. CAMKII, Ca 2 + /calmodulin-dependent protein kinase II; eNOS, endothelial nitric oxide synthase; ERK, extracellular-signal-regulated kinase; JAK, Janus kinase; JNK, c-JUN N-terminal kinase; MAPK, p38 mitogen-activated protein kinase; MEK, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; NHE-1, sodium-hydrogen antiporter 1; NO, nitric oxide; p^{70}S6K, ribosomal protein S6 kinase beta-1; PIK3, phosphatidylinositol 3-kinase; PKC, protein kinase C; PyK2, proline-rich tyrosine kinase 2; Rac1, Ras-related C3 botulinum toxin substrate; STAT3, signal transducer and activator of transcription 3.

Interestingly, it has been reported that the ACE2 deficient mouse model shows increased glomerular damage as a result of increased oxidative stress, proinflammatory and profibrotic changes. The administration of ACE2 in this model upregulated renal ANP expression, with a 3-fold increase within 10 days compared to controls. ANP gene expression increased even further when Ang II was added to AT1R blockers, probably as a consequence of a rise of local unbound Ang II then degraded to Ang (1-7) by ACE2. This study suggested that ACE2 may directly regulate renal ANP production, independently from volume expansion and pressure overload (Bernardi et al., 2012).

The interaction between ACE2 and NPs was confirmed in another study which investigated the influence of sacubitril/valsartan (S/V), the main molecule of the ARNi pharmacological class, on the expression of RAAS genes and proteins in an animal model (Zhao et al., 2019). It is known that the concomitant selective inhibition of NEP and AT1R upon ARNi administration produces an increase of NPs, particularly of ANP (Rubattu et al., 2018; Ibrahim et al., 2019), level and prevents the potential effect of an excess of Ang II. In the mentioned study, S/V significantly upregulated ACE2 mRNA expression, compared to valsartan alone, probably as a consequence of the ANP increase in addition to the AT1R blockade (Zhao et al., 2019).

The stimulation of NPs may reveal crucial in conditions characterized by a defect of ACE2 and excessive Ang II, such as that observed in the SARS-CoV-2 infection.

POTENTIAL PROTECTIVE ROLE OF NPs IN COVID-19

As previously mentioned, at the vascular level, NPs regulate cellular growth and proliferation, preserving endothelial function

and integrity as well as vascular tone. On the other hand, they oppose inflammation and atherosclerosis progression (Volpe et al., 2014; Forte et al., 2019; Rubattu and Volpe, 2019). In addition, ANP opposes blood clotting. In fact, it reduces *in vitro* the plasminogen activator inhibitor 1 (PAI-1) expression, a known modulator of fibrinolysis and a promoter of blood clotting (Bouchie et al., 1998). Evidence of a significant inverse relationship between ANP and PAI-1 levels was also reported in humans (Barbato et al., 2011).

Besides their well-described systemic hemodynamic and autocrine/paracrine functions within the cardiovascular system, NPs play an important protective role in the lungs. Herein, ANP reduces the secretion of inflammatory mediators and of endothelial and leukocyte-derived soluble adhesion molecules in response to lipopolysaccharide (LPS) and tumor necrosis factor α (TNFα). As a consequence, ANP is able to reduce lung endothelial permeability caused by inflammation and oxidative stress, avoiding the development of acute respiratory distress syndrome and improving arterial oxygenation during mechanical ventilation (Baron et al., 1989; Mitaka et al., 1998). As a further support to their relevant anti-inflammatory action, NPs have been shown to inhibit LPS/ATP-induced interleukin-1β secretion and to regulate the nuclear factor NF-kB/ERK pathway, THP-1 monocytes, and all elements of the NLR Family Pyrin Domain Containing 3/Apoptosis-associated Speck-like protein containing a Caspase-recruitment domain (NALP3/ASC)/caspase-1 inflammasome cascade (Mezzasoma et al., 2017).

Natriuretic and diuretic effects may also limit pulmonary edema and kidney damage. Moreover, based on the above described effects on blood clotting, NPs can contribute to inhibit the coagulopathy associated with COVID-19.

Furthermore, these hormones exert a well-known cardioprotective action toward myocarditis and acute cardiac dysfunction that may develop during infections (Currie et al., 2020). SARS-CoV-2 can certainly infect the heart, although the exact mechanism of cardiac involvement in COVID-19 has not been clearly understood. Based on both experimental and clinical evidence, both direct and indirect infections can take place. ACE2 is the receptor known to favor virus entry into the cells. However, a recent study demonstrated that extracellular vesicles from lung epithelial cells overexpressing SARS-CoV-2 were able to enter directly human cardiomyocytes *in vitro*, transferring viral RNA fragments into these cells and promoting inflammation (Kwon et al., 2020). Of note, Tavazzi et al. recently described a case of acute cardiac injury directly linked to myocardial localization of SARS-CoV-2, demonstrating low-grade myocardial inflammation and viral particles in the myocardium at the endomyocardial biopsy (Tavazzi et al., 2020). The precise role of NPs at the heart level in the context of SARS-CoV-2 infection remains to be established. The only study investigating a potential link of NPs with SARS-CoV-2 within the heart was conducted by performing a single-cell RNA sequencing (scRNA-seq) in both normal and failing hearts (Ma et al., 2021). Herein, ACE2 was found to be expressed in cardiomyocytes, vascular endothelial cells, fibroblasts, smooth muscle cells and immune cells in normal hearts, and its expression further increased in several cell subsets of the failing hearts (Ma et al., 2021). Importantly, BNP and ANP expression was upregulated in the more vulnerable ACE2-positive cardiomyocytes of failing hearts, along with a subset of genes favoring the viral infection (Ma et al., 2021). Although the latter evidence did not establish the exact type of relationship between ACE2 and NPs expression toward the virus entry and infection of the heart, it further suggested that NPs and ACE2 are tightly linked and may play an important role in the SARS-CoV-2 disease of HF patients. In particular, based on our knowledge, we can interpret the observed rise of ANP and BNP within the more vulnerable ACE2-positive cardiomyocytes as a reaction to the acute myocardial injury and as a protective response toward the inflammatory insult.

In fact, according to the available evidence, it has been proposed that COVID-19 patients with a deficiency of the NPs system, particularly obese subjects and black people, may have an increased risk of developing severe complications (Currie et al., 2020). This hypothesis certainly warrants further investigation.

USE OF NPs AS PROGNOSTIC MARKERS IN COVID-19

The role of NPs as potential prognostic biomarkers during COVID-19 infection represents an interesting issue and has attracted increasing attention in the medical community.

The cardiac release of NPs in patients with pneumonia or systemic infections may be explained by several pathophysiological mechanisms, such as increased myocardial wall stress as a consequence of hypoxia-induced pulmonary hypertension, development of cardiac injury due to the activation of inflammatory system, oxidative stress, demand-supply mismatch or the direct virus invasion, occurrence of renal failure and reduced NPs clearance (Thomas-Rüddel et al., 2019).

Current available studies have shown that increased NT-proBNP level is associated with an adverse clinical course during COVID-19 disease.

In a retrospective analysis by Guo et al. conducted in 187 patients, NT-proBNP level increased significantly during hospitalization only in those patients who died, without significant dynamic changes among survivors, thus predicting mortality independently from sex, age, hypertension, coronary heart disease, chronic obstructive pulmonary disease, myoglobin, creatin kinase-MB, high sensitivity troponin-I, white blood cells count, lymphocytes count, C-reactive protein, and procalcitonin (Guo et al., 2019). These findings were confirmed by a meta-analysis including 13 observational studies and a total of 2248 patients, which showed that elevated NT-proBNP level on admission was associated with a worse prognosis (Sorrentino et al., 2020). Patients with high BNP level had more severe cardiac injury with elevated troponin I level, a higher incidence of respiratory failure and a significantly increased mortality rate. Moreover, markers of coagulative disturbance were positively correlated with BNP level (Sorrentino et al., 2020). Another study conducted on 111 patients confirmed that BNP level was increased in patients with in-hospital mortality and it significantly correlated with age and previous CVD, whereas

increased troponin I level correlated with age, PaO2/FIO2 and D-dimer (Arcari et al., 2020).

Accordingly, NPs level may be used as an indicator of clinical severity for SARS-CoV-2 infection, suggesting a more accurate cardiac evaluation, to exclude a direct or indirect myocardial involvement, to support clinical judgement and to tailor medical therapy.

POTENTIAL THERAPEUTIC IMPLICATIONS OF ARNi IN COVID-19

As stated above, S/V, the available component of the novel ARNi class of drugs, combines the inhibition of AT1R and of NEP, the latter responsible of NPs degradation. In addition, NEP degrades other vasodilator peptides such as bradykinin, substance P, enkephalins and adrenomedullin. Therefore, the effect of ARNi depends on a complex neuro-hormonal modulation with potentially greater beneficial effects compared to the selective RAAS inhibition (Bayes-Genis et al., 2016; Singh et al., 2017).

With regard to the trend of the different NPs plasma level after the initiation of S/V, NT-proBNP level decreases, as a consequence of the improvement of cardiac function and hemodynamic status representing a useful biomarker of treatment response, BNP level slightly increases due to its relatively low affinity to NEP, whereas ANP level consistently and substantially increases both in humans and experimental models, mediating most of the benefits of NEP inhibition (Rubattu et al., 2018; Ibrahim et al., 2019).

Importantly, S/V has been demonstrated to inhibit the secretion of granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage chemoattractant protein-1 (MCP-1), responsible of the so-called cytokine storm and of adverse clinical course (D'Elia et al., 2017).

Due to all beneficial properties dependent from its mechanisms of action, S/V has been already proposed as an early therapeutic strategy in all COVID-19 patients with the aim to reduce the progression of the disease, the need for intensive treatment with ventilators and other major complications and mortality (Acanfora et al., 2020; Mohammed El Tabaa and Mohammed El Tabaa, 2020). However, it should be underlined that, although S/V is recognized as a cornerstone of the therapeutic management of HFrEF, due to the impressive benefits on cardiovascular death and HF hospitalization, recommendations for its use apart from this clinical subset do not exist (McMurray et al., 2014; Ponikowski et al., 2016; Yancy et al., 2017).

In fact, a more specific, targeted approach to test the expected beneficial role of S/V in COVID-19 patients would be, first of all, to retrospectively investigate existing registries of hospitalized COVID-19 patients to find out whether, among subjects affected by HFrEF, those treated with S/V presented a lower disease incidence, a better clinical course (particularly in terms of intensive care unit access, mechanical ventilation and death) and a better prognosis, compared to patients who received other medications, including ACEI/ARBs (Rubattu et al., 2020). This key issue should become the target of future investigation during the ongoing COVID-19 pandemic.

AUTHOR CONTRIBUTIONS

SR, GG, and MV substantially contributed to the conception and design, acquisition of data, or analysis and interpretation of data, drafted the article and approved the final version to be published. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by a grant from the Italian Ministry of Health.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 19 August 2021
doi: 10.3389/fphys.2021.693099

Molecular Signaling Mechanisms and Function of Natriuretic Peptide Receptor-A in the Pathophysiology of Cardiovascular Homeostasis

*Kailash N. Pandey**

Department of Physiology, School of Medicine, Tulane University Health Sciences Center, New Orleans, LA, United States

The discovery of atrial, brain, and C-type natriuretic peptides (ANP, BNP, and CNP) and their cognate receptors has greatly increased our knowledge of the control of hypertension and cardiovascular homeostasis. ANP and BNP are potent endogenous hypotensive hormones that elicit natriuretic, diuretic, vasorelaxant, antihypertrophic, antiproliferative, and antiinflammatory effects, largely directed toward the reduction of blood pressure (BP) and cardiovascular diseases (CVDs). The principal receptor involved in the regulatory actions of ANP and BNP is guanylyl cyclase/natriuretic peptide receptor-A (GC-A/NPRA), which produces the intracellular second messenger cGMP. Cellular, biochemical, molecular, genetic, and clinical studies have facilitated understanding of the functional roles of natriuretic peptides (NPs), as well as the functions of their receptors, and signaling mechanisms in CVDs. Transgenic and gene-targeting (gene-knockout and gene-duplication) strategies have produced genetically altered novel mouse models and have advanced our knowledge of the importance of NPs and their receptors at physiological and pathophysiological levels in both normal and disease states. The current review describes the past and recent research on the cellular, molecular, genetic mechanisms and functional roles of the ANP-BNP/NPRA system in the physiology and pathophysiology of cardiovascular homeostasis as well as clinical and diagnostic markers of cardiac disorders and heart failure. However, the therapeutic potentials of NPs and their receptors for the diagnosis and treatment of cardiovascular diseases, including hypertension, heart failure, and stroke have just begun to be expanded. More in-depth investigations are needed in this field to extend the therapeutic use of NPs and their receptors to treat and prevent CVDs.

Keywords: natriuretic peptides, natriuretic peptide receptors, gene-targeting, cardiovascular disorders, genetic mouse models

OPEN ACCESS

Edited by:
Amie Moyes,
Queen Mary University of London,
United Kingdom

Reviewed by:
Marcelo Roberto Choi,
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina
Alessandro Cataliotti,
University of Oslo, Norway

***Correspondence:**
Kailash N. Pandey
kpandey@tulane.edu

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *09 April 2021*
Accepted: *26 July 2021*
Published: *19 August 2021*

Citation:
Pandey KN (2021) Molecular Signaling Mechanisms and Function of Natriuretic Peptide Receptor-A in the Pathophysiology of Cardiovascular Homeostasis. Front. Physiol. 12:693099. doi: 10.3389/fphys.2021.693099

INTRODUCTION

The natriuretic peptides (NPs) family contains a group of hormones that are pivotal in the control of cardiovascular, endocrine, renal, and vascular homeostasis (Brenner et al., 1990; Drewett and Garbers, 1994; de Bold et al., 2001; McGrath et al., 2005; Pandey, 2005a; Rubattu et al., 2006; Kishimoto et al., 2011). Atrial and brain natriuretic peptides (ANP, BNP) are the members of the

NPs hormone family. A third member of the NPs family, C-type, natriuretic peptide (CNP) was also isolated and identified but each NP was found to be derived from a separate gene (Rosenzweig and Seidman, 1991; LaPointe, 2005; Schulz, 2005). ANP and BNP exhibit diuretic, natriuretic, vasorelaxant, antiproliferative, antiinflammatory, and antihypertrophic effects that are directed toward the reducing and controlling of body fluid volume, blood pressure (BP) and cardiovascular diseases (CVDs) (McGrath et al., 2005; Ellmers et al., 2007; Wang T.J. et al., 2007; Pandey, 2011; Volpe et al., 2014; Cannone et al., 2019). Although the possibility of NPs role in metabolic regulation has been given limited consideration but NPs potently affect lipid and glucose metabolism, may also contribute to the pathophysiological link between metabolic and CVDs (Schlueter et al., 2014; Jordan et al., 2018). ANP has been shown to induce lipolysis and lipid oxidation and to ameliorate insulin resistance (Birkenfeld et al., 2008; Coue and Moro, 2016). All three NPs (ANP, BNP, and CNP) have highly homologous sequence structures, bind to cognate cell surface receptors, and elicit discrete biological and physiological functions (Brenner et al., 1990; Koller and Goddel, 1992; Anand-Srivastava and Trachte, 1993; Khurana and Pandey, 1993; Pandey, 2008). Each member of the family of endogenous NPs, including ANP, BNP, CNP, and urodilatin, has an integral role in BP, renal dysfunction, and CVDs (de Bold, 1985; Levin et al., 1998; Pandey, 2011; Rubattu and Volpe, 2014).

Importantly, NPs not only regulate BP and CVDs, but also maintain natural antagonistic actions to the renin-angiotensin-aldosterone system (RAAS), exert antimitogenic effect, and inhibit myocardial hypertrophy and fibrosis. Moreover, NPs play roles in endothelial cell function, cartilage growth, immunity, and mitochondrial biogenesis (Pandey, 2005a; Vollmer, 2005; Garbers et al., 2006; Ellmers et al., 2007). The demonstration of structurally related NPs indicated that the physiological control of BP and cardiovascular homeostasis is both complex and multifactorial. Studies of a combination of cellular, biochemical, molecular, genetic, and pharmacological properties of NPs and their prototype receptors have suggested the hallmark functions with physiological and pathophysiological responsiveness, including cardiovascular, endocrine, renal, neuronal, skeletal, and immunological importance in health and disease (Kuhn, 2005; Vollmer, 2005; Kishimoto et al., 2011; Misono et al., 2011; Jordan et al., 2018; Pandey, 2018).

Different NPs receptors have been classified namely; NP receptor-A (NPRA), NP receptor-B (NPRB), and NP receptor-C (NPRC). NPRA and NPRB exhibit an intrinsic intracellular guanylyl cyclase (GC) catalytic domain and are designated as GC receptor-A (GC-A/NPRA) and GC receptor-B (GC-B/NPRB) (Drewett and Garbers, 1994; Levin et al., 1998; Pandey, 2005a). Both ANP and BNP bind and activate NPRA, which produces intracellular second messenger cGMP in response to hormone binding. CNP activates NPRB and also produces cGMP. All three NPs (ANP, BNP, CNP) bind to NPRC, which lacks a GC catalytic domain and does not increase levels of intracellular cGMP (Fuller et al., 1988; Garbers, 1992; Koller and Goddel, 1992; Khurana and Pandey, 1993; Matsukawa et al., 1999). NPRA is a principal loci involved in the regulatory action of ANP and BNP (Lucas et al., 2000; Tremblay et al., 2002; Pandey, 2005a). Determining the insight into the intricacies of ANP-BNP/NPRA/cGMP signaling is of primary importance if we are to understand both the receptor biology and CVDs arising from cell- and tissue-specific abnormal hormone-receptor interplay. The binding of ANP and BNP to the extracellular domain of NPRA seem to exerts a conformational shift or change, whereby the signal is transmitted to the intracellular GC catalytic region of NPRA, which then activates generation of the second messenger cGMP in target cells and tissues (Pandey and Singh, 1990; Garbers, 1992; Koller and Goddel, 1992).

Although, great importance has been placed on the functional roles of NPs and their receptors in renal, cardiovascular, endocrine, neuronal, skeletal, and adipose homeostasis, in-depth research is still attempting to fully understand their potential molecular and therapeutic targets in the diseases states. We expect that future research on NPs and their receptors will yield new therapeutic targets and novel loci for the prevention and treatment of hypertension, stroke, and cardiovascular events. Earlier studies focused on elucidating the biochemical, cellular, molecular, and genetic aspects of the mode of functioning of NPRA, which still are not fully understood (Kishimoto et al., 2011; Misono et al., 2011; Pandey, 2011). Studies of cultured cells *in vitro* and transgenic and gene-targeted (gene-knockout and gene-duplication) mouse models *in vivo* have greatly advanced our understanding of NPs and their receptors by delineating the normal and abnormal control of physiological and pathophysiological functions in CVDs (Pandey, 2005a, 2019; Ellmers et al., 2007; Volpe et al., 2019a).

NATRIURETIC PEPTIDE HORMONE FAMILY

Almost four decades ago, it was firmly established that atrial homogenate contains diuretic and natriuretic activity and simultaneously identified and characterized the hormone atrial natriuretic factor (ANF) now designated as ANP in cardiac myocytes (de Bold et al., 1981; de Bold, 1985). ANP, the initial member of the NP hormone family, is predominantly synthesized and secreted from the cardiac atrium, which led to classification of the heart as an endocrine organ. The primary structure deduced from the cDNA sequence, demonstrated that ANP is synthesized as a 152-amino acid pre-pro-ANP that contains the sequences of biologically active hormone in the carboxyl-terminal region and has the major form of circulating ANP as a 28-amino acid peptide molecule (Maki et al., 1984). It was found that a 17-amino acid disulfide-bonded ring structure of circulating ANP is essential for its physiological functions. Indeed, disruption of the ring structure of ANP abolished its functional activity (Misono et al., 1984; de Bold, 1985; Brenner et al., 1990). BNP is synthesized as a 134-amino acid pre-pro-BNP that produces a 108-amino acid prohormone. Processing of pro-BNP yields a 75-residue amino-terminal-BNP (NT-pro-BNP) and a biologically active 32-amino acid circulating BNP molecule (Sudoh et al., 1988; Seilhamer et al., 1989). CNP is synthesized as a 103-amino acid pre-pro-CNP cleaved to a 53-amino acid

peptide by the protease furin. It is subsequently processed to yield a 22-amino-acid biologically active CNP (Wu et al., 2003).

Cellular, biochemical, molecular, and immunohistological studies have suggested that three specific NPs (ANP, BNP, CNP) and their three distinct receptor subtypes (NPRA, NPRB, NPRC) have widespread cell and tissue distributions, indicating the pleotropic actions at the systemic and local levels (Pandey et al., 1986, 1988; Leitman et al., 1988; Brenner et al., 1990; Levin et al., 1998). ANP and BNP exhibit the most variability in primary sequence structure across species, while CNP is highly conserved among species. Subsequently, a 32-amino acid peptide, urodilatin (URO) was identified in urine. However, URO is not detected in the circulation. It appears to be a unique intrarenal NP with largely unexplored physiological functions (Schulz-Knappe et al., 1988; Saxenhofer et al., 1990; Feller et al., 1990; Goetz, 1991). Immunohistological staining has indicated that URO is mainly synthesized in renal cortical tubules around the collecting ducts; however, its exact role has yet to be determined (Sugimoto et al., 1993; Forssmann et al., 1994; Meyer et al., 1996). Additionally, another member of the NPs hormone family, the D-type natriuretic peptide (DNP), was initially isolated from the venom of green mamba (*Dendroaspis angusticeps*) as a 38-amino acid biologically active peptide molecule (Schweitz et al., 1992; Lisy et al., 1999). ANP shows a rapid clearance rate in the circulation; its half-life ranges from 0.5 to 3.5 min in experimental animals (Nakao et al., 1986; Yandle et al., 1986; Ruskoaho, 1992). In human, the half-life ranges between 2 and 2.5 min (Nakao et al., 1986). The clearance of BNP in humans occurs with both a short half-life of 3–4 min and a long half-life of 20–23 min (Mukoyama et al., 1991; Holmes et al., 1993). CNP has a half-life of 2−3 min in humans and approximately 1.5−2.0 min in experimental animals (Hunt et al., 1994; Charles et al., 1995).

The design of chimeric NPs has led to synthesis of the biologically active novel NPs of clinical importance, which represent single-chemical molecule with combined structural and functional properties (Lisy et al., 2008; Ichiki et al., 2019). Chimeric NPs exert the actions of more than one NP molecule, often with reduced undesirable or adverse hypotensive effects. Previously, a chimeric NP, namely CD-NP containing 22 residues of CNP and 15 residues at the carboxyl-terminus of DNP was synthesized, which showed vasorelaxant properties and cardiac and renal protective effects (Lisy et al., 2008; Lee et al., 2009; Ichiki et al., 2019). The synthetic CD-NP showed high resistance to NP degrading enzyme, neutral endopeptidases, making it clinically useful than endogenous naturally occurring NPs (Ichiki et al., 2019). Interestingly, it is expected that endogenous GC-A/NPRA activators and designer NPs will be powerful tools for preserving renal and cardiovascular functions and decreasing mortality among patients with cardiac events (Chen et al., 2019).

NATRIURETIC PEPTIDES LEVELS IN HUMANS

The cardiac hormones, ANP and BNP are released from the heart in response to atrial stretch and distension. The ANP concentration ranges at the level of 50−100-fold greater than BNP. The primary sites of synthesis and secretion of ANP and BNP is cardiac atrium. Both ANP and BNP are also synthesized in the ventricle; however, 100−1,000-fold lower than does the atrium. In cardiac disease states, the ventricle becomes the primary site of synthesis and release of BNP. A recent study showed that super-enhancer cluster controls the *Nppa* and *Nppb* promoters in a competitive mode instead in a cooperative manner (Man et al., 2021). These authors suggested that the super-enhancer cluster selectively regulates the expression of *NPPA* and *NPPB* during CVDs, resulting in an increased expression of *NPPA* after the *NPPB* region is deleted, which augments the stress-induced expression of NPRA and prevention of premature cardiac hypertrophy in human. Circulating levels of ANP and BNP are greatly elevated in the early stages of cardiac infarction (Tomoda, 1988; Phillips et al., 1989; Morita et al., 1993). The expression of ANP and BNP also increases in the atrium and ventricle during the initiation of cardiac hypertrophy and congestive heart failure (CHF) (Mukoyama et al., 1991; Volpe, 2014; Nakagawa et al., 2019). In patients with severe CHF, the levels of ANP and BNP increase more than do control levels but the BNP concentration increases to a level 10−25-fold greater than fold increased in ANP levels (Mukoyama et al., 1991). A recent study found differential regulation of ANP and BNP in patients with acute decompensated heart failure (ADHF) (Reginauld et al., 2019). These authors suggested that a deficiency of ANP might exhibit a unique characteristics of CHF. However, the exact mechanism of this type of ANF deficiency with elevated BNP levels is not yet know. Those findings suggested that the heart plays a potential compromised compensatory endocrine role with differential regulation of ANP and BNP in ANP-deficient ADHF subpopulation (Reginauld et al., 2019). Previously, it had been suggested that obese hypertensive men, despite having high BP, have lower than anticipated ANP level in the plasma (Asferg et al., 2013). Similarly, a previous study indicated that a lack of compensatory ANP elevation occurs in the advanced phase of hypertension (Macheret et al., 2012). This study also showed the prevalence of impaired synthesis and secretion of BNP, pro-BNP, and NT-pro-BNP in hypertensive patents.

ANP and BNP show similar hemodynamic and physiological responses. But because of longer half-life of BNP (12−20 min) than ANP (0.5−4), BNP exhibits extended action and leads to enhanced natriuretic and diuretic actions as compared with ANP (Yoshimura et al., 1991; Omland et al., 1996). The cardiac atrium expresses almost 50–100- fold higher ANP mRNA levels than do extra cardiac tissues (Gardner et al., 1986). Although, in normal subjects; circulating BNP levels are far less than ANP levels, the increases in BNP concentrations in plasma can be 5−10-fold higher than the fold increases in the levels of ANP in CHF patients (Mukoyama et al., 1991; Hanford et al., 1994; Hanford and Glembotski, 1996). The half-life of NT-proBNP is much longer (90−120 min) than the half-life of ANP and BNP (Weber and Hamm, 2006).

Because of the longer half-life and stability, the measurement of NT-proBNP as a diagnostic marker is preferred over measurements of BNP and ANP. Moreover, the NT-proBNP molecule is considered to be a predictive marker after cardiac

transplantation (Gardner et al., 2006). In contrast, CNP does not seem to behave as a cardiac hormone and its levels in the circulation are very low (Igaki et al., 1996). CNP is largely present in the vascular endothelial cells and central nervous system (Ogawa et al., 1992; Suga et al., 1992, 1993; Tamura et al., 1996; Chen and Burnett, 1998).

STRUCTURAL DOMAINS AND SIGNALING MECHANISMS OF NPS RECEPTORS

Initially, using photoaffinity labeling and GC activity assay, three distinct types of NP receptors were identified and classified in different cell types (Pandey et al., 1988). Using molecular cloning, GC-A/NPRA was sequenced from rat brain, human placenta, and murine testis (Chinkers et al., 1989; Lowe et al., 1989; Pandey and Singh, 1990). Three distinct sub-types of NPs receptors (NPRA, NPRB, and NPRC) constitute the NPs receptor family; however, these receptors are variable in their ligand-binding specificity, GC activity, and signal transduction mechanisms (Chinkers et al., 1989; Garbers, 1992; Koller and Goddel, 1992; Khurana and Pandey, 1993). NPRC lacks the intracellular GC catalytic domain and by default, has been designated as NP clearance receptor (Maack et al., 1987; Fuller et al., 1988). Both ANP and BNP selectively activate NPRA, whereas CNP primarily binds to NPRB; all three NPs show binding affinity to NPRC (Koller et al., 1991; Suga et al., 1992; Khurana and Pandey, 1993). ANP binding to its receptor *in vivo* probably exerts a chloride-dependent feedback-control on receptor function (Misono, 2000; Misono et al., 2005). The general structural topology of NPRA and NPRB is consistent with the GC-receptor family, containing four distinct regions: an extracellular ligand-binding domain, a single transmembrane spanning region, an intracellular protein kinase-like homology domain (protein-KHD), and an intracellular carboxyl-terminal GC catalytic domain. The biologically dominant form of the NP receptors is NPRA, which is distributed in several peripheral and visceral cells and tissues and mediates most of the known physiological and pathophysiological actions of ANP and BNP.

Solubilization of the extracellular domain of NPRA provided a starting point for the studies of its ligand-binding domain (Pandey and Kanungo, 1993; Huo et al., 1999). NPRA was crystalized as a dimer of two receptor molecules with a tendency to dimerize spontaneously (van den Akker et al., 2000). Based on the crystal structure of NPRC, the hormone-induced structural changes in NPRA differ from those in NPRC. Ligand-binding to NPRC causes the dimer to bend between the two subdomains of the receptor creating a clamping motion to capture the ligand (He et al., 2001). Conformational flexibility at the intra-molecular hinge region in NPRC seems to be a critical factor for the broad-specificity of this receptor protein in binding all three NPs, including ANP, BNP, and CNP (He et al., 2001). Based on crystallographic modeling, two polypeptide chains of NPRA are required to activate the functional receptor molecule (van den Akker et al., 2000). It is predicted that the transmembrane GC receptors function as homodimers and that the dimerization region of NPRA is located between the protein-KHD and GC catalytic domains, forming an amphipathic alpha helix structure (de Lean et al., 2003; Misono et al., 2005). NPRB, which is mainly localized in the brain and vascular tissues, is thought to mediate CNP-dependent actions in the endothelial cells of the vasculature and central nervous system (Schulz, 2005). NPRC consists of a large extracellular domain of 496-amino acids, a single transmembrane domain, and a very short 37-amino-acid cytoplasmic tail that contains no homology to any other known receptor domain (Fuller et al., 1988; Matsukawa et al., 1999).

After ligand binding, both NPRA and NPRC are internalized and redistributed into subcellular compartments (Rathinavelu and Isom, 1991; Koh et al., 1992; Pandey, 1992, 2005b; Mani et al., 2015; Mani and Pandey, 2019). The endocytosis and intracellular sequestration of NPRA involves a series of sequential sorting steps through which ligand-receptor complexes are eventually internalized, sequestered, redistributed, and degraded in the lysosomes. However, a population of ligand-receptor complexes recycles back to the plasma membrane, and intact ligand is released outside the cell, while the receptor is anchored on the cell surface (Pandey, 1993; Pandey et al., 2002, 2005; Mani et al., 2015; Mani and Pandey, 2019). Trafficking of NPRB occurs in a ligand-dependent manner in response to stimulation with CNP. NPRB is internalized and recycled back to the plasma membrane (Brackmann et al., 2005). Degradation of the majority of internalized ligand-receptor complexes of NPRA, NPRB, and NPRC occurs into the lysosomes. Two specific sequence motif in the carboxyl terminal domain of NPRA, namely GDAY (Gly^{920} – Asp^{921}-Ala^{922}-Tyr^{923}) and FQQI (Phe^{790}-Gln^{791}-Gln^{792}-Ile^{793}) have been identified, which serve as consensus internalization signal motifs for endocytosis and intracellular trafficking of this receptor protein (Pandey et al., 2005; Mani et al., 2016; Mani and Pandey, 2019). However, a specific sequence motif has not yet been identified for the internalization of either NPRB or NPRC. The internalization of NPRA also involves micro-RNA and clathrin-dependent pathways (Somanna et al., 2013, 2018).

Ligand-binding seems to allosterically regulate increased specific activity of the GC catalytic domains of NPRA and NPRB, which catalyzes cGMP production (Drewett and Garbers, 1994; Pandey et al., 2000; Pandey, 2005a; Burczynska et al., 2007). ANP markedly and dose-dependent increases second messenger cGMP in target cells and tissues (Waldman et al., 1984; Pandey et al., 1985, 1988, 2002, 2005). Confocal immunofluorescence analyses have demonstrated that cGMP is continuously produced in parallel during the internalization of ligand-bound NPRA in the subcellular compartments (Mani et al., 2015, 2016; Mani and Pandey, 2019). The generation of intracellular cGMP by NPRA stimulates at least three known cGMP effector protein molecules: cGMP-dependent protein kinases (PKGs), cGMP-dependent phosphodiesterases (PDEs), and cGMP-dependent ion channels (Pfeifer et al., 1998; Kaupp and Seifert, 2002; Maurice et al., 2003; Rybalkin et al., 2003; Schlossmann et al., 2005; Reinhart et al., 2006; Pandey, 2008). The activation of cGMP-dependent effector molecules leads to a wide range of signaling mechanisms leading to physiological functions, including excretion of salt and water, vasorelaxation, antiinflammation, antifibrosis, antihypertrophic actions, and immune suppressive responses (**Figure 1**). Overall,

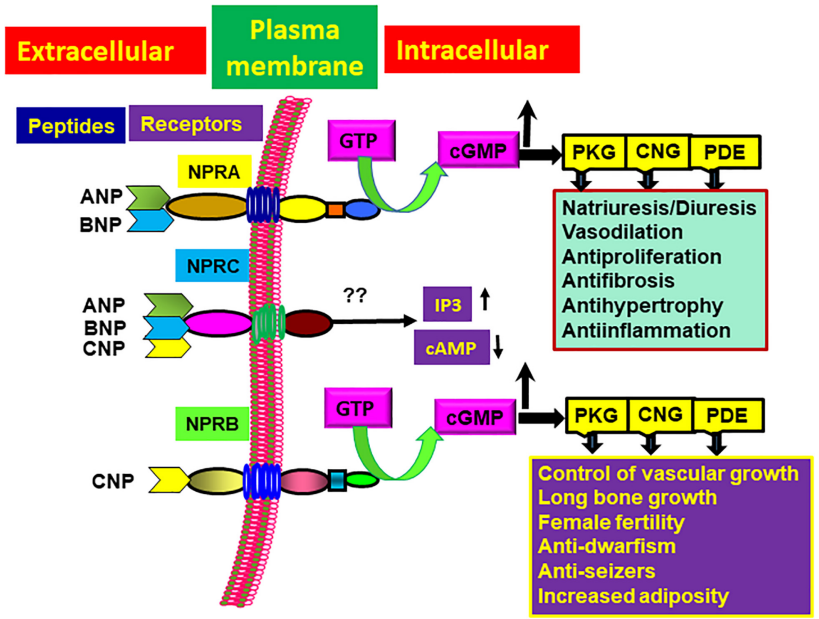

FIGURE 1 | Ligand-binding specificity of different NPs (ANP, BNP, and CNP) with their cognate receptors (NPRA, NPRB, NPRC) and intracellular signaling mechanisms leading to the physiological responses. The diagram depicts that specific NP ligands activate their cognate receptor(s) and generate intracellular signals. The extracellular ligand binding domain (LBD) and intracellular protein kinase-like homology domain (protein-KHD) and guanylyl cyclase (GC) catalytic domain of GC-A/NPRA and GC-B/NPRB are shown. Both NPRA and NPRB produce intracellular second messenger cGMP, which activates effector molecules, including cGMP-dependent protein kinase (PKG), gated ion channels (CNG), and phosphodiesterase (PDE) to produce specific biological and physiological responsiveness.

the signaling mechanisms of NPs and their receptors, lead to diverse physiological and pathophysiological actions, which together provide the protection to CVDs.

NPs AND THEIR RECEPTORS IN CARDIOVASCULAR DISORDERS

Several lines of evidence suggest that from the perspective of pathophysiology, a characteristic hallmark of CVDs is the elevation of ANP and BNP concentrations in patients with chest pain and cardiovascular dysfunction (Burnett et al., 1986; Maisel et al., 2002; Richards and Troughton, 2004; Reginauld et al., 2019). The elevation of ANP and BNP concentrations in CHF seem to be serve as a beneficial compensatory responsiveness that offset cardiac events (Stevens et al., 1995; Wang et al., 2014). However, the emerging paradigm in hypertension and CVDs is the existence of ANP and BNP deficiency with high BP and CVDs. From the point of view of the roles of ANP, BNP, and their cognate receptor NPRA in hypertension and CVDs, this section has been classified in the follow categories:

(a) Hypertension and Cardiovascular Diseases
(b) Renovascular Dysfunction
(c) Cardiac remodeling and dysfunction
(d) Metabolic Syndrome and Diabetic Conditions
(e) Immunogenic Responses and Cardiovascular Disorders

(a) Hypertension and Cardiovascular Diseases

The activation of ANP-BNP/NPRA signaling lowers fluid volume and BP by causing excretion of salt and water from the kidneys and inhibition of the vasoconstriction of vascular smooth muscle cells. The mutations in genes encoding ANP, BNP, and NPRA seems to exert deleterious effects and trigger high BP and CVDs (Ellmers et al., 2007; Pandey, 2008, 2018; Kishimoto et al., 2011). Several studies have demonstrated a link of ANP (*NPPA*), BNP (*NPPB*), and NPRA (*NPR1*) polymorphisms with hypertension and CVDs in humans (Rubattu et al., 2006; Webber and Marder, 2008; Xue et al., 2008; Newton-Cheh et al., 2009; Vandenwijngaert et al., 2019). In mice, the gene-knockout phenotypes of *Nppa*, *Nppb*, *Nppc*, *Npr1*, *Npr2*, and *Npr3* are provided in **Table 1**.

Genetically modified mouse models have helped to delineate the functions of ANP, BNP, and NPRA in the physiological and pathophysiological conditions of high BP and CVDs (John et al., 1995; Oliver et al., 1997; Shi et al., 2003; Vellaichamy et al., 2005a; Ellmers et al., 2007; Pandey, 2019). Transgenic mice overexpressing ANP developed sustained hypotension; also their mean arterial pressure (MAP) was decreased by 25−30 mmHg compared with the non-transgenic control groups (Steinhelper et al., 1990; Melo et al., 1999). Somatic delivery of the proANP cDNA to a spontaneously hypertensive rat (SHR) model induced a sustained reduction in systolic BP (Lin et al., 1995). Similarly, overexpression of proANP in hypertensive mice lowered systolic BP, suggesting that the proANP is an ideal gene-therapy candidate for the treatment of human hypertension and CVDs (Schillinger et al., 2005).

Several cardiovascular pathways, including NPs, RAAS, and the adrenergic systems, are considered to regulate BP and cardiovascular events. However the genetic determinants

TABLE 1 | Tissue distribution of natriuretic peptides (ANP, BNP, and CNP) along with their cognate receptors (NPRA, NPRB, and NPRC) and gene-knockout phenotypes in mice.

Gene	Protein	Tissue-distribution	Gene-knockout phenotype	References
Nppa	ANP	Atrium, brain, kidney, ovary, testis, ventricle	Hypertension, cardiac hypertrophy	Steinhelper et al., 1990; John et al., 1995; Lin et al., 1995; Melo et al., 1999
Nppb	BNP	Atrium, brain, ventricle	Ventricular fibrosis, skeletal abnormalities, vascular complications	Ogawa et al., 1994; Tamura et al., 2000
Nppc	CNP	Aorta, brain, heart, testis, vascular endothelium	Inhibition of long bone growth, dwarfism, abnormal chondrocyte growth	Chusho et al., 2001; Yasoda et al., 2004; Wang Y. et al., 2007; Moyes et al., 2020
Npr1	NPRA	Adrenal glands, brain, heart, kidney, liver, lung, olfactory neurons, ovary, pituitary gland, placenta, testis, thymus, vasculature, liver	Hypertension, cardiac hypertrophy and fibrosis, inflammation, volume overload, reduced testosterone	Lopez et al., 1995; Oliver et al., 1997; Shi et al., 2003; Vellaichamy et al., 2005a; Reinhart et al., 2006; Ellmers et al., 2007; Zhao et al., 2007; Das et al., 2010; Vellaichamy et al., 2014; Subramanian et al., 2016; Das et al., 2020
Npr2	NPRB	Brain, cartilage, heart, lung, ovary, pituitary gland, placenta, testis, thymus, vasculature	Dwarfism, decreased adiposity, female sterility, seizures, vascular complications	Tamura et al., 2004; Langenickel et al., 2006
Npr3	NPRC	Brain, heart, intestine, kidney, liver, vasculature	Bone deformation, skeletal over-growth, long bone overgrowth	Matsukawa et al., 1999

ANP, atrial natriuretic peptide; Nppa, coding for pro-atrial natriuretic peptide; BNP, brain natriuretic peptide; Nppb, coding for pro-brain natriuretic peptide; CNP, C-type natriuretic peptide; Nppc, coding for pro-C-type natriuretic peptide. NPRA, natriuretic peptide receptor-A; Npr1, coding for NPRA; NPRB, natriuretic peptide receptor-B; Npr2, coding for NPRB; NPRC, natriuretic peptide receptor-C; Npr3, coding for NPRC.

contributing to inter-individual differences in BP regulation have been linked with only the NPs and their receptors (Newton-Cheh et al., 2009). Previously, it was indicated that the polymorphism in the *NPPA* caused left ventricular cardiac hypertrophy in the Italian patients suffering from high BP and these patients also showed significantly reduced levels of proANP (Rubattu et al., 2006). The relationship between BP and cardiovascular disorders indicated that in the absence of ANP-BNP/NPRA signaling, even small increases in BP had excessive and detrimental effects. Mutation of a single allele in the human *NPR1* was found to decrease levels of NPRA protein by 70% and showed susceptibility to BP, kidney dysfunction, and LVH (Nakayama et al., 2000). The single allele mutation in *NPR1* in human could reflect parallel characteristics in the haplotype genotype of *Npr1*$^{/}$ in mice (Shi et al., 2003; Vellaichamy et al., 2005a; Kumar et al., 2017). It has been reported that a positive association exists between the polymorphisms of *NPPA, NPPB, and NPR1* causing essential hypertension and LHV (Newton-Cheh et al., 2009; Vandenwijngaert et al., 2019). Those previous studies indicated that genetic polymorphisms in *NPPA, NPPB, and NPR1* seem to be linked with a family history of high BP, LVH, cardiac mass index, and paraventricular septal wall thickness. More studies will be required to characterize the functionally significant markers of *NPPA, NPPB,* and *NPR1* polymorphism variants in a larger human population.

Global ablation of *Nppa* in mice can increase BP and cause hypertension (John et al., 1995). The genetic mouse model with ablation of *Nppa*, has suggested that ANP has a central role in hypertension. In *Nppa* null mutant (*Nppa*$^{/}$) mice fed standard or intermediate-salt diets, BP was elevated by 8−10 mmHg. Haplotype (*Nppa*$^{/}$) mice on a standard-salt diet contained a normal amount of plasma ANP and had normal BP, while on a high-salt diet these animals were hypertensive, and their BP was elevated by 25−27 mmHg. Global deletion of the *Nppa* allele can lead to salt-sensitive hypertension even when plasma ANP level are not significantly decreased. Global ablation of *Npr1* increases BP by 36−40 mmHg in null mutant (*Npr1*$^{/}$; 0-copy) mice as compared with wild-type (*Npr1*$^{/}$; 2-copy) mice (Oliver et al., 1997; Shi et al., 2001, 2003; Das et al., 2010, 2020; Vellaichamy et al., 2014). In contrast, increased expression of global *Npr1* in gene-duplicated (*Npr1*$^{/}$; 3-copy and *Npr1*$^{/}$; 4-copy) mice significantly reduced BP and enhanced kidney and heart function (Oliver et al., 1998; Shi et al., 2003; Zhao et al., 2013; Das et al., 2020).

Earlier, we examined the mechanisms that may mediate the function of increasing numbers of *Npr1* gene copies, determining the excretion of urine and sodium, renal blood flow (RBF), glomerular filtration rate (GFR), and BP after blood volume expansion in *Npr1* 0-copy, 2-copy, and 4-copy mice in a gene-dose-dependent manner (Shi et al., 2003). The volume expansion with whole blood infusion, increased MAP in *Npr1* gene-knockout (0-copy), wild-type (2-copy), and gene-duplicated (4-copy) mice. In addition, *Npr1* null mutant (0-copy) mice retained significantly higher levels of Na^+ and water; however, gene-duplicated (4-copy) mice as compared to wild-type (2-copy) mice had greatly reduced levels of Na^+ and water. Our findings demonstrated that the ANP/NPRA axis is predominantly responsible for regulating the renal hemodynamics and Na^+ excretory responses to intravascular blood volume expansion.

(b) Renovascular Dysfunction

ANP-BNP/NPRA system primarily affects glomerulus, tubular, and vascular functions in the kidneys (Nonguchi et al., 1987; Kremer et al., 1988; Light et al., 1989; Appel, 1990; Cermak et al., 1996; Kumar et al., 1997b, 2014; Pandey et al., 2000; Tripathi and Pandey, 2012; Das et al., 2020). ANP prevents contraction of mesangial cell and vascular smooth muscle cells in response to vasoconstrictors, including Ang II, endothelin, vasopressin, and agonists of the adrenergic systems (Appel, 1990; Kumar et al., 1997a; Levin et al., 1998; Pandey, 2018). NPRA antagonists, A71915 and HS-121-1 abolished the renal effect of infused ANP,

including a decrease in urinary cGMP (von Geldern et al., 1990; Sano et al., 1992). In the kidneys, a combination of hemodynamic and tubular transport effects seem to be responsible for ANP-BNP/NPRA-induced natriuresis and diuresis, exerting both direct and indirect effects on tubules, including the proximal tubules, as well as cortical and innermedullary collecting ducts (Sonnenberg et al., 1986; Brenner et al., 1990; Zhao et al., 2010; Prieto et al., 2012). ANP actions facilitate the excretion of salt and water with an increase in GFR and RBF in the kidneys (Burnett et al., 1984; Camarago et al., 1984; Freeman et al., 1985; Meyer and Forsmann, 1997; Villrreal and Freeman, 1997; Melo et al., 2000; Shi et al., 2003; Pandey, 2008; Zhao et al., 2010). ANP- and BNP-induced natriuresis and diuresis causes a direct inhibition of tubular transport processes. This occurs without dependence on alterations in GFR and RBF, which favor both hemodynamic and tubular effects (Sonnenberg et al., 1986; Meyer et al., 1997). The natriuretic and diuretic actions of ANP and BNP lead to the direct inhibition of tubular transport mechanisms. It has been proposed that ANP-BNP/NPRA system also interacts with another local natriuretic system, by enhancing the action of renal dopaminergic system (Choi et al., 2014). These authors suggested that part of the inhibitory effects of ANP on sodium and water reabsorption depends on dopaminergic system. Those previous studies also indicated that ANP/NPRA signaling stimulates dopamine uptake in tubular cells and also reduces the dopamine catabolism. Similarly, urodialatin also seem to exert similar effect on renal dopaminergic system (Citarella et al., 2009; Choi et al., 2013). Confocal immunofluorescence microscopic studies have indicated that the epithelial sodium channel (ENaC) is regulated by ANP/NPRA-dependent second messenger cGMP in *Xenopus* 2F3 cells (Guo et al., 2013). The authors of those previous studies suggested that NPRA, but not NPRB or NPRC, is involved in the regulation of ENaC activity. Further, with the increasing concentrations of intracellular cGMP, ENaC activity was inhibited in epithelial cells from patients with cystic fibrosis (Poschet et al., 2007).

The ANP-BNP/NPRA signaling cascade plays critical roles to counteract both systemic and local RAAS (Meyer and Forsmann, 1997; Shi et al., 2001; Pandey, 2005a, 2011; Vellaichamy et al., 2007). ANP/NPRA markedly lowers renin secretion from the kidneys and also reduces plasma renin concentrations (Burnett et al., 1984; Obana et al., 1985; Paul et al., 1988; Meyer and Forsmann, 1997; Shi et al., 2003). Further, using genetically modified mouse models, ANP/NPRA signaling has been shown to suppress renin activity and other RAAS components as well as to decrease BP (Obana et al., 1985; Brenner et al., 1990; Levin et al., 1998; Shi et al., 2001; Pandey, 2005a; Vellaichamy et al., 2007; Periyasamy et al., 2019).

Studies from our laboratory using *Npr1*$^{-/-}$ null mutant mice have demonstrated that at the birth, the absence of NPRA allows increased renin mRNA expression and greater renin and Ang II levels than occur in *Npr1*$^{-/-}$ wild-type mice (Shi et al., 2001). On the contrary, in adult *Npr1*$^{-/-}$ mice, both circulating and intrarenal concentrations of renin and Ang II levels were significantly lower than those in wild-type control animals. The decrease in renin concentrations in adult *Npr1*$^{-/-}$ mice was found to be due to progressive increases in BP, which lead to inhibition of renin synthesis and secretion from juxtaglomerular cells in the kidney. It has been suggested that an increased levels of ANP released into the circulation in response to blood volume expansion, was mainly responsible for the natriuretic and diuretic actions (Paul et al., 1988; Antunes-Rodrigues et al., 1992). We have examined the quantitative contributions and possible mechanisms mediating renin synthesis and release using the varying *Npr1* gene copy of *Npr1*$^{-/-}$ (0-copy), *Npr1*$^{+/+}$ (2-copy), and *Npr1*$^{++/++}$ (4-copy) mice in a gene-dose-dependent manner (Shi et al., 2003). Our studies have demonstrated that NPRA ablation increases the expression of AT_1R and angiotensin-converting enzyme 1 (ACE 1) in the kidneys of *Npr1*$^{-/-}$ null mutant mice (Periyasamy et al., 2019).

ANP-BNP/NPRA signaling inhibits the synthesis and secretion of aldosterone in adrenal glomerulosa cells suggesting that ANP is important for natriuretic and diuretic responses (Atarashi et al., 1984; Brenner et al., 1990; Anand-Srivastava and Trachte, 1993; Pandey, 2005a). The NPRA antagonist HS-142-1 was found to eliminated the suppressive effects of ANP on aldosterone synthesis and secretion in adrenal glomerulosa cells (Oda et al., 1992). The ANP/ NPRA system increases cGMP concentrations, resulting in an activation of cAMP-dependent phosphodiesterases, which decreases both cAMP and aldosterone levels in adrenal glomerulosa cells (MacFarland et al., 1991; Levin et al., 1998; Pandey, 2005a).

Interestingly, adrenal renin content and renin mRNA along with Ang II and aldosterone levels were found to be increased in the adult *Npr1*$^{-/-}$ mice than with *Npr1*$^{+/+}$ control animals (Shi et al., 2001; Zhao et al., 2007). ANP-BNP/NPRA signaling opposes all the actions of Ang II in the phathophysioogical states (Pandey, 2005a, 2010). Our studies demonstrated that ablation of *Npr1* exhibits in chronic elevation of BP in mice fed a high-salt diet (Oliver et al., 1998; Zhao et al., 2007, 2013). On the other hand, adrenal Ang II and aldosterone concentrations were decreased in *Npr1* gene-duplicated animals kept on the high-salt diet; however, a low-salt diet increased the adrenal Ang II and aldosterone concentrations in *Npr1* mice in a gene-dose-dependent manner (Zhao et al., 2007, 2013). Previously, it was indicated that BP of *Npr1*$^{-/-}$ mice remained at higher levels and unchanged in response to high-salt diets (Lopez et al., 1995). Our findings indicated that NPRA signaling is protective against the effects of a high-salt diet in the kidneys and heart of *Npr1* gene-duplicated mice (Zhao et al., 2007, 2013).

(c) Cardiac Remodeling and Dysfunction

Plasma ANP and BNP levels are markedly elevated in the pathophysiological and clinical conditions of cardiac dysfunction and remodeling, including fibrosis, diastolic dysfunction, cardiac hypertrophy, pulmonary embolism, and CHF leading to severe conditions of CVDs (Vellaichamy et al., 2005a, 2007; Felker et al., 2006; Jaffe et al., 2006; Reinhart et al., 2006; See and de Lemos, 2006; Ellmers et al., 2007; Zhao et al., 2013; Rubattu and Volpe, 2014; Pandey, 2019). ANP and BNP exert their cardioprotective functions not only as the circulating peptide hormones but also as local autocrine and paracrine factors (Chen et al., 2019; Pandey, 2019). ANP-BNP/NPRA signaling serves cardiac protective role by various mechanisms (**Table 2**). The concentrations of ANP and BNP are increased proportion to

TABLE 2 | Effect of ANP-BNP/NPRA signaling in different cardiovascular target organs and tissues.

Tissues	Parameters	References
Heart	Increased fractional shortening Decreased heart weight/body ratio Decreased LVDS and LVDD Inhibition of cardiac hypertrophy Inhibition of fibrosis Inhibition of inflammation	Oliver et al., 1997; Ellmers et al., 2002; Vellaichamy et al., 2005a; Zhao et al., 2013; Vellaichamy et al., 2014; Subramanian et al., 2016
Kidney	Increased GFR and RBF Increased natriuresis/diuresis Decreased Na and water transport Decreased Na absorption Decreased solute concentration Inhibition of renin release Inhibition of hypertrophy Inhibition of remodeling Inhibition of fibrosis Inhibition of inflammation	de Bold et al., 1981; Burnett et al., 1984; Freeman et al., 1985; Obana et al., 1985; Sonnenberg et al., 1986; Light et al., 1989; Appel, 1990; Meyer and Forsmann, 1997; Shi et al., 2001; Shi et al., 2003; Das et al., 2010; Kumar et al., 2017; Gogulamudi et al., 2019; Das et al., 2020
Vasculature	Increased smooth muscle relaxation Increased endothelial permeability Decreased intravascular volume Inhibition of cell growth	Garcia et al., 1984; Abell et al., 1989; Itoh et al., 1990; Appel, 1990, 1992; Sharma et al., 2002; Sabrane et al., 2005; Sen et al., 2016; Arise et al., 2020
Adrenal gland	Inhibition of aldosterone release Inhibition of renin synthesis	Shi et al., 2001; Airhart et al., 2003

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; NPRA, natriuretic peptide receptor-A; LVDS, left ventricular dimension systolic; LVDD, left ventricular dimension diastolic; RBF, renal blood flow; GFR, glomerular filtration rate.

the severity of cardiac dysfunction and remolding in humans and experimental animal models (Nakao et al., 1996; Chen and Burnett, 1999; Reinhart et al., 2006; Ellmers et al., 2007; Pandey, 2008; Rubattu et al., 2019). The concentrations of ANP and BNP are also markedly elevated in the cardiac tissues of patients with CHF. In humans, both of these hormones appear to reduce the preload and afterload of the heart. The high plasma levels of ANP and BNP tend to predict cardiac events and CHF but BNP levels rise 10−25-fold higher than the fold increases in ANP concentrations (Mukoyama et al., 1991; Tsutamoto et al., 1993; Chen and Burnett, 1999; Felker et al., 2006; Reinhart et al., 2006; Pandey, 2019; Volpe et al., 2019a). *NPPA*, *NPPB*, and *NPR1* genes are overexpressed in hypertrophied failing heart, suggesting that the autocrine and/or paracrine effects of NPs predominate and acts endogenously to protect against maladaptive pathology of CVDs (Knowles et al., 2001; Vellaichamy et al., 2005a, 2007, 2014; Felker et al., 2006; Ellmers et al., 2007; Xue et al., 2008; Subramanian et al., 2016; Pandey, 2019; Volpe et al., 2019a,b).

The longer half-life of BNP has favored its diagnostic use over that of ANP to evaluate NPs as important indicator of CHF in patents with chest pain in emergency conditions (Reinhart et al., 2006; Rubattu and Volpe, 2014). The ventricular expression of *Nppa*, *Nppb*, and *Npr1* is more closely associated with local cardiac hypertrophic and fibrotic events than are plasma ANP levels and systemic BP (Vellaichamy et al., 2005a, 2007). Nevertheless, NT-pro-BNP seems to be a stronger predictor of cardiovascular risk (Doust et al., 2005; Khan et al., 2006; Girsen et al., 2007; Kocylowski et al., 2009). In hypertrophied and failing hearts, the expression of *Nppa* and *Nppb*, which serve as endogenous protective mechanisms against maladaptive cardiac events and dysfunction is markedly increased (Knowles et al., 2001; Zahabi et al., 2003; Vellaichamy et al., 2005a; Pandey, 2019; Rubattu et al., 2019). The alterations in *Nppa* promoter seem to be linked with cardiac hypertrophy and dysfunction (Deschepper et al., 2001). Mice lacking NPRA develop cardiac hypertrophy and fibrosis, independent of BP (Oliver et al., 1997; Nakanishi et al., 2005; Vellaichamy et al., 2005a, 2007, 2014; Ellmers et al., 2007; Scott et al., 2009; Zhao et al., 2013). Our studies have demonstrated that the global ablation of *Npr1* in mice triggers the expression of hypertrophic and fibrotic markers and matrix metalloproteinases (MMP-2, MMP-9) in the cardiac tissues (Vellaichamy et al., 2005a, 2007, 2014; Pandey, 2008, 2011; Subramanian et al., 2016). It has been indicated that ANP/NPRA signaling antagonizes Ang II-induced collagen systhesis via suppression and activation of MMP-2, MMP-9 (Parthasarathy et al., 2013).

The expression of Ca^{2+}-ATPase-2a (SERCA-2a) is progressively decreased and the level of cytosolic $Ca2^{+}$ is increased in the hypertrophied heart of *Npr1*$^{-/-}$ mice (Vellaichamy et al., 2005a; Pandey and Vellaichamy, 2010). ANP-BNP/NPRA signaling mechanism is known to decrease the cytosolic levels of $Ca2^{+}$ and inosital trisphosphate in different cells and tissues (Pandey, 2014). Further, both ACE 1 and AT_1R were found to be significantly increased leading to cardiac hypertrophy and fibrosis in *Npr1*$^{-/-}$ mice, but not *Npr1*$^{+/+}$ control mice (Vellaichamy et al., 2007). In failing hypertrophied hearts, ANP-BNP/NPRA signaling antagonizes Ang II- and AT_1R receptor-mediated cardiac dysfuction and remodeling (Li et al., 2002; Kilic et al., 2007; Vellaichamy et al., 2007; Zhao et al., 2013). The endothelial cell-specific arteries from *Npr1* gene-disrupted mice showed significant elevaion in systolic BP (Sabrane et al., 2005). The conditional inactivation of *Npr1* in cardiac myocytes, exhibited mild cardiac hypertrophy; however, ANP levels were markedly increased (Holtwick et al., 2003). Both ANP and BNP levels were elevated in the global *Npr1*$^{-/-}$ and haplotype *Npr1*$^{+/-}$ mice with myocardial infarction. These mice showed a higher incidence of CHF and significantly greater mortality rates than did wild-type mice (Nakanishi et al., 2005; Vellaichamy et al., 2005a, 2007). ANP-BNP/NPRA signaling provides cardiac protective mechanism against maladaptive cardiac disorders and remodeling of CVDs (**Figure 2**).

(d) Metabolic Syndrome and Diabetic Conditions

The growing evidence suggest that ANP-BNP/NPRA signaling regulates whole body metabolism and controls diabetic conditions (Wang et al., 2004; Wang T.J. et al., 2007; Moro, 2013; Coue et al., 2015). The previous studies have indicated that a decreased plasma levels of ANP might be associated with the obesity, metabolic syndrome, insulin resistance, energy balance, and glucose homeostasis in humans (Wang et al., 2004; Wang T.J. et al., 2007; Birkenfeld et al., 2008; Coue and Moro, 2016; Cannone et al., 2019). It has also been shown that NPs signaling activates the peroxisome proliferator-activated

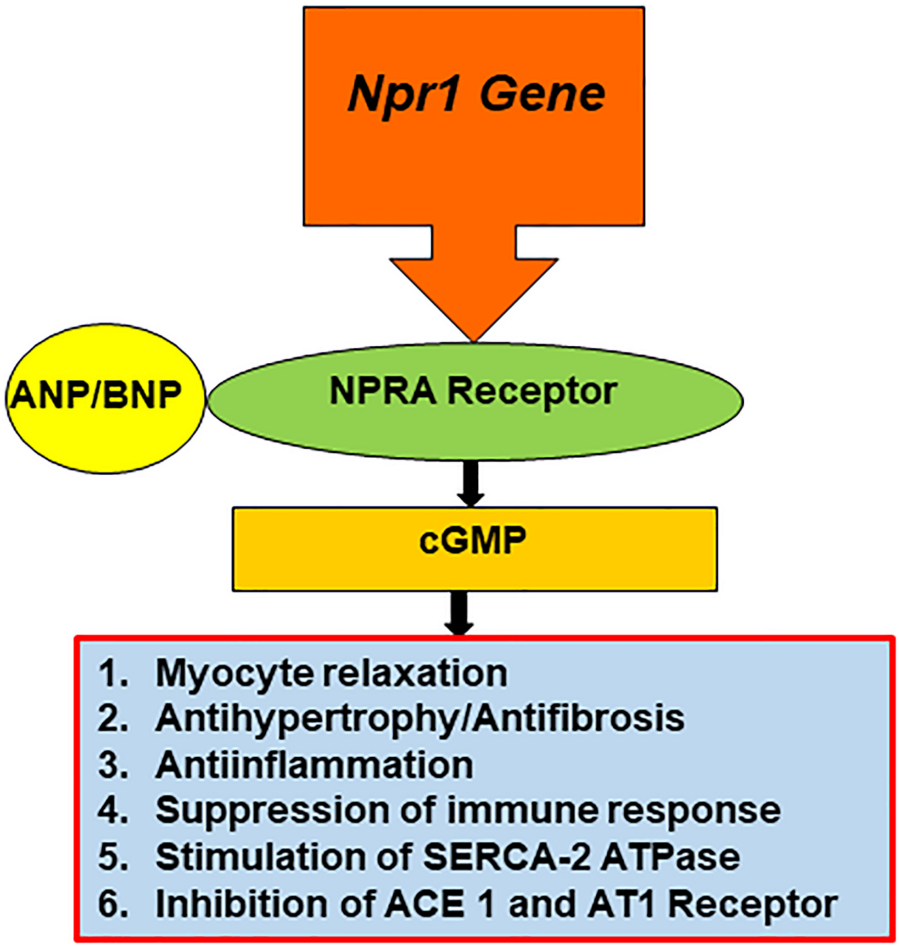

FIGURE 2 | The schematic representation of the NP-dependent activation and signaling of ANP/NPRA in the heart. To maintain the cardiac homeostasis, *Npr1* gene is transcribed and translated to produce mature NPRA protein. Specifically, NPRA is activated by ANP and BNP; in turn produces intracellular second messenger cGMP that activates various physiological responsiveness in the heart tissues and cells.

receptor-γ (PPAR-γ) and enhances the fat oxidation and mitochondrial biogenesis in the muscle tissues (Mitsuishi et al., 2008; Miyashita et al., 2009). The ANP/NPRA signaling also enhances the PPAR-γ coactivator-1α (PGC-1α), which stimulates oxidative phosphorylation (OXPHOS) in skeletal muscle tissues (Engeli et al., 2012). The previous studies have demonstrated that ANP/NPRA system accelerates lipid mobilization, mitochondrial oxidative metabolism, and fat oxidation (Birkenfeld et al., 2008, 2012; Tsukamoto et al., 2009). In fact, ANP/NPRA action modulates energy expenditure and supply of fatty acids for cardiac and skeletal muscle metabolic utilization (Engeli et al., 2012; Schlueter et al., 2014).

Furthermore, ANP/NPRA signaling seems to regulate obesity, type 2 diabetes, and resistance to insulin (Moro, 2013; Coue et al., 2015). On the other hand, previous studies have indicated that NPRC exhibits lipolytic effect of NPs in mouse adipose tissues (Sengenes et al., 2002; Bordicchia et al., 2012). Mice challenged to the low temperature environment showed stimulated release of ANP, but exhibited a reduced level of NPRC in both brown and white adipocytes (Bordicchia et al., 2012). Interestingly, ANP has been shown to enhance the browning of human white adipose tissues (Miyashita et al., 2009; Enerback, 2010). It has been suggested that insulin enhances the expression of NPRC in adipocytes in a glucose-dependent manner (Bordicchia et al., 2016). It is believed that a defective ANP-BNP/NPRA signaling might promote a maladaptive metabolic disorders, which could trigger lipid accumulation, decreased mitochondrial function, hyperglycemia, and insulin resistance leading to hypertension and CVDs in humans. It is believed that an increased circulating concentrations of ANP might serve as protective mechanisms, nevertheless, the human subjects having low circulating levels of ANP might have a greater risk of cardiometabolic syndrome (Pereira et al., 2015).

(e) Immunogenic Responses and Cardiovascular Disorders

Proinflammatory cytokines play a central role in the development of hypertension, cardiac hypertrophy, and CHF in experimental animal models and in humans (Hirota et al., 1995; Testa et al., 1996; Vanderheyden et al., 2005; Vellaichamy et al., 2005a, 2014; Das et al., 2010, 2020; Subramanian et al., 2016; Gogulamudi et al., 2019). Elevated circulating and myocardial

levels of proinflammatory cytokines, including tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) have been reported in the plasma of cardiomyopathic patients, correlating with the severity of their disease (Testa et al., 1996; Vanderheyden et al., 2005). Myocardial cells are capable of producing substantial amounts of proinflammatory cytokines in response to ischemia and experimental load-induced stress (Baumgarten et al., 2002; Palmieri et al., 2002). Inappropriate activation of TNF-α, IL-1β, and IL-6 has been shown to induce phenotypic changes in CVDs, encompassing the myocyte hypertrophy, myocardial apoptosis, extracellular matrix deposition, and contractile dysfunction (Thaik et al., 1995; Sekiguchi et al., 2004; Vellaichamy et al., 2005a, 2014; Subramanian et al., 2016).

Investigations in our laboratory have indicated that the ANP-BNP/NPRA system acts as a negative regulator of inflammation and hypertrophic growth in the kidneys and heart (Vellaichamy et al., 2005a,b, 2007, 2014; Das et al., 2010, 2020; Subramanian et al., 2016; Kumar et al., 2017). ANP inhibited TNF-α production in interferon-gamma (IFN-γ)-activated macrophages and blocked TNF-α-induced adhesion molecule expression in endothelial cells (Tsukagoshi et al., 2001; Vollmer, 2005). We have used *Npr1* gene-knockout and gene-duplicated mouse models in efforts to determine whether genetically determined differences in *Npr1* expression changes the levels of cardiac proinflammatory cytokines (Zhao et al., 2013; Vellaichamy et al., 2014; Subramanian et al., 2016). Our findings suggested that ablation of *Npr1* triggered a sustained activation of proinflammatory cytokines gene expression and protein levels associated with exaggerated ventricular remodeling and cardiac hypertrophy leading to CVDs. However, gene-duplication of *Npr1* in mice attenuated cardiac proinflammatory cytokines expression and protected against cardiac remodeling (Vellaichamy et al., 2005a, 2007, 2014; Subramanian et al., 2016).

Increased expression of TNF-α, IL-6, TGF-β, LT-β, and IFN-γ mRNA and protein levels were found in the hearts of adult $Npr1^{-/-}$ mice as compared with the hearts of adult $Npr1^{+/+}$ mice (Vellaichamy et al., 2014). In parallel, cytokine receptor protein levels were also increased in the heart of null mutant mice indicating increased expression of gb-130, TNF-α receptor 1 (TNF-αR1), and TNF-β receptor 1 (TGF-βR1). In contrast, significant decreases in IL-6, TNF-α, and TGF-β1 cytokine protein levels were found in the gene-duplicated hearts of $Npr1^{++/++}$ mice as compared with the hearts of wild-type mice.

The activated ANP-BNP/NPRA system may serve a protective function by inhibiting the ventricular expression of proinflammatory cytokines in CVDs (Vellaichamy et al., 2005a,b, 2014). Some previous observations advanced the notion that disruption of the ANP/NPRA/cGMP signaling pathway can augment activation of proinflammatory cytokines leading to extracellular matrix remodeling, pathological hypertrophy, and CHF. Expression of myocardial proinflammatory cytokine genes, including TNF-α, IL-1β, and IL-6 was found to be significantly higher in patients with compensated CHF condition, along with increased cytokine gene expression, which could play an adaptive role in left ventricular remodeling and other CVDs (Oral et al., 2003; Vanderheyden et al., 2005). It has been shown that cardiac-specific over expression of proinflammatory cytokines in mouse hearts leads to cardiac hypertrophy and ventricular disorders similar to those in human heart disease, indicating that proinflammatory cytokines are critically involved in the cardiac remodeling process in CVDs (Hirota et al., 1995; Krown et al., 1996; Oral et al., 2003; Vellaichamy et al., 2005a, 2014; Subramanian et al., 2016). Blockade of the action of proinflammatory cytokines has been shown to prevent hypertension, cardiac hypertrophy, and diastolic dysfunction in experimental animal models (Villarreal and Dillmann, 1992; Krown et al., 1996; Isono et al., 1998; Kurrelmeyer et al., 2000; Li et al., 2000; Vellaichamy et al., 2005a, 2007).

Evidence suggests that the ANP-BNP/NPRA system has antiinflammatory activity, and could inhibit the bacterial toxin (LPS)- and IFN- gamma- induced expression of proinflammatory cytokines and nuclear factor-kappa B (NF-κB) in macrophages (Tsukagoshi et al., 2001; Vollmer, 2005). Ablation of *Npr1* also enhances the expression and activation of transcription factors, NF-κB and activating protein-1 (AP-1), which seem to be associated with cardiac hypertrophy, fibrosis, and extracellular matrix remodeling (Vellaichamy et al., 2005a, 2007, 2014). *Npr1* gene-disruption activates NF-κB and leading to cardiac remodeling and hypertrophic conditions (**Figure 3**). A significant increases in mRNA expression and protein levels of NF-κB and IKK-β isoforms were observed in the hearts and kidneys of $Npr1^{-/-}$ and $Npr1^{+/-}$ mice as compared with $Npr1^{+/+}$mice, suggesting that the NF-κB signaling pathway is activated in the hearts and kidneys of mutant mice (Vellaichamy et al., 2005a, 2014; Subramanian et al., 2016). The increased NF-κB binding activity was positively correlated with increased expression of proinflammatory cytokine genes in *Npr1* null mutant mice with initiation and development of CVDs (Vellaichamy et al., 2005a, 2014; Subramanian et al., 2016).

Previous studies indicated that the activation of NF-κB and proinflammatory cytokines serve as a causal event in the development of cardiac hypertrophy and fibrosis (Frantz et al., 2003; Purcell and Molkentin, 2003; Li et al., 2004; Vellaichamy et al., 2005a; Subramanian et al., 2016). The sustained activation of proinflammatory cytokines contributes to the pathological forms of ventricular remodeling and exaggerating the CVDs in experimental animals (Leitman et al., 1988; Hirota et al., 1995; Baumgarten et al., 2002; Palmieri et al., 2002; Vellaichamy et al., 2005a, 2014; Zhao et al., 2013). Our findings suggested that enhanced ANP-BNP/NPRA signaling can protect the heart by inhibiting ventricular expression of NF-κB, a master regulator of proinflammatory cytokines in relation to increasing *Npr1* gene copies (Vellaichamy et al., 2014; Subramanian et al., 2016).

PERSPECTIVES

In the past four decades, a large body of research has provided a unique perspective on the biochemical, cellular, molecular, genetic, and clinical aspects of NPs and their receptors in relation to CVDs. The physiological and pathophysiological roles

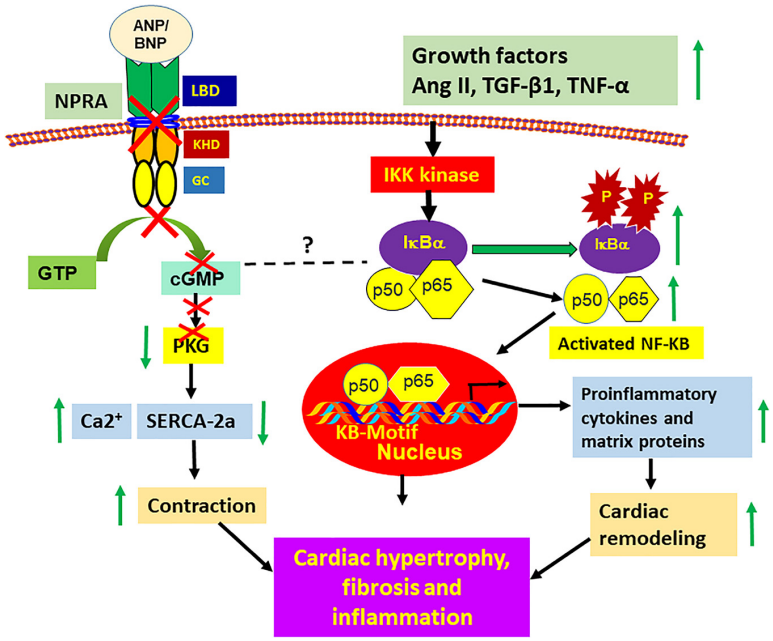

FIGURE 3 | Ablation of *Npr1* triggers the activation of proinflammatory pathways that promotes cardiac hypertrophy, fibrosis, and remodeling. Disruption of ANP/NPRA signaling causes unbalanced activation of master transcription factor NF-κB that initiates the expression of proinflammatory cytokines and matrix proteins; thereby promotes specific cardiac structural and molecular changes in *Npr1*$^{-/-}$ mice heart. The activated NF-κB translocates into the nucleus and promotes the genes transcription and expression of various proinflammatory cytokines and extracellular matrix proteins, including MMP-2 and MMP-9. Increased activation of NF-κB, thereby promotes medial thickening and perivascular fibrosis, in turn leads to the cardiac hypertrophy, fibrosis, remodeling and heart failure. On the other hand, the absence of ANP/NPRA signaling also simultaneously inhibits SERCA-2a that increases the cytosolic $Ca2^{+}$ levels, thereby results in the increased contractile activity of the heart, which also promotes cardiac hypertrophy and remodeling. Ang II, angiotensin II; TGF-β1, transforming growth factor-β1; TNF-α, tumor necrosis factor-α; IKK, inhibitory kappa kinase; IKB, inhibitory kappa B, NF-κB, nuclear factor-kappa B; PKG, cGMP-dependent protein kinase; SERCA-2a, sarcolemmal endoplasmic reticulum $Ca2^{+}$-ATPase-2a; MMP-2 and -9, matrix metalloproteinase-2 and −9.

of ANP-BNP/NPRA signaling are implicated with protective mechanisms in various organ systems, including the heart, kidneys, lungs, central nervous system, gonads, adrenal glands, and vasculature. Cardiac hormones, ANP and BNP are considered to be diagnostic markers of CHF, but we need to determine their therapeutic potentials for the treatment and prevention of CVDs such as hypertension, renal insufficiency, cardiac hypertrophy, CHF, and stroke. Intraperitoneal delivery of recombinant ANP (Carperitide) facilitated the recovery of increased blood flow in ischemic conditions and exerted antihypertrophic and antifibrotic effects (Suwa et al., 2005; Park et al., 2008; Nishikimi et al., 2013). Recombinant BNP (Nesiritide) exhibited natriuretic and vasodilatory action in CHF patients (Colucci et al., 2000). In *Npr1*$^{-/-}$ gene ablated mice, impaired recovery of blood flow after hand-limb ischemia was significantly inhibited by both ANP and BNP (Tokudome et al., 2009). Genetic molecular approaches have delineated of the functions of NPRA by decreasing and/or increasing *Npr1* gene-copies (gene-knockout and/or gene-duplication) in mice by genetically altering protein product levels. It has been found that common genetic variants of *NPPA*, *NPPB*, and *NPR1* are associated with circulating ANP and BNP levels and BP, contributing individual variations in the regulation of BP and CVD risk factors (Wang, 2018; Reginauld et al., 2019).

The strategy of enhancing endogenous NPs and their protective roles in CHF is achieved with the drug sacubitril/valsartan, which augments circulating ANP and BNP by inhibiting of neprilysin that degrades NPs (McMurray et al., 2014). This drug combines dual properties; inhibiting both neprilysin and Ang II type 1 receptor (AT_1R) blocker, which is also referred to as Ang II receptor-neprilysin inhibitor (ARNi). Enhancing endogenous ANP-BNP/NPRA signaling has proven to be critical in the first line of therapeutic targets for hypertension, cardiac dysfunction, and CHF in decades (McMurray et al., 2014).

The apparent action of ANP-BNP/NPRA system in antagonizing the RAAS, there seem to be immense potential in using the NPs as a novel therapeutic axis in treating hypertension, renal inefficiencies, and CVDs. The clinical trials have suggested both the benefits and risks of using the synthetic ANP (anaritide and carperitide) and BNP (nesiritide) for the treatment of hypertension, renal diseases and CVDs. Unfortunately, synthetic NPs as a drug have not yet been recommended for the therapeutic treatments in the United States (Kuhn et al., 2009; Saito, 2010; Hayek and Nemer, 2011; Dohi and Ito, 2014; McMurray et al., 2014). However, the success of the drug sacubitril/valsartan in treating the CHF has heightened the enthusiasm and interest in NPs as a therapeutic target for hypertension, renal diseases, and CVDs. This drug combines an inhibitor of the NPs degrading

enzyme neprilysin (sacubitril) with an antagonist of Ang II receptor 1 (AT_1R) (Valsartan), which lower BP and pulse pressure (Vellaichamy et al., 2005a; Ruilope et al., 2010; Williams et al., 2017). Interestingly, the new chimeric natriuretic peptide-based therapies have a great potential in treating hypertension and CVDs. Furthermore, molecular mimicking of the action of BP-lowering Npr1 expression and receptor signaling could be effective in identifying the novel mechanisms for the new therapeutic treatment strategies.

Future studies are expected to lead a better understanding of the genetic and molecular basis of the ANP-BNP/NPRA system in regulating CVDs, including high BP, stroke, CHF, and neurological disorders. The results of future investigations of NPs and their receptors should certainly help resolve the complexities of CVDs. These future studies need to be designed to elucidate the genetic and molecular basis of *Npr1* function in both normal and disease states. Both ongoing and future clinical studies will be needed to identify more functionally significant markers of *NPPA, NPPB*, and *NPR1* variants in a larger human population. The chimeric designer peptides of ANP, BNP, and DNP have opened new avenues for studies of CHF, cardiac disorders, and remodeling therapy. The future progress in this area of research should significantly strengthen and advance our understanding of the genetic and molecular approaches used to evaluate diverse pathophysiological conditions in CVDs. We need to expand the potential clinical implications of ongoing investigations on the next generation personalized medicine and pharmacogenomics of NPs and their receptors that are currently in progress. The resulting knowledge will yield novel therapeutic targets and new treatment strategies for CVDs.

AUTHOR CONTRIBUTIONS

KP wrote the manuscript and complied information and references.

FUNDING

The research works in the author's laboratory were supported by the National Institutes of Health grant (HL-062147), Tulane Carol Levin Bernick Research award, and Tulane ByWater Fellowship grant award.

ACKNOWLEDGMENTS

The author sincerely acknowledges past and present members of his laboratory who participated in research related to this review. The author also thanks Mrs. Kamala Pandey for her assistance in the preparation of this manuscript.

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frontiers in Physiology

REVIEW
published: 23 August 2021
doi: 10.3389/fphys.2021.652163

Sacubitril/Valsartan as a Therapeutic Tool Across the Range of Heart Failure Phenotypes and Ejection Fraction Spectrum

Giovanna Gallo, Massimo Volpe, Allegra Battistoni, Domitilla Russo, Giuliano Tocci and Maria Beatrice Musumeci*

Cardiology Unit, Department of Clinical and Molecular Medicine, Faculty of Medicine and Psychology, Sant'Andrea Hospital, Sapienza University of Rome, Rome, Italy

Heart failure (HF) is a complex syndrome caused by a variety of structural or functional cardiac abnormalities as a consequence of several involved pathophysiological pathways. In the last decades, left ventricular ejection fraction (LVEF) has represented the principal criterion used to stratify HF, to interpret ventricular function and to identify therapeutic strategies. However, this chimeric parameter oversimplifies the multiple pathways and mechanisms underlying the progression of HF. Indeed, HF should be more appropriately considered as the final stage of multiple disease states, characterized by distinct phenotypes on the basis of key clinical and molecular variables, such as underlying etiologies and conditions, demographic and structural features and specific biomarkers. Accordingly, HF should be viewed as a continuous spectrum in which the specific phenotypes need to be accurately identified with the aim to improve the disease management with a more tailored approach. In such a complex and heterogeneous scenario, the clinical benefits of an angiotensin receptor neprilysin inhibition strategy, namely in the single pill sacubitril/valsartan (S/V), have been shown across the entire HF continuum, representing a fundamental therapeutic strategy, although with different magnitudes depending on the severity and the stage of the clinical syndrome. In this viewpoint paper we have reconsidered the role of S/V in the light of different HF phenotypes and on the basis of HF considered as a whole spectrum.

Keywords: heart failure, left ventricular ejection fraction continuous spectrum, heart failure phenotypes, sacubitril/valsartan, ARNI

OPEN ACCESS

Edited by:
Rainer Schulz,
University of Giessen, Germany

Reviewed by:
Klaus-Dieter Schlüter,
University of Giessen, Germany
Sang-Bing Ong,
The Chinese University of Hong Kong, China

****Correspondence:***
Massimo Volpe
massimo.volpe@uniroma1.it

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *11 January 2021*
Accepted: *05 August 2021*
Published: *23 August 2021*

Citation:
Gallo G, Volpe M, Battistoni A, Russo D, Tocci G and Musumeci MB (2021) Sacubitril/Valsartan as a Therapeutic Tool Across the Range of Heart Failure Phenotypes and Ejection Fraction Spectrum. Front. Physiol. 12:652163. doi: 10.3389/fphys.2021.652163

INTRODUCTION

The current approach to clinical investigation in heart failure (HF) has been substantially focused on the left ventricular ejection fraction (LVEF) viewed as a dichotomous variable to dissect the disease phenotype expression with two distinct categories, namely HF with reduced EF (HFrEF, LVEF < 40%) and HF with preserved EF (HFpEF, LVEF > 50%) (Metra and Teerlink, 2017). This gross subdivision has been instrumental to a simplified approach in randomized controlled trials designed to identify whether a certain pharmacological or non-pharmacological treatment of HF would be beneficial. However, this simplistic approach to the biologic complexity of HF has

two major drawbacks: (1) EF is not a categorical dichotomic variable, but rather is a continuous variable which reflects the cardiac pumping properties which, in turn, is the final result of other important factors (inotropism, ventricular synergic contraction, heart rate, pre-load and after-load, ventricular interdependence, myocardial bioenergetics and active myocyte recruitment); (2) while HFrEF mostly reflects its major etiology, i.e., ischemic heart disease, HFpEF is a "garden variety" condition which resembles multiple and very different conditions (aging HF, hypertension mediated LV hypertrophy, diabetic cardiomyopathy, infiltrative cardiomyopathy) which cannot be viewed as a unique disease. Furthermore, it appears not reasonable or biologically plausible to consider patients ranging from 45 to 65% as a whole unique group. In fact, recent European Society of Cardiology Guidelines identified a group with EF ranging from 40 to 49% as HF with mid-range EF (HFmrEF) (Ponikowski et al., 2016).

In this viewpoint paper we have reconsidered the role of sacubitril/valsartan (S/V), an angiotensin receptor blocker/neprilysin inhibitor (ARNI) in the light of different HF phenotypes and on the basis of HF considered as a whole spectrum.

Heart Failure: From Pathophysiology to Clinical Phenotypes Across a Continuous Spectrum

HF is a progressive syndrome characterized by complex pathophysiological pathways and caused by a variety of structural or functional cardiac abnormalities that lead to elevated intracardiac pressures or to a reduced cardiac output at rest or during stress (Braunwald, 2013).

Cardiac injury with loss of myocyte cells, increased myocardial strain, fibrosis, progressive LV dilatation, change in ventricular shape resulting in cardiac remodeling, leading to increased myocardial oxygen consumption and reduced efficiency of myocardial contraction and arrhythmias are more frequently involved in the development of systolic dysfunction and failure of the pumping properties of the heart (Katz and Rolett, 2016).

On the other hand, vasoconstriction, pro-inflammatory and pro-thrombotic states contribute to impaired ventricular and active atrial relaxation and filling capacities, increased cardiomyocyte passive stiffness, reduced arterial compliance and abnormal ventricular-arterial coupling which finally lead to LV diastolic dysfunction as a final expression (Packer et al., 2020).

Although the above-mentioned pathophysiological mechanisms are frequently shared by the different HF clinical presentations, it may be reasonable to consider HF as the final stage of multiple disease states. A precise determination of distinct phenotypes on the basis of key clinical and molecular variables may help to identify d a more tailored approach rather than a "one-size-fits-all" management strategy (Johnson et al., 2017). Demographic characteristics (such as age, sex, comorbidities, and ethnicity), structural features (such as ventricular wall thickness and cardiac chambers dimensions and compliance) and specific biomarkers of different pathophysiological pathways (such as myocardial stretch, fibrosis, and injury or cardiorenal syndrome) should be combined to improve phenotypic classification and accurately stratify HF patients (Francis et al., 2014; **Table 1**).

Indeed, for too long HF has been arbitrarily stratified only according to LVEF, a criterion chosen mostly to reflect binomial pathophysiological categories, though obviously oversimplifying the multiple pathways and mechanisms underlying the progression of HF. The level of LVEF has been extensively used to provide a simple clinical marker with a high prognostic predictive value, a numerical indicator for therapeutic decision making and finally to distinguish the two main phenotypes (HFrEF and HFpEF) (Triposkiadis et al., 2019). This simple, though not comprehensive, parameter has driven most of evidence based medicine and clinical trials in HF for the last 30 years. On the positive side, this has generated key information for the contemporary management of HF. On the other side, the nature of the information derived from a mere stratification and classification of patients on the basis of EF has probably prevented a more precise therapeutic approach target of the different pathophysiological phenotypes underlying HF in each single individual.

Indeed, the biological basis of a physiological variable together with a growing body of literature speak against the distinction of HF into categories based on the LVEF values and in turn support the hypothesis that HF should be rather viewed as a continuous variable reflecting the whole spectrum of the properties of the LV. Each phenotype and disease trajectory depends on demographic features and risk factors, etiology, functional and structural changes of the heart and therapeutic strategies, which include the potential bidirectional LVEF transition through the recognized classes (De Keulenaer and Brutsaert, 2011; Triposkiadis et al., 2016; Konstam and Abboud, 2017; Lupón et al., 2018). Thus, a dichotomous vision of clinical presentations of HF cannot be adopted anymore in clinical practice to define borders and boundaries of patient classification.

Indeed, although EF remains a key parameter to interpret LV function, it has the disadvantage of being a chimeric index which does not sufficiently represent the major determinants of systolic function including inotropy, lusitropy, preload and afterload, heart rate, and LV synchrony.

Moreover, several studies have demonstrated that systolic and diastolic dysfunction, coronary microvascular dysfunction, endothelial inflammation, oxidative stress, fibrosis, and cardiomyocyte loss may coexist independent of LVEF (Tan et al., 2009; DeVore et al., 2017; von Roeder et al., 2017; Camici et al., 2020). In addition, the detrimental upregulation of renin-angiotensin-aldosterone system (RAAS) has been described in both HF categories although with different degrees of over-activation (Braunwald, 2013; Katz and Rolett, 2016; Ponikowski et al., 2016; Metra and Teerlink, 2017). On the other hand, the natriuretic peptide (NP) system, which may counterbalance the increase of sodium and water reabsorption and the increased vasoconstriction caused by RAAS and SNS, is enhanced across the HF spectrum and may contribute to reduce cardiac hypertrophy and inflammation (McMurray, 2015).

Therefore, it is not surprising that pharmacological strategies able to block the neurohormonal activation, such

TABLE 1 | Association between main HF etiologies and LVEF phenotypes.

Common HF etiologies	LVEF	Phenotypes
Coronary artery disease	HFrEF, HFmrEF, less commonly HFpEF	Remodeling characterized by wall thinning and dilatation, fibrosis, and scar; regional wall motion abnormalities. Subendocardial or transmural LGE distribution.
Hypertension	HFpEF, less commonly HFrEF and HFmrEF	Concentric or eccentric hypertrophy. Diastolic dysfunction with increased left filling pressure. Reduced GLS.
Diabetes mellitus	HFpEF, HFrEF, and HFmrEF (particularly in the presence of coronary artery disease)	Thicker LV walls, small indexed LV end-diastolic and end-systolic volumes, high E/e′ ratio, abnormal LV geometry. Endothelial dysfunction, coronary disease, increased fibrosis and deposition of advanced glycation end products.
Aortic stenosis	HFmrEF, HFpEF, less commonly HFrEF	Concentric hypertrophy, impaired LV myocardial deformation, impaired flow reserve, myocardial fibrosis.
Mitral regurgitation	HFrEF, HFmrEF, HFpEF	Increased LA volume and LV preload, eccentric hypertrophy, reduced forward stroke volume, marked LA pressure elevation, pulmonary hypertension.
Idiopathic dilated cardiomyopathy	HFrEF and HFmrEF	LV and/or RV dilatation and dysfunction, diastolic dysfunction with increased left filling pressure.
Hypertrophic cardiomyopathy	More commonly HFpEF	Asymmetric LV hypertrophy, diastolic dysfunction with increased left filling pressure, left atrial dilatation, systolic anterior movement of mitral leaflets with mitral regurgitation. Multi-pattern LGE distribution.
Infection (e.g., myocarditis, Chagas) and systemic immune-mediated disease	More commonly HFrEF and HFmrEF	LV dilatation and systolic dysfunction. Specific immune cell infiltration, myocardial inflammation or diffuse myocardial fibrosis. Subepicardial LGE distribution.
Drugs (e.g., chemotherapy) and toxic agents (e.g., alcohol, cocaine, steroids)	More commonly HFrEF and HFmrEF	LV dilatation and systolic dysfunction, impaired longitudinal and circumferential strain.
Infiltrative myocardial diseases (amyloidosis, hemochromatosis)	More commonly HFpEF	Granular appearance on echocardiography, LV symmetric hypertrophy. RV hypertrophy, increased thickness of the atrio-ventricular valves, thickening of the interatrial septum, small pericardial effusion, restrictive filling Doppler pattern. Diffuse subendocardial LGE distribution.
Chronic kidney disease	HFpEF, HFrEF, and HFmrEF particularly in the presence of coronary artery disease	LV hypertrophy with LV stiffness, diastolic dysfunction with increased left filling pressure, reduced GLS.

GLS, global longitudinal strain; HFmrEF, heart failure with mid-range ejection fraction; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; LGE, late gadolinium enhancement; LV, left ventricle; RV, right ventricle.

as inhibitors of the angiotensin converting enzyme (ACEi), angiotensin receptor blockers (ARBs), and mineralocorticoid receptor antagonists (MRA) associated to significant benefits in cardiovascular outcomes (cardiovascular death and re-hospitalization for HF) in HFrEF, repeatedly showed evident trends toward a reduction in morbidity and mortality in other HF subsets characterized by a preserved LVEF (Ponikowski et al., 2016).

In a recent meta-analysis, conducted on 30,882 patients with HFpEF, we showed that treatments based on neurohormonal inhibitors significantly reduce the risk of the primary composite outcome of mortality and hospitalizations for HF and the secondary analysis of HF hospitalizations, without reaching significant benefits on the separate end-point of mortality (Gallo et al., 2020).

On the basis of these pathophysiological considerations, the entire HF spectrum could be better reflected by integrating LVEF with clinical phenotypes.

In this view, the inhibition of the involved neurohormonal systems may represent a fundamental therapeutic strategy through the entire LVEF continuum, although with different magnitudes depending on the severity and the stage of the clinical syndrome.

Implications of the Unique Mechanism of Action of Sacubitril/Valsartan

As previously mentioned, the pathophysiology of HF is complex, involving the activation of different neuro-hormonal systems such as RAAS. A potential counterbalancing mechanism, the natriuretic peptides (NPs), is also activated in response to increased myocardial wall stress, volume or pressure overload. These latter peptides have recognized diuretic, natriuretic, vasorelaxant, anti-proliferative and anti-hypertrophic properties, and modulate the RAAS (Volpe et al., 2014). However, their role in HF is apparently overridden by the vasoconstriction and sodium retaining actions of RAAS. The action of NPs is mediated through their cell membrane receptors, which are coupled to the particulate guanylate cyclase-cyclic guanosine monophosphate (cGMP) intracellular signaling (Packer et al., 2015). The activation of the effector molecule, protein kinase G (PKG), is linked to the main cardio-protective effects of NPs which potentially inhibit inflammation and leukocyte

recruitment, smooth muscle proliferation, vasoconstriction, coronary microvascular impairment, platelet aggregation, fibrosis and hypertrophy. NP cleavage is mostly catalyzed by the neutral endopeptidase neprilysin (NEP) (Bayes-Genis et al., 2016). On the basis of a conceptually meaningful role of NPs catabolism and the theoretically protective significance of its inhibition, development of drugs inhibiting NEP was considered a new attractive strategy to contrast HF development.

The NEP inhibitors were in fact developed to prevent NP degradation and the consequent alteration of the balance between the RAAS and NP system. Since NEP is not specific for NP catabolism and is involved in the degradation of other biological active peptides, such as angiotensin II (Ang II), NEP inhibition alone increases NPs levels, but this effect may be offset by a concomitant rise of Ang II and other peptides. Hence, the concomitant selective blockade of Ang II receptors with an ARB prevents the potential effect of excess Ang II, whereas combining an NEP inhibitor with an ACE-i has been shown to cause unacceptably high rates of angioedema since both NEP and ACE contribute to breakdown of bradykinin (Packer et al., 2002). The composite drug S/V may also reduce the degradation of vasodilator peptides such as bradykinin, substance P, C-type natriuretic peptides, enkephalins, and adrenomedullin resulting in a complex neurohormonal modulation with potentially greater beneficial effects compared with those limited the RAAS inhibition alone (Packer et al., 2002; Volpe et al., 2015; Bayes-Genis et al., 2016; Muiesan et al., 2017). Indeed, urinary cGMP has been found to be elevated after treatment with S/V suggesting that inhibition of NEP together with Ang II receptor blockade may promote the effective binding of NPs to guanylate cyclase-coupled receptors.

The combination of the concomitant inhibition of type 1 Ang II receptor (AT1R) and of NEP achieved with S/V results in a synergistic effect. The AT1R blockade reduces the signal transduction pathways mediated by Gq/11-proteins activating the Ca^{2+} signal, by numerous tyrosine phosphorylated proteins, including the JAK kinase family (JAK2 and Tyk2), and by the phosphokinase- C (PKC)-mediated system, responsible of cellular proliferation, hypertrophy and fibrosis. The increase in NPs levels also produces favorable biological effects mediated by the soluble guanylyl cyclase (sGC)/cGMP pathway (Packer et al., 2002; Volpe et al., 2015; Bayes-Genis et al., 2016; Muiesan et al., 2017).

The Role of Sacubitril/Valsartan Across LVEF Spectrum

In this complex and heterogeneous scenario, the proposed role of S/V has been tested through the entire HF continuum generating non-univocal data throughout different phenotypes in terms of clinical benefits. The effects on cardiovascular outcomes of S/V have been investigated in a large proof-of-concept study, the PARADIGM-HF (Prospective Comparison of ARNI with ACEI to Determine Impact on Global Mortality and Morbidity in Heart Failure) (McMurray et al., 2014). This double-blind trial randomized 8,442 patients with HFrEF and NYHA (New York Hear Association) class from II to IV to receive S/V or the largely used and validated ACEi enalapril in addition to standard treatment. The trial was stopped before the prespecified term due to outstanding beneficial results obtained in the S/V group. S/V was demonstrated to reduce the risk of the composite primary outcome of cardiovascular death or hospitalization for HF by 20% [hazard ratio 0.80; 95% confidence interval (CI), 0.73–0.87; $P < 0.001$] compared to enalapril. Considering the components of the primary end-point separately, the rate of death from cardiovascular causes and of hospitalization for HF was, respectively, 20 and 21% lower in the group treated with S/V (McMurray et al., 2014). In a *post hoc* analysis of the study, however, S/V maintained its efficacy across the EF spectrum, remaining worthwhile also in patients with a poorer prognosis related to a progressive decline of ventricular function (Solomon et al., 2016a).

Patients treated with lower dosages of S/V had a higher risk of CV events compared to those who maintained the maximal recommended dose of 200 mg twice daily, suggesting a proportional relationship between drug concentrations and achieved benefits (Vardeny et al., 2016).

Subgroup analysis of PARADIGM-HF demonstrated that the benefits of S/V over enalapril were consistent across etiologic categories (infective/viral, alcoholic, valvular, drug-related, peripartum–related), history and timing of previous HF hospitalizations, age categories (also in patients aged $\geq$ 75 years, although with a higher incidence of hypotension, renal impairment and hyperkalaemia), baseline risk estimated using the MAGGIC (Meta-Analysis Global Group in Chronic Heart Failure) and EMPHASIS-HF (Eplerenone in Mild Patients Hospitalization and Survival Study in Heart Failure) risk scores, the number of signs of congestion (jugular venous distention, edema, rales, and third heart sound) and Charlson co-morbidity index (Jhund et al., 2015; Simpson et al., 2015; Solomon et al., 2016b; Rodil Fraile et al., 2018; Balmforth et al., 2019; Selvaraj et al., 2019). Furthermore, efficacy of S/V was independent from background medications including diuretics, digitalis glycoside and MRA (Okumura et al., 2016).

In this view, S/V may represent a reasonable therapeutic strategy across different HF phenotypes influenced by several determinants, including but not being limited to LVEF.

On the wave of these exciting results, the benefits of a S/V-based therapeutic strategy based were also investigated in 4,822 patients with LVEF of 45% or higher, randomly assigned to S/V or valsartan in the PARAGON-HF (Angiotensin-Neprilysin Inhibition in Heart Failure with Preserved Ejection Fraction) trial (Solomon et al., 2019). Although with a promising trend toward significance, S/V did not reach the statistical power in the overall study population in the reduction of the composite primary outcome of cardiovascular death and total hospitalization for HF (Solomon et al., 2019). Regarding the secondary end-points, a significant improvement of functional status (NYHA class) and of quality of life (Kansas City Cardiomyopathy Questionnaire) was shown in patients treated with S/V compared to valsartan alone (Solomon et al., 2019). Several mechanisms have been proposed to explain the apparently different results obtained in these two above-mentioned studies. First of all, HFpEF represents a complex and heterogeneous phenotypic set,

including patients with several different comorbidities (such as diabetes, atrial fibrillation, chronic kidney disease) and with different clinical characteristics, such as those subjects with wild-type transthyretin (TTR) amyloidosis (Mohammed et al., 2014; Russo et al., 2020) who often remain underdiagnosed or misdiagnosed, being inappropriately enrolled in clinical trials and showing an unsatisfactory response to neurohormonal inhibitors (Maurer et al., 2018). Another explanation has been related to the limited sample and follow-up duration of PARAGON-HF, speculating that a larger trial with a more prolonged observational period might have been able to show significant advantages in HFpEF. Moreover, it has been proposed that the less impressive benefits derived from targeting neprilysin in HFpEF may be a consequence of the lower measured circulating levels of the biological NPs substrate in this subset. A pooled analysis of both trials, including an overall sample of 13,195 subjects (8,399 from PARADIGM-HF and 4,796 from PARAGON-HF) was recently performed aimed to investigate the efficacy of S/V across the ejection fraction spectrum (Solomon et al., 2020). In the overall population, S/V was associated to a significant reduction of the combined end-point of cardiovascular death and first hospitalization for

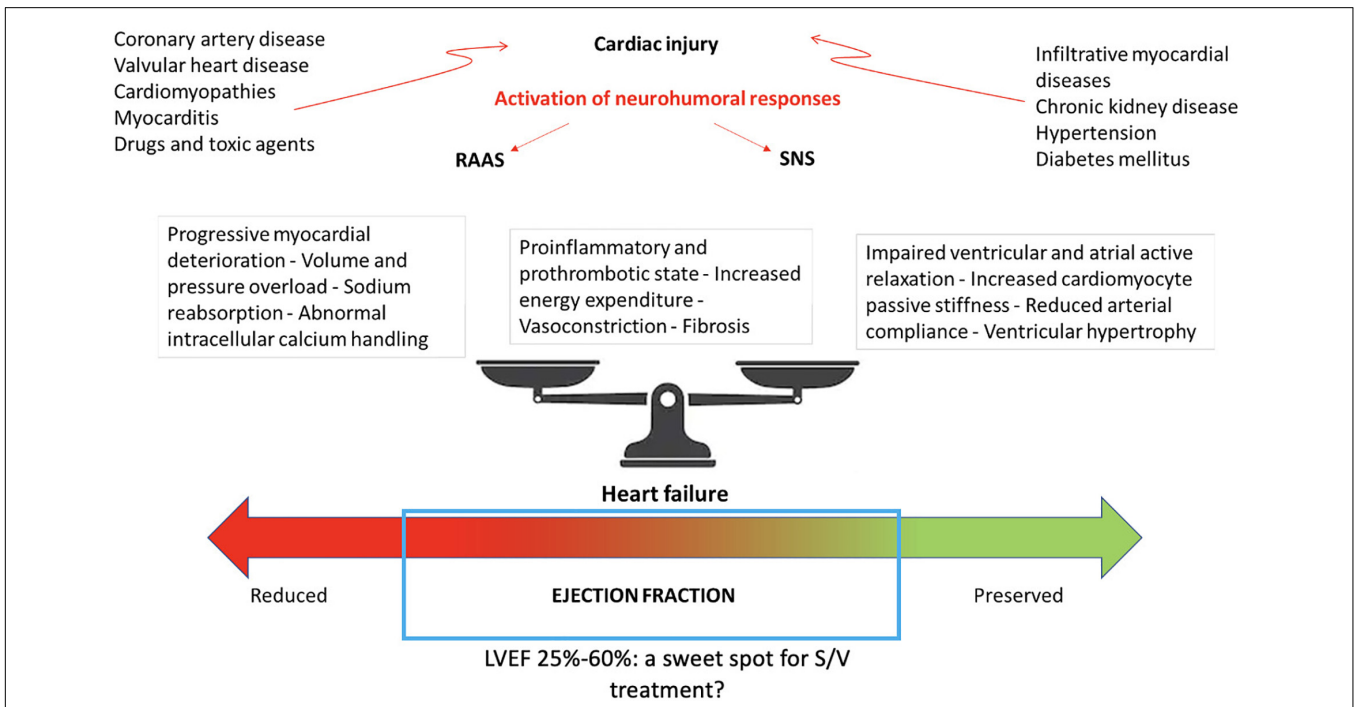

FIGURE 1 | Bidirectional disease trajectories in HF and potential involved pathophysiological mechanisms. LV, left ventricular ejection fraction; RAAS, renin-angiotensin-aldosterone system; SNS, sympathetic nervous system; S/V, sacubitril/valsartan.

TABLE 2 | Efficacy of S/V in different categories.

Benefits of S/V in different clinical subsets
LVEF: S/V has shown efficacy across the LVEF spectrum, despite an increased burden of events in patients with lower LVEF.
BP: Also patients with lower SBP generally well tolerate S/V and have the same benefit as patients with higher baseline SBP.
HF etiology: The benefit of S/V is consistent across etiologic categories (ischemic, idiopathic, hypertensive or other non-ischemic causes such as infective/viral, alcoholic, valvular, drug-related, peripartum–related).
Age: S/V has efficacy across various age categories including aged ≥ 75 years, albeit with a higher incidence of hypotension, renal impairment and hyperkalemia.
Prior decompensation: Despite the increased risk associated with more recent hospitalizations, the superiority of S/V does not differ among patients who have never been hospitalized, who have remote HF hospitalizations or who have been more recently hospitalized.
Concomitant medications: Efficacy of S/V does not vary according to concomitant medications.
Renal function: The effect of S/V is not modified by baseline eGFR, with a lower rate of decrease in eGFR compared to RAS inhibitors.
CV risk: The benefit of S/V is maintained across the spectrum of risk estimated with both MAGGIC and other HF-risk scores.
Diabetes: S/V is beneficial irrespective of glycemic status, with a reduced incidence of diabetes and a low percentage of subjects requiring oral anti-hyperglycemic therapy or new insulin use.
Signs of congestion: S/V reduces CV death and HF hospitalization irrespective of the number of signs of congestion (jugular venous distention, edema, rales, and third heart sound) at baseline and during follow-up.

BP, blood pressure; CV, cardiovascular; eGFR, estimated glomerular filtration rate; HF, heart failure; LVEF, left ventricular ejection fraction; MAGGIC, Meta-Analysis Global Group in Chronic Heart Failure; MRA, mineralocorticoid receptor antagonists; S/V, sacubitril/valsartan.

HF (−16%), cardiovascular death (−16%), first hospitalization (−16%), all-cause death (−12%), total HF hospitalizations and cardiovascular death (−18%), and total HF hospitalizations (−19%). The incidence of the primary composite outcome, HF hospitalizations, cardiovascular death, and all-cause mortality was inversely associated to LVEF. However, the most consistent benefits of the treatment with S/V was observed in a LVEF range between 25 and 50% (Solomon et al., 2020). This could correspond to a "sweet spot" for S/V-based therapeutic strategy, thus confirming that ventricular function may represent a significant effect modifier (**Figure 1**). The data derived by this composite analysis suggest that patients with HFmrEF could be a reasonably successful target for S/V-based treatment, this extending the current recommendations for this pharmacological class (Volpe et al., 2021). Accordingly, considering that the mean LVEF of the population included in the PARAGON-HF was 57% (Solomon et al., 2019), the evidence obtained in this pooled analysis seems to confirm the lack of benefits in a subset of patients with clearly preserved ventricular function. However, the limitations of classifying patients by LVEF cut-offs should be highlighted also in this context (Marwick, 2018). In particular, the beneficial effects of S/V have been demonstrated to extend to higher LVEF in women, suggesting a sex influence, probably related to smaller heart dimensions, to a different adipose-tissue distribution of NPs pharmacokinetic characteristics and possibly to a higher neurohormonal activation (McMurray et al., 2020). Moreover, a recent meta-analysis of 6 studies, with a total of 5,503 patients showed that SV significantly reduced the rate of HF hospitalization (RR, 0.84; 95% CI, 0.77−0.91; $p < 0.001$) and improved the NYHA class (RR, 1.25; 95% CI, 1.10−1.43; $p = 0.001$) in HFmEF and HFpEF patients compared with ACEi and ARB, without a significant increase in side effects (Nie et al., 2020).

According to these results, the US Food and Drug Administration (FDA) has approved an expanded indication for S/V in HFpEF patients, making it the first drug indicated for HF independently from LVEF (Novartis, 2021).

Consistently, future therapeutic strategies in HF, among which S/V represents a first-line choice, should not be based on a single numeric LVEF value, but rather on HF etiology and clinical phenotype (**Table 2**).

CONCLUSION

Lately, the distinction of HF in separate categories according to arbitrary LVEF cut-off seems to be out-of-date, due to the overlapping characteristics of the proposed subgroups and to the complexity of the pathophysiological mechanisms involved in the development of this syndrome, which should be more correctly considered as a unique spectrum composed by different specific phenotypes. In this context, the benefits of a S/V-based strategy have been demonstrated along most of the HF continuum, in which the neurohormonal dysfunction has a pivotal role in the development and progression of the disease.

We still have a long way to go to cover all the unmet needs in HF and a crucial effort should be performed by the medical community to optimize the available pharmacological regimens, particularly selecting a tailored therapeutic approach according to each patient specific phenotypes.

AUTHOR CONTRIBUTIONS

GG, DR, AB, and MV contributed to the conception and design, acquisition of data, and analysis and interpretation of data and drafted the article. MV, GT, and MBM contributed to the conception and design and critically revised the manuscript. All authors approved the final version to be published.

FUNDING

This work was supported by a 2019 grant from Sapienza University of Rome.

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Conflict of Interest: MV reports honoraria for lectures and/or consulting in Advisory Board from Amgen, Astra Zeneca, Daiichi-Sankyo, Menarini Int, MSD, Novartis Pharma, Novo Nordisk, Servier.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers in Physiology

REVIEW
published: 13 October 2021
doi: 10.3389/fphys.2021.650124

Toward a New Paradigm for Targeted Natriuretic Peptide Enhancement in Heart Failure

*Olof Gidlöf**

Department of Cardiology, Clinical Sciences, Lund University, Lund, Sweden

The natriuretic peptide system (NPS) plays a fundamental role in maintaining cardiorenal homeostasis, and its potent filling pressure-regulated diuretic and vasodilatory effects constitute a beneficial compensatory mechanism in heart failure (HF). Leveraging the NPS for therapeutic benefit in HF has been the subject of intense investigation during the last three decades and has ultimately reached widespread clinical use in the form of angiotensin receptor-neprilysin inhibition (ARNi). NPS enhancement via ARNi confers beneficial effects on mortality and hospitalization in HF, but inhibition of neprilysin leads to the accumulation of a number of other vasoactive peptides in the circulation, often resulting in hypotension and raising potential concerns over long-term adverse effects. Moreover, ARNi is less effective in the large group of HF patients with preserved ejection fraction. Alternative approaches for therapeutic augmentation of the NPS with increased specificity and efficacy are therefore warranted, and are now becoming feasible particularly with recent development of RNA therapeutics. In this review, the current state-of-the-art in terms of experimental and clinical strategies for NPS augmentation and their implementation will be reviewed and discussed.

Keywords: heart failure, natriuretic peptide system, ARNI, epigenetics, RNAi, gene therapy, non-coding RNAs

OPEN ACCESS

Edited by:
Speranza Rubattu, Istituto Neurologico Mediterraneo Neuromed, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Italy

Reviewed by:
Mariann Gyöngyösi, Medical University of Vienna, Austria
Francisco Altamirano, Houston Methodist Research Institute, United States

***Correspondence:**
Olof Gidlöf
olof.gidlof@med.lu.se

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *06 January 2021*
Accepted: *21 September 2021*
Published: *13 October 2021*

Citation:
Gidlöf O (2021) Toward a New Paradigm for Targeted Natriuretic Peptide Enhancement in Heart Failure. Front. Physiol. 12:650124. doi: 10.3389/fphys.2021.650124

INTRODUCTION

Heart failure (HF) is a serious and complex clinical condition characterized by inadequate ventricular filling or ejection of blood (Yancy et al., 2013). It arises as the result of structural or functional impairments of the heart, caused by a wide range of underlying diseases, including e.g., hypertension, ischemic heart disease and valvular disease (Ziaeian and Fonarow, 2016). An estimated 37.7 million people suffer from HF (Vos et al., 2012), and the number of patients is rapidly rising as a result of shifts in global age distribution. Symptoms of HF include fatigue, dyspnea, poor exercise tolerance, and fluid retention and often lead to impaired quality of life and frequent hospitalizations. An important clinical distinction is made between HF patients with reduced and preserved left ventricular ejection fraction (LVEF). The two groups are approximately equal in size, but with significant differences with regards to risk factors, pathophysiology, prognosis, and response to treatment (Ponikowski et al., 2016). HF with reduced (<40%) ejection fraction (HFrEF) is characterized by a weakened myocardium, typically with a dilated left ventricle, resulting in inefficient pumping during systole. HF with preserved (>50%) ejection fraction (HFpEF) is associated with a stiff myocardium with left ventricular hypertrophy and increased filling pressures which result in insufficient relaxation and filling of the heart during diastole.

Standard medical treatment for HFrEF is aimed at reducing cardiac workload and increase diuresis through inhibition of the renin-angiotensin-aldosterone (RAAS) and sympathetic nervous system (SNS), and can confer substantial improvements with regards to mortality and quality of life (Garg and Yusuf, 1995; Packer et al., 1996). Still, morbidity remains high for many patients and the 5-year survival rate after hospitalization is comparable to that of many cancers (Murphy et al., 2020). Moreover, in the case of HFpEF there are currently no evidence-based treatments available. Thus, there is an urgent need for new and improved therapeutic strategies for both HFpEF and HFrEF.

The natriuretic peptide system (NPS)constitutes a beneficial compensatory mechanism in all forms of HF, effectively reducing cardiac pre- and afterload in response to increased cardiac filling pressures by promoting natriuresis, diuresis, and vasodilation (Goetze et al., 2020). Thus, harnessing the NPS for therapeutic benefit has long been considered an attractive strategy and, as a result of extensive research over the last three decades, has recently reached widespread clinical use. The success of angiotensin receptor/neprilysin inhibition (ARNi) in treating HFrEF (McMurray et al., 2014) has been attributed to increased NP activity (Packer et al., 2015; Ibrahim et al., 2019). However, its use is complicated by the fact that neprilysin degrades a number of other vasoactive peptides in the circulation, e.g., glucagon, substance P, and bradykinin, which can cause hypotension and angioedema (McMurray et al., 2014) and raises concerns about long-term adverse effects through accumulation of potentially pathogenic peptides (e.g., amyloid-β) (Galo et al., 2020). Moreover, ARNi has proven ineffective in patients with HFpEF (Solomon et al., 2019). Therefore, alternative approaches to enhance the NPS with increased specificity and potency could lead the way toward more refined and efficacious HF therapies. The aim of this review is to provide an overview of the current state-of-the art in terms of experimental, preclinical and clinical strategies to enhance the NPS for therapeutic benefit in HF.

THE NATRIURETIC PEPTIDE SYSTEM

The NPS is a fundamental homeostatic mechanism regulating blood pressure and extracellular fluid volume. Atrial and b-type natriuretic peptides (ANP and BNP) are hormones produced and released from cardiomyocytes in response to mechanical stimuli and stimulates diuresis, natriuresis, and vasodilation (Goetze et al., 2020). Urodilatin is an ANP isoform with four additional N-terminal residues produced primarily in the kidney (Schulz-Knappe et al., 1988). Within the cardiovascular system, C-type natriuretic peptide (CNP) is mainly synthesized by the endothelium, where it is released in response to various stimuli (e.g., increased shear stress and cytokine signaling) and exerts vasodilatory and anti-proliferative effects locally within the vasculature (Fu et al., 2018). Whereas ANP and BNP are intimately associated with HF pathogenesis and progression, CNP is only modestly increased in HF (Wei et al., 1993a; Cargill et al., 1994; Del Ry et al., 2005) and mechanistically not a direct therapeutic target in HF. Therefore, CNP will not be discussed in detail in this review.

Synthesis and Secretion of Natriuretic Peptides

Atrial and b-type natriuretic peptides are transcribed from the *NPPA* and *NPPB* genes, respectively, which are located within approximately 10 kb of each other on chromosome 1p36.22. The locus is subject to highly coordinated and rigorous spatiotemporal control during development and disease, which is exerted both by epigenetic regulators (Rubattu et al., 2020) and transcription factors (Man et al., 2018). In healthy adults, *NPPA* expression is restricted to atrial cardiomyocytes whereas cardiac *NPPB* expression is generally low (Litvinukova et al., 2020; Tucker et al., 2020). However, the locus is subject to extensive changes in the local chromatin environment (Hohl et al., 2013; Sergeeva et al., 2016) resulting in a coordinated upregulation of both genes in atrial as well as ventricular tissue upon mechanical and neurohormonal stimulation (Saito et al., 1989; Feldman et al., 1991; Sergeeva et al., 2014). Numerous hallmarks of HF, including hemodynamic overload and increased wall stretch, as well as increased neurohormonal signaling through angiotensin II (Lako-Futo et al., 2003; Majalahti et al., 2007), endothelin (Archer et al., 2017), and alpha adrenergic (Knowlton et al., 1991) stimuli all result in potent transcriptional activation of the *NPPA*/*NPPB* locus in both atrial and ventricular tissue. HF-related activation of *NPPA*/*NPPB* gene expression has been demonstrated to be driven partly through mitogen-activated protein kinases (MAPKs), specifically mediated via extracellular signal-related kinase- (ERK-) signaling (Bueno et al., 2000; Kehat et al., 2011; Koivisto et al., 2011), with GATA4(Liang et al., 2001; Kerkela et al., 2002; Tenhunen et al., 2004; van Berlo et al., 2011), NFAT (Molkentin et al., 1998), Myocardin (Kuwahara et al., 2010) being some examples of key transcription factors involved.

Translation of *NPPA* and NPPB mRNA results in 151- and 134-amino acid preprohormones, respectively, which are processed into proANP and proBNP by enzymatic removal of their respective signal peptides (Fu et al., 2018). Biologically active BNP is produced intracellularly by the subtilisin-like proprotein convertase Furin (Nishikimi et al., 2015), whereas proANP is cleaved to form biologically active ANP after secretion by the membrane-bound serine protease Corin (Yan et al., 2000). Under physiological conditions, ProANP and processed BNP are stored together in specific atrial granules and released together in response to mechanical (Mangat and de Bold, 1993) and hormonal stimuli (Ogawa et al., 1999). In contrast, sustained pressure overload and wall stress leads to production and release of these NPs from both atrial and ventricular tissue, reflected in the marked elevation of both ANP and BNP in the circulation of HF patients.

Natriuretic Peptide Signaling

The natriuretic system includes three known receptors: NPR-A, -B, and -C. The biological functions of ANP and BNP are mediated by binding to natriuretic peptide receptor A (NPR-A), whereas CNP binds primarily to NPR-B. NPR-A and -B are membrane bound guanylate cyclases that, upon binding of their respective ligands produce cyclic guanosine monophosphate (cGMP) which acts as an intercellular second messenger activating protein

kinase PKG and phosphodiesterase (PDE) to regulate numerous pathways, including ion channels, protein phosphorylation, nuclear translocation and gene expression (Schlossmann et al., 2005). It has been reported that ANP is 10-fold more potent in stimulating NPR-A than BNP (Koller and Goeddel, 1992). NPR-A expression is particularly high in the arterial system, kidney, adipose tissue, adrenal gland, and lung (Sarzani et al., 1996; Nagase et al., 1997; Mele et al., 2015). In the kidney, NPR-A activation leads to increased glomerular filtration rate (Marin-Grez et al., 1986), inhibition of sodium and water reabsorption (Kishimoto et al., 1996) and reduced secretion of renin (Kurtz et al., 1986). In the arterial system, NPR-A mediates vasorelaxation by decreasing intracellular calcium levels and calcium sensitivity in vascular smooth muscle cells through PKG-I (Carvajal et al., 2000). In the adrenal gland, ANP/NPR-A inhibits adrenocorticotropin- and angiotensin-induced synthesis of aldosterone (Chartier et al., 1984; Kudo and Baird, 1984). Together, these physiological effects mediated by NPs constitute a key homeostatic counterweight to dysregulated SNS- and RAAS-signaling in HF. Interestingly, at concentrations observed in mild HF, ANP is more potent than BNP in inhibiting the aldosterone response to Angiotensin II (Hunt et al., 1996). Additionally, the natriuretic peptide system exerts direct effects in the heart, inhibiting cardiac remodeling and fibrosis. ANP and BNP have been shown to inhibit angiotensin II- and norepinephrine-induced proliferation of cardiac fibroblasts (Fujisaki et al., 1995; Calderone et al., 1998). Genetic approaches aimed at reducing or abrogating ANP/NPR-A signaling receptor in mice leads to a blood pressure-independent exacerbation of cardiac hypertrophy, fibrosis, and left ventricular dysfunction in animal models of HF (Kishimoto et al., 2001; Knowles et al., 2001; Kuhn et al., 2002; Holtwick et al., 2003; Wang et al., 2014). Moreover, carriers of genetic variants in the *NPPA* promoter associated with decreased circulating proANP levels showed increased left hypertrophy (Rubattu et al., 2006).

Natriuretic Peptide Clearance and Metabolism

Once released from their cell of origin, NPs are rapidly cleared from the circulation. ANP has an estimated half-life of ∼ 2 min in healthy human subjects (Nakao et al., 1986; Yandle et al., 1986), whereas the half-life of BNP is slightly longer, ∼20 min (Mukoyama et al., 1991; Holmes et al., 1993). There are two recognized mechanisms for NP clearance: receptor-mediated degradation and enzymatic proteolysis. The NPR-C receptor, which is bound by all NPs (Suga et al., 1992) and has a similar tissue expression profile as NPR-A (Sarzani et al., 1996), lacks the intercellular GC domain (Sudoh et al., 1990) and is believed to function mainly as a clearance receptor, internalizing natriuretic peptides for lysosomal degradation (Nussenzveig et al., 1990; Cohen et al., 1996). Enzymatic degradation of circulating NPs is mainly carried out by Neprilysin (NEP), a membrane-bound metalloprotease expressed in a wide variety of tissues and cells but is particularly abundant in the kidney (Pavo et al., 2020). Besides NPs, NEP degrades numerous additional vasodilatory peptides including bradykinin (Gafford et al., 1983), substance P (Skidgel et al., 1984), adrenomedullin (Lisy et al., 1998), as well as vasoconstrictors such as angiotensins (Gafford et al., 1983) and endothelins (Skolovsky et al., 1990). NPs are also cleaved by insulin-degrading enzyme (IDE) (Muller et al., 1991, 1992), another zinc-dependent metalloprotease with a wide repertoire of target peptides (Tundo et al., 2017). It should be noted however that BNP is a poor substrate for both NEP and IDE (Potter, 2011), and additional proteases are likely involved in its degradation. A number of animal studies have assessed the relative contribution of Natriuretic peptide receptor C (NPRC)- and NEP-mediated degradation of ANP. Results consistently show that the combination of NPRC-blocking peptides and NEP inhibitors (NEPi) cause a greater increase in circulating ANP (and its downstream physiological effects) than either of the individual treatments alone (Koepke et al., 1989; Kukkonen et al., 1992; Okolicany et al., 1992; Charles et al., 1996). Although these results suggest that both pathways are equally important in degrading ANP, it has been postulated that NEP plays a predominant role in the clearance of patho-physiological levels of ANP, when clearance receptors are believed to be saturated (Hashimoto et al., 1994).

CURRENT THERAPIES TARGETING THE NATRIURETIC PEPTIDE SYSTEM

The concept of utilizing NPs for clinical benefit in HF dates back to the 1980s, after the potent natriuretic and vasodilatory properties of ANP was discovered (de Bold et al., 1981; Kangawa and Matsuo, 1984). To date, two principal approaches for therapeutic augmentation of the NP system have been utilized: (1) administration of synthetic NPs and (2) inhibition of NP degradation.

Synthetic Natriuretic Peptides

Findings that infusion of human alpha-atrial natriuretic peptide led to increased natriuresis and arterial pressure in healthy subjects (Richards et al., 1985) and reduced pulmonary arterial wedge pressure, systemic vascular resistance and increased stroke volume in patients with congestive HF (Crozier et al., 1986; Riegger et al., 1986; Saito et al., 1987; Goy et al., 1988) spurred the development of synthetic NPs for clinical use. Intravenous injection of Carperitide, recombinant ANP, was approved for the treatment of acute decompensated HF in Japan in 1995, but evidence for its long-term benefit on cardiac function, clinical symptoms or prognosis have not been confirmed in large-scale, randomized clinical trials, and the drug has not reached widespread clinical use. Ularitide, a chemically synthesized analogue of the urodilatin, showed short-term beneficial hemodynamic effects in patients with congestive and decompensated HF with both reduced and preserved EF in early clinical trials (Elsner et al., 1995; Mitrovic et al., 2005, 2006) but failed to reduce long-term cardiovascular mortality in the larger, double-blind TRUE-AHF trial, including patients with acute HF (of which 35% had preserved LVEF) (Packer et al., 2017). Nesiritide, a 32 amino acid form of recombinant BNP effectively reduced cardiac pre- and afterload in patients with congestive

HFrEF (Mills et al., 1999) and was subsequently approved for the treatment of acute decompensated HF in the United States in 2001. However, since the large-scale, randomized clinical ASCEND-HF trial failed to show any benefit on mortality or re-hospitalization of Nesiritide compared to placebo in acute HF, it is no longer recommended for routine use (O'Connor et al., 2011).

Taken together, the results of numerous clinical trials (summarized in **Table 1**) over the last three decades shows that while administration of exogenous, synthetic NPs can provide short-term effects on hemodynamics and natriuresis in patients with acute HF, their use is limited by lack of long-term effects on mortality or hospitalization, a requirement for intravenous administration, and side effects such as severe hypotension.

Inhibition of Natriuretic Peptide Degradation

While infusion of exogenous NPs has thus far been ineffective in treating HF, increasing the amount of biologically active NPs in the circulation through NEPi has eventually proven to be a more successful therapeutic strategy (**Table 2**). The first proof-of-concept was established in 1989, when intravenous infusion of thiorpan, a selective NEPi, resulted in increased plasma ANP, increased natriuresis/diuresis and reduced pulmonary arterial wedge pressure and atrial pressure in patients with mild chronic HFrEF (Northridge et al., 1989). Beneficial effects was later seen with another intravenously administered NEPi, candoxatril, in patients with more severe HFrEF (Munzel et al., 1992), but the compound failed to reduce blood pressure and systemic vascular and pulmonary resistance in subsequent clinical trials and development was discontinued (McDowell and Nicholls, 1999). Similarly, a dose-ranging trial with the oral NEPi ecadotril failed to show significant effects on neuroendocrine measures and symptoms in chronic HFrEF patients (Cleland and Swedberg, 1998). These initial failures were attributed to the fact that NEPi-mediated NP augmentation was counteracted by the parallel increase in other vasoactive peptides degraded by NEP, in particular vasoconstrictors such as Angiotensin I, II, and Endothelin-1 (Gafford et al., 1983; Skolovsky et al., 1990). In line with these observations, it was also noted that the effect of angiotensin converting enzyme inhibitors (ACEi) on exercise capacity in patients with mild chronic HFrEF was improved with the addition of candoxatril (Newby et al., 1998). These insights led to the development of the orally active, combined ACEi-NEPi compound omapatrilat (Robl et al., 1997). Although proving to be more potent in reducing blood pressure and improving hemodynamics than candoxatril (McClean et al., 2000; Rouleau et al., 2000), omapatrilat failed to show meaningful benefit with regards to mortality and hospitalization compared to ACEi alone in the large, randomized double-blind OVERTURE trial, which included >5,700 HFrEF patients (Packer et al., 2002). In addition, omapatrilat was shown to be associated with increased occurrence and severity of angioedema compared to ACEi alone in the OCTAVE trial (Kostis et al., 2004), which ultimately meant the drug never reached clinical use. The increased risk of angioedema was attributed to elevated levels of bradykinin, a result of the simultaneous inhibition of three bradykinin-degrading proteases: ACE, NEP, and aminopeptidase (Sulpizio et al., 2005), resulting in excessive vasodilation and vascular permeability. This problem was circumvented by instead combining NEPi with angiotensin receptor blockade (ARB), which was shown to provide NP augmentation and inhibition of angiotensin signaling without disrupting ACE-mediated bradykinin degradation (Hegde et al., 2011). The orally active, first-in-class angiotensin receptor NEP inhibitor (ARNi) LCZ696 (sacubitril-valsartan) was safe and well tolerated and associated with increased plasma NP levels, increased diuresis and lowered blood pressure in phase I and II studies (Gu et al., 2010; Solomon et al., 2012). A phase III trial also showed that LCZ696 reduced diastolic and systolic blood pressure compared to valsartan alone in patients with mild to moderate hypertension. Importantly, LCZ696 was well tolerated and not associated with increased risk of angioedema (Ruilope et al., 2010). The PARADIGM-HF phase III, randomized, double-blind trial included >8,000 HFrEF patients was designed to compare the effects of LCZ696 and the ACEi enalapril on a composite end point of death from cardiovascular causes or hospitalization for HF. The trial was terminated early because the pre-specified limit for an overwhelming benefit of LCZ696 had been reached. Compared with enalapril, LCZ696 reduced death from any cardiovascular cause by 20% and hospitalization from HF by 21%. In secondary analyses, LCZ696 was also shown to provide reduced risk of non-fatal clinical deterioration compared to enalapril (Packer et al., 2015). Patients in the LCZ696 group were less likely to require intensified HF treatment or to be hospitalized for worsening HF compared to patients in the enalapril group. There was also a trend toward patients on LCZ696 being less likely to require mechanical assist device implantation or cardiac transplantation. On the molecular level, a marked and sustained decrease of plasma NT-proBNP and troponin, biomarkers of cardiac wall stress and injury respectively, was observed in the LCCZ696 group. Interestingly, a separate study found that LCZ696 also reduced markers of fibrosis (sST2, TIMP-1, Gal-3, PNP, and PIINP) and collagen degradation (MMP-2 and -9) compared to enalapril, providing indirect evidence that NEPi-mediated NP-augmentation also benefits the myocardium, which may contribute to improved clinical outcomes (Zile et al., 2019). Indeed, compared to enalapril, LCZ696 was recently shown to reduce the risk of sudden cardiac death (Shen et al., 2017), a complication of HF to which fibrosis is a known risk factor (Gulati et al., 2013). More evidence for a beneficial effect of ARNi on the myocardium was published in two separate studies recently, showing that LCZ696 improved indices of ventricular volume and function (left ventricular end-diastolic and -systolic volume index, left atrial volume index and early diastolic annular velocity) compared to enalapril both short-(Desai et al., 2019) and long term (Januzzi et al., 2019).

The favorable effects of ARNi have been attributed to increased NP availability and signaling, but the contribution of each individual NP family member has not been elucidated. Differences in the affinity of NEP for the individual NPs

TABLE 1 | Clinical trials—synthetic NPs in HF.

Study	Synthetic NP	Patient group (n)	Main outcomes
Crozier et al., 1986	ANP	CHF (7)	PCWP/RAP ↓
			CO ↑
Saito et al., 1987	ANP	CHF (6)	PCWP ↓
			SV ↑
			Diuresis/Natriuresis ↑
Goy et al., 1988	ANP	HFrEF (8)	PCWP ↓
			CI ↑
Elsner et al., 1995	Urodilatin	HFrEF (12)	SBP/CVP ↓
			Diuresis/Natriuresis ↑
Mitrovic et al., 2005	Urodilatin	DHF (24)	PCWP ↓
			RAP ↓
Mitrovic et al., 2006	Urodilatin	DHF (221)	SVR ↓
			CI ↑
Packer et al., 2017	Urodilatin	AHF (2157, 477 HFpEF)	SBP ↓
			Cardiovascular death—NS
			Hospitalization—NS
Mills et al., 1999	BNP	HFrEF (103)	PCWP ↓
			RAP ↓
			SVR ↓
			CI ↑
			SV ↑
O'Connor et al., 2011	BNP	AHF (7141, 1048 HFpEF)	Death or rehospitaliztion for HF—NS

CHF, congestive heart failure; HFrEF, heart failure with reduced ejection fraction; DHF, decompensated heart failure; AHF, acute heart failure; HFpEF, heart failure with preserved ejection fraction; PCWP, pulmonary capillary wedge pressure; RAP, right atrial pressure, CO, cardiac output; SV, stroke volume; CI, cardiac index; SBP, systolic blood pressure; CVP, central venous pressure; SVR, systemic vascular resistance; NS, not significant.

TABLE 2 | Clinical trials—inhibition of NP degradation in HF.

Study	Drug	Mode of action	Patient group (n)	Main outcomes
Northridge et al., 1989	Thiorpan	NEPi	HFrEF (6)	Diuresis/Natriuresis ↑
				PCWP ↓
Munzel et al., 1992	Candoxatril	NEPi	HFrEF (9)	CI ↑
				Diuresis/Natriuresis ↑
				PCWP ↓
Cleland and Swedberg, 1998	Ecadotril	NEPi	HFrEF (279)	Plasma/urinary cGMP ↑
				Symptoms/QoL scores—NS
Packer et al., 2002	Omapatrilat	ACEi-NEPi	HFrEF (5770)	Death/Hospitalization in HF—NS
McMurray et al., 2014	Sacubitril-Valsartan	ARNi	HFrEF (8442)	Death/Hospitalization in HF ↓
Solomon et al., 2019	Sacubitril-Valsartan	ARNi	HFpEF (4822)	Death/Hospitalization in HF—NS
Zile et al., 2019	Sacubitril-Valsartan	ARNi	HFrEF (2067)	Profibrotic biomarkers ↓
Desai et al., 2019	Sacubitril-Valsartan	ARNi	HFrEF (464)	LAVI ↓
				LVEDVI ↓
				LVESVI ↓
				E/e' ↓
Januzzi et al., 2019	Sacubitril-Valsartan	ARNi	HFrEF (794)	NT-proBNP ↑
				LVEF ↑
				LVEDVI ↓
				LVESVI ↓
				LAVI ↓
				E/e' ↓

NEPi, neprilysin inhibition; ACEi; angiotensin converting enzyme inhibition; ARNi, angiotensin receptor neprilysin inhibition; HFrEF, heart failure with reduced ejection fraction; HFpEF, heart failure with preserved ejection fraction; PCWP, pulmonary capillary wedge pressure; CI, cardiac index; QoL, quality of life; NS, not significant; LAVI, left atrial volume index; LVEDVI, left ventricular end-diastolic volume index; LVESVI, left ventricular end-systolic volume index; LVEF; left ventricular ejection fraction.

(CNP ≥ ANP >> BNP)(Kenny et al., 1993; Watanabe et al., 1997) suggests that NEPi would affect plasma levels of CNP and ANP to a greater extent than BNP. Nevertheless, the investigators of the PARADIGM-HF trial associated the beneficial effects of LCZ969 with increased levels of BNP, based on a relatively modest (16%) increase in the levels of the prohormone NT-proBNP (McMurray et al., 2014). However, the substantially larger (63%) increase in plasma cGMP suggested that other NPs contributed to the overall effect of ARNi. Ibrahim et al. (2019) recently carried out a comprehensive analysis of plasma NPs and a range of NP cleavage products over time in HFrEF patients on LCZ696 using a wide variety of assays. While the difference in BNP was inconsistent and showed considerable inter-assay variability and the levels of NT-proBNP actually decreased, ANP was elevated in a rapid, potent and sustained manner in response to ARNi. CNP levels were generally low and did not appear to be affected by the treatment. These results points toward ANP as an important mediator of the beneficial effects of ARNi.

Despite the undoubted success of ARNi in the treatment of HF, there are a number of limitations and precautions to consider. First, while LCZ696 has proven effective in reducing mortality and hospitalization in HFrEF, ARNi appears to be less effective in HFpEF. The PARAGON-HF trial assessed the effect of LCZ696 compared to valsartan alone in HF patients (NYHA class II–IV) with EF >45% (Solomon et al., 2019). After a median of 35 months follow-up, no significant differences were observed with regards to the primary endpoint (a composite of hospitalization for HF and cardiovascular death) between the treatment groups. In secondary analyses, LCZ696 showed significant benefit in women and in patients with mid-range EF (46–57%), suggesting that ARNi might be effective in certain subgroups of HFpEF patients. Second, there is a concern regarding the numerous other vasoactive peptides targeted by Neprilysin, and the potential side effects that ARNi might cause. Although safety and tolerability of NEPi therapy was considerably improved by the combination with ARB, the occurrence of angioedema and hypotension is still higher with ARNi compared to ACEi (McMurray et al., 2014; Solomon et al., 2019). Moreover, serious concerns have been raised regarding the risk of NEPi and Alzheimer's disease (AD). Amyloid-β is a well-established NEP substrate (Shirotani et al., 2001; Kanemitsu et al., 2003) and NEPi in animals results in a substantial increase in amyloid-β and the appearance of plaque-like deposits in the brain, similar to those associated with AD in humans (Iwata et al., 2000). Although no increase in incidence of AD or neurological adverse effects were observed during the 4.3 year follow-up of PARADIGM-HF (McMurray et al., 2014) or the 36-week time course of the PARAMOUNT-HF trial (Solomon et al., 2012), the development of AD occurs over a considerably longer period of time. A multi-center, randomized, double-blind trial (PERSPECTIVE, ClinicalTrials.gov identifier: NCT02884206) to comprehensively evaluate the longitudinal effects of LCZ696 on cognitive function (including memory, executive function, and attention) in patients with HFpEF is currently ongoing and will hopefully be able to address these concerns.

NOVEL APPROACHES FOR THERAPEUTIC NATRIURETIC PEPTIDE AUGMENTATION

As discussed above, ARNi represents a shining example and proof-of-concept of how harnessing the NP system can be used for therapeutic benefit in HF. However, the ubiquitous and promiscuous nature of Neprilysin makes it less than ideal as a drug target and the potential long-term side effects of ARNi have yet to be fully characterized. Currently, a wide range of alternative therapeutic strategies, involving regulation of NP synthesis, processing and signaling are being explored in experimental, preclinical and clinical studies with the aim to provide more specific and efficacious NP augmentation therapy (summarized in **Figure 1**).

Epigenetic Approaches to Natriuretic Peptide Augmentation

Epigenetic drugs, i.e., drugs modulating the state of chromatin, via targeting e.g., DNA methyltransferases, histone deacetylases (HDACs) and Bromodomain and Extra-terminal motif (BET) proteins have reached widespread clinical use in cancer and chronic inflammatory diseases (Nebbioso et al., 2018; Nemtsova et al., 2019). The use of epigenetic drugs have also shown promising effects in experimental models of HF. For example, both the BET inhibitor JQ1 and the broad-spectrum HDAC inhibitor trichostatin A have been shown to suppress pathological cardiac hypertrophy and fibrosis in pressure-overload models of HF (Kong et al., 2006; Anand et al., 2013; Duan et al., 2017). The genetic locus encompassing *NPPA* and *NPPB* is subject to extensive epigenetic regulation in response to mechanical and neurohormonal stimuli, both via changes in DNA methylation and through modifications of chromatin, allowing a more permissive transcriptional environment. In experimental models of phenylephrine- and myocardial infarction-induced cardiac hypertrophy and failure, activation of the *Nppa* promoter was shown to be mediated by the histone acetyltransferase (HAT)-activity of p300 (Gusterson et al., 2003; Miyamoto et al., 2006). A marked increase in H3K9-acetylation was also observed in the promoter of the *Nppa* and *Nppb* genes in response to pressure overload in mice (Sayed et al., 2013). Moreover, the histone demethylase JMJD2A was shown to mediate angiotensin II and endothelin-1- induced increases in *NPPB* expression in human induced pluripotent stem (iPS) cell derived cardiomyocytes (Rosales and Lizcano, 2018) and its expression was also found to be upregulated in human failing myocardium, accompanied by a decrease in the repressive chromatin modification H3K9me2 and H3K9me3 in the *NPPA* and *NPPB* promoters of human failing hearts (Hohl et al., 2013). An epigenome-wide analysis of cardiac tissue from HF patients with dilated cardiomyopathy (DCM) identified significant hypomethylation across the *NPPA*/*NPPB* locus, indicative of transcriptional activation (Meder et al., 2017). NP receptors are also subject to epigenetic control. Overexpression of HDAC1 and HDAC2 significantly enhanced *Npr1* promoter activity and expression of *Npr1* in primary mouse mesangial cells (Kumar et al., 2014b). Moreover, HDAC

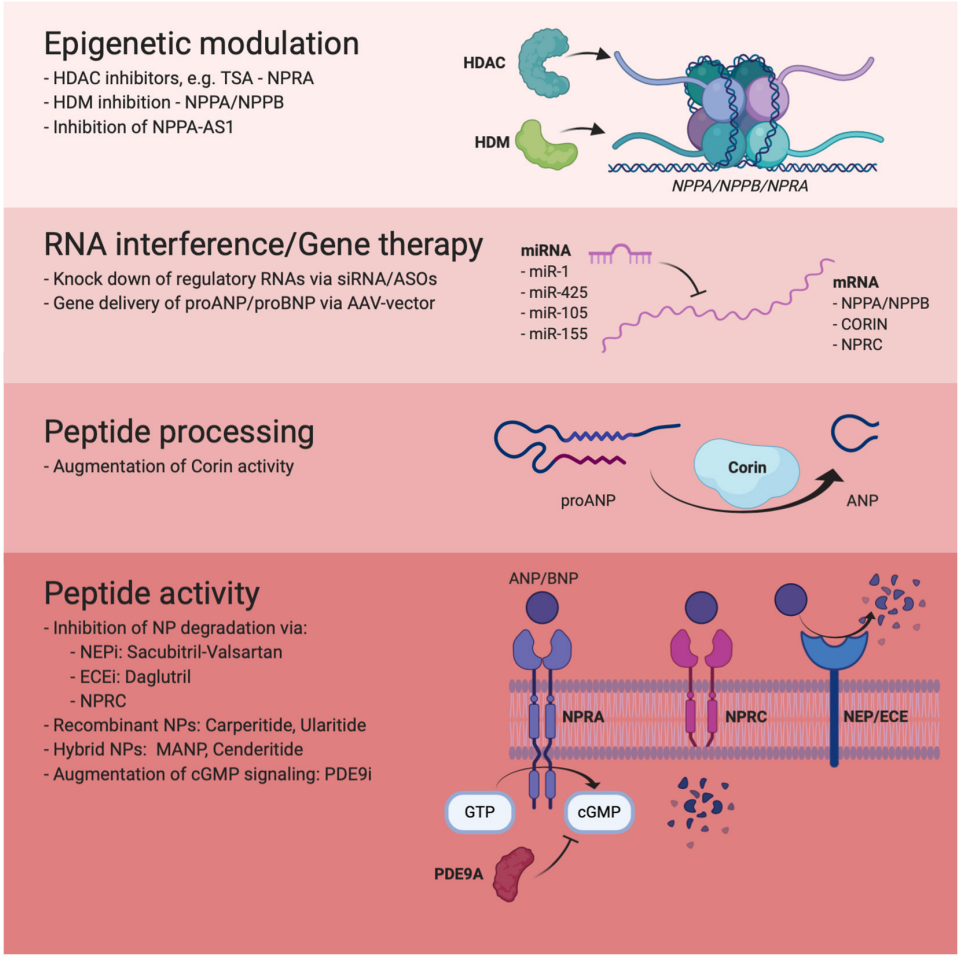

FIGURE 1 | Overview of novel approaches to therapeutic NP augmentation included in this review.

inhibition promoted recruitment of HATs to the *Npr1* promoter and significantly elevated renal *Npr1* expression, GC activity and cGMP levels *in vivo* (Kumar et al., 2014a). These studies show that epigenetic mechanisms actively regulate the NP system at multiple levels, that many of these mechanisms are active in the failing myocardium and that they could potentially be exploited for therapeutic NP augmentation. However, pharmacological tools for precise tuning of the chromatin environment within the heart are not yet available (Napoli et al., 2020).

RNA-Based Therapeutic Targets Within the Natriuretic Peptide System

The concept of RNA as therapeutic targets in cardiovascular medicine is gaining increasing traction, with RNA-based drugs for hypercholesterolemia (Inclisiran) and cardiac amyloidosis (Patisiran) being FDA- and EMA-approved or in late stage clinical trials (Leiter et al., 2019; Minamisawa et al., 2019).

The advantages of RNA as drug targets include ease of design and production as well as the ability to target any gene with high specificity and the potential to affect targets that are "undruggable" on the protein level. In theory, once a disease-associated target transcript has been identified, a chemically modified, complementary antisense oligonucleotide (ASO) or small interfering RNA (siRNA) can be synthesized and, once delivered to the cell or tissue of interest, modulate the expression, translation or splicing of the target (Levin, 2019). By leveraging genomic information, therapeutic antisense oligonucleotides with high specificity for the target transcript can be produced, minimizing the risk for off-target effects. One class of transcripts of particular interest as therapeutic targets are non-coding, regulatory RNAs (Gomes et al., 2020). This class encompasses both short (microRNAs) and long (long non-coding RNAs, lncRNAs) which regulate gene expression. MicroRNAs bind to complementary sequences, primarily in the 3' untranslated region (UTR), of mRNAs to repress translation or mediate mRNA degradation (Bartel, 2009), whereas lncRNAs regulate gene expression through a wide range of mechanisms, including antisense binding, transcription factor recruitment and modulation of the local chromatin environment (Palazzo and Koonin, 2020). Tissue- and context-specific expression and a high degree of conservation make regulatory RNAs attractive

as potential therapeutic targets. Across the various stages of NP synthesis, processing, and signaling, there are a number of potential RNA targets that could be harnessed for therapeutic benefit in HF. The first approach that will be covered here is targeting regulatory RNAs with negative effects on NP synthesis and processing. The NP genes are themselves subject to such regulation by both short and long regulatory RNAs. Arora et al. (2013) were the first to describe a regulatory RNA involved in NP synthesis. A genetic variant associated with blood pressure, situated in the 3'UTR of *NPPA*, was found to alter the binding and regulatory capacity of microRNA-425 (miR-425). Thus, miR-425 silenced *NPPA* expression in an allele-specific manner and was proposed as a potential target for specific upregulation of ANP. Subsequently, through a comprehensive transcriptomic and genetic screening and various *in vitro* assays performed by the same constellation of researchers, miR-105 and miR-155 were also shown to regulate *NPPA* expression in a similar manner (Wu et al., 2016; Vandenwijngaert et al., 2018). Interestingly, a recent clinical trial found the expression of miR-425 in atrial tissue to be significantly elevated in black individuals compared to white participants (Patel et al., 2019), an observation which might in part explain what has been touted as an NP-deficient state of black individuals (Gupta et al., 2015a,b, 2017; Bajaj et al., 2018). Overlapping the *NPPA* gene is a natural antisense transcript, *NPPA-AS1,* with potential regulatory capacity (Halley et al., 2013). Our research group showed that *NPPA-AS1* expression is localized to cardiomyocyte nuclei, is responsive to mechanical stimuli and is elevated in the myocardium of HF patients. Mechanistically, *NPPA-AS1* was shown to negatively regulate *NPPA* expression through interaction and recruitment of the repressive transcription factor RE1-silencing transcription factor (REST) to the *NPPA* promoter. *In vivo* inhibition of mouse Nppa-as1 resulted in increased cardiac and circulating Anp, reduced blood pressure and increased renal cGMP signaling (Celik et al., 2019b). In our view, this makes *NPPA-AS1* an interesting target for NP augmentation and work is currently ongoing to elucidate the therapeutic benefit of *Nppa-as1* knock down in models of HFpEF and HFrEF.

While no evidence has been published showing direct regulation of *NPPB* by non-coding RNAs, there are a number of studies showing how miRNAs negatively affect processing of proBNP. Two separate studies have demonstrated that *FURIN* expression is regulated by miR-24, but neither investigated the potential downstream effects on BNP synthesis and signaling (Luna et al., 2011; Wang et al., 2012). Nakagawa et al. (2017) found that miR-30 regulates the expression of GalNAc-transferases 1 and 2, and thereby the extent to which proBNP is glycosylated at threonines 48 and 71, and as a result, the amount of secreted proBNP. With regards to processing of proANP, our group recently performed a functional screening to identify microRNA regulators of Corin activity in human iPS-derived cardiomyocytes. miR-1, a cardiac-enriched microRNA, was identified as a particularly potent inhibitor of Corin activity through direct binding to a target site in *CORIN* mRNA. Interestingly, miR-1 was also found to have multiple additional targets involved in the transcription and processing of ANP (Celik et al., 2019a).

An alternative approach for RNA-based NP augmentation is targeting NP clearance receptor expression and/or function. Two microRNAs that regulate NPR-C expression have been discovered thus far. Using a combination of bioinformatic screening and transcriptome analysis, Wong et al. (2015) identified miR-100 as a potential regulator of *NPR3* expression and subsequently validated a direct miRNA:mRNA interaction *in vitro*. Later, Wang et al. (2018) described a mechanism whereby miR-143 exerts repressive effects on *NPR3* expression in cardiomyocytes. Of note, the authors also showed that the levels of miR-143 were elevated in the circulation of HF patients. Based on these results, both miR-100 and miR-143 could constitute potential RNA-based targets to achieve an increased level of circulating NPs. A more direct approach was taken by Venkatesan et al. (2016), who used an siRNA-based strategy to directly target the *Npr3* gene. In an isoproterenol-induced model of HF, intramyocardial injection of *Npr3* siRNA resulted in increased circulating levels of Anp and reduced cardiac hypertrophy and fibrosis. While promising, these findings should be taken with caution as NPR-C has been shown to play roles beyond NP clearance (Rubattu et al., 2010), for example with regards to bone growth (Matsukawa et al., 1999) and cardiac conductance (Rahmutula et al., 2019).

Although potent *in vivo* modulation of gene expression can be achieved relatively easily through administration of ASOs or siRNAs, there are a number of considerations and concerns that must be taken into account. For example, delivery of RNA-based drugs to organs other than the liver has proved to be challenging. Conjugation of ASOs or siRNAs with peptides (Ammala et al., 2018), sugar derivatives (Akinc et al., 2010), or aptamers (Catuogno et al., 2019) can provide tissue-specific targeting in some contexts, but an effective route for myocardial delivery has yet to be discovered.

Another issue, which is particularly relevant with regards to the targeting of regulatory RNAs, is specificity. As discussed above, microRNAs are inherently pleiotropic, with the potential of binding a wide repertoire of mRNA targets. The effects of long regulatory RNAs are more heterogenous by nature, exerting influence over a number of genes in a specific locus through modulation of the chromatin conformation (Khalil et al., 2009), or acting on a single target gene through antisense binding (Faghihi et al., 2008). Thus, careful evaluation of transcriptome-wide effects of therapies based on regulatory RNAs is warranted. Based on the studies included here (summarized in **Table 3**) numerous interesting targets for RNA-based augmentation of the NP system exist, but important challenges remain before they can reach clinical use.

Modulation of Enzymatic Activity in Natriuretic Peptide Synthesis and Processing

Inhibition of the metalloprotease Neprilysin is the obvious example of how altering enzymatic activity can be utilized to enhance the NP system, but other enzymes within the NP pathway have also been touted as potential therapeutic targets. There is evidence to suggest that the expression and activity

TABLE 3 | Putative targets for RNA-based augmentation of the NP system.

Target	Type of target	Effect	References
miR-425	microRNA	Repression of *NPPA*	Arora et al., 2013
miR-105	microRNA	Repression of *NPPA*	Wu et al., 2016
miR-155	microRNA	Repression of *NPPA*	Vandenwijngaert et al., 2018
miR-1	microRNA	Repression of *CORIN*	Celik et al., 2019a
miR-24	microRNA	Repression of *FURIN*	Luna et al., 2011; Wang et al., 2012
miR-30	microRNA	Regulates proBNP glycosylation/secretion	Nakagawa et al., 2017
miR-100	microRNA	Repression of *NPR3*	Wong et al., 2015
miR-143	microRNA	Repression of *NPR3*	Wang et al., 2018
NPPA-AS1	lncRNA	Repression of *NPPA*	Celik et al., 2019b
NPR3	mRNA	siRNA-mediated knock down of *NPR3*	Venkatesan et al., 2016

of Corin is decreased in the development and progression of HF, (Chen et al., 2010; Dong et al., 2010; Ibebuogu et al., 2011; Tripathi et al., 2016) resulting in dysregulated proANP processing and less biologically active ANP in the circulation. Restoring Corin activity can therefore be viewed as a potential therapeutic strategy in HF. Experimental support for this approach was provided in a study by Gladysheva et al. (2013), where transgenic mice overexpressing Corin showed reduced pulmonary congestion as well as improved systolic function and survival compared to wild type mice in a DCM-like model of HF. However, it is important to acknowledge that enhancement of Corin activity could lead to accumulation of other, yet unidentified target peptides, and with that, potentially undesired effects.

Endothelin-converting enzyme (ECE) is a transmembrane metalloprotease primarily known to produce biologically active Endothelin-1 (ET-1) from its precursor peptide PPET1. ET-1 is a highly potent vasoconstrictor which counteracts the beneficial effects of the NP system in HF (Giannessi et al., 2001). ECE shares structural features with Neprilysin and was shown both in experimental (Johnson et al., 1999) and physiological (Nakayama et al., 2012) settings to also actively degrade ANP. Thus, inhibition of ECE (ECEi) could provide beneficial neurohormonal outcomes by simultaneously decreasing ET-1 and increasing ANP. Combined ECEi and NEPi was subsequently shown to result in sustained improvement in systolic function and reduced cardiac remodeling compared to NEPi alone in ischemic and hypertensive models of HF (Mulder et al., 2004; Emoto et al., 2005; Kalk et al., 2011). In clinical trials, administration of the oral ECEi-NEPi Daglutril in healthy human subjects resulted in a significant increase in preproET-1 and ANP (Seed et al., 2012) and reduced pulmonary capillary wedge pressure and atrial pressure in patients with HFrEF (Dickstein et al., 2004). While these studies have established proof-of-concept, the long-term effects of ECE-NEPi on survival and hospitalization in HF have not been investigated.

Natriuretic Peptide Gene Therapy

With the advent of recombinant adeno associated viral (AAV) vectors, gene therapy is now a clinical reality for the treatment of diseases such as hemophilia and spinal muscular atrophy (High and Roncarolo, 2019). Cardiomyocytes, being terminally differentiated, non-dividing cells, should in theory constitute promising target cells for AAV-vectors, and the idea of using gene therapy to elevate cardiac NP expression has been explored in a number of experimental studies during the last 25 years. In pioneering work, Lin et al. (1995) showed that injection of naked plasmid DNA encoding ANP caused a potent and sustained lowering of blood pressure in young (but not adult) spontaneously hypertensive rats (SHR). Later, the same research group showed that adenoviral delivery of human ANP increased diuresis and natriuresis, lowered blood pressure and attenuated cardiac hypertrophy in Dahl salt-sensitive rats on a high-salt diet (Lin et al., 1998). More recently, Cataliotti et al. (2011) designed a myocardium-tropic AAV serotype 9 vector encoding preproBNP that, upon injection in SHR produced cardiomyocyte-specific overexpression of BNP and elevated plasma BNP as well as a sustained reduction in systemic blood pressure and improved diastolic and systolic function. In a later study, injection of an AAV9-vector encoding rat proBNP inhibited worsening of cardiac function and significantly prolonged survival in SHR (Tonne et al., 2014). Although these animal studies have shown promising results, targeting the heart with gene therapy has thus far been a challenge in humans. The experience from the CUPID and CUPID2b trials, where the effect of AAV1-mediated delivery of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase 2a (SERCA2a) on hospitalization for or ambulatory treatment for worsening HFrEF, showed that while safe, the treatment did not confer significant clinical benefit (Jaski et al., 2009; Jessup et al., 2011; Zsebo et al., 2014; Greenberg et al., 2016). The lack of effect has been attributed to inefficient delivery of the vector to cardiac cells, with an estimated <2% of cardiomyocytes containing a vector in the group receiving the highest dose. Thus, increasing transduction efficiency must be improved before myocardial gene therapy becomes clinically useful as a means of increasing cardiac NP production.

Designer NPs

The limited clinical success of infusion of recombinant, native NP (discussed above) has prompted the development of engineered hybrid NPs to improve pharmacological profiles and

minimize undesirable effects. Vasonatrin (VNP) is a synthetic peptide consisting of the full-length 22-amino acid CNP and the 5-AA C-terminus of ANP (Wei et al., 1993b). It has been shown to stimulate both NPR-A and -B (Jiang et al., 2014) and to be a more potent vasorelaxant than ANP (Wei et al., 1993b). In an animal model of ischemic cardiomyopathy, infusion of VNP was shown to improve hemodynamic parameters through a cGMP/PKG-dependent mechanism (Shi et al., 2015).

Cenderitide, a chimeric NP consisting of the full-length 22 AA human CNP and the 15-AA C-terminus of DNP, an NP isolated from *dendroaspis*, was designed in order to reduce the risk of systemic hypotension, a common and serious side effect of recombinant ANP and BNP, while retaining potent renal effects (Lisy et al., 2008). Infusion of Cenderitide was shown to be safe and to increase plasma cGMP and urinary sodium excretion in healthy subjects (Lee et al., 2009) and in a recent randomized clinical trial, increased plasma cGMP and urinary cGMP excretion without affecting blood pressure in patients with HFrEF (Kawakami et al., 2018).

Mutant ANP (mANP) is the result of a familial frame-shift mutation in exon 3 of *NPPA*, which gives rise to an ANP isoform with 12 additional C-terminal amino acids (Hodgson-Zingman et al., 2008). mANP is more resistant to NEP-mediated degradation (Dickey et al., 2009) and produces more potent natriuretic, diuretic and hemodynamic effects than ANP when administered *in vivo* (McKie et al., 2009). In a canine model of acute Ang II-induced hypertension with elevated cardiac filling pressures, infusion of mANP caused a significantly lowered pulmonary wedge pressure, artery pressure and right atrial pressure, increased urinary cGMP and reduced aldosterone levels as compared to infusion with human BNP (McKie et al., 2010). Importantly, the increased stability of mANP makes it suitable for subcutaneous administration (Chen et al., 2020), and does not require intravenous infusion like native recombinant NPs. In a randomized, double-blind, placebo-controlled trial, subcutaneous administration of mANP (ZD100) resulted in a sustained decrease in blood pressure and a reduction in aldosterone levels in patients with resistant hypertension, a major driver of HF (Chen et al., 2016). An additional phase I clinical trial to evaluate the cardiovascular properties of mANP administration in African Americans with resistant hypertension is currently recruiting (ClinicalTrials.gov identifier: NCT04542681), but the long-term effects of mANP on blood pressure as well as potential outcomes in patients with established HF has yet to be elucidated.

Modulation of Downstream Signaling

Augmentation of GC-A/cGMP/PKG signaling downstream from the NPR-A receptor could represent another possible approach for utilizing the NP system therapeutically in HF. Stimulation of the related guanylate cyclase sGC, the downstream effector of nitric oxide (NO)-signaling, via oral administration of Vericiguat was recently shown to reduce death and hospitalization in patients with HFrEF (Armstrong et al., 2020). While activation of the NO/cGMP pathway can be achieved with NO-independent sGC-stimulators, similar pharmacologic tools are currently unavailable for stimulating GC-A. Phosphodiesterases (PDEs) negatively regulate the cGMP signal by hydrolyzing cGMP to GMP, and could be considered therapeutic targets with relevance to the NP pathway. Lee et al. (2015) revealed that PDE9A is upregulated in the failing human heart and inhibits NP- rather than NO-dependent cGMP signaling. Inhibition of PDE9A was subsequently shown to protect the myocardium from neurohormonal and hemodynamic stress. Interestingly, oral PDE9A inhibitors are available and well tolerated in humans (Schwam et al., 2014; Moschetti et al., 2016; Brown et al., 2019) and might constitute a future approach to HF treatment.

SUMMARY AND FUTURE PERSPECTIVES

Heart Failure is a disease characterized by neurohormonal imbalance, and the NP system has been recognized as a beneficial equipoise to RAAS- and SNS-activation and a potential therapeutic target for three decades. Finding the right approach to enhance the NP system has however been a long and arduous endeavor, and even though the success of ARNi means that it has finally found its way to widespread clinical use (in HFrEF), alternative routes for more refined, precise and potent NP augmentation should still be explored. As evident from the many experimental and clinical studies presented here, the NP system can be targeted on multiple levels with diverse therapeutic modalities. Importantly, each approach also comes with specific challenges. A common hurdle for epigenetic, RNAi- and gene therapy-based therapies is the difficulties of cardiac drug delivery. The identification of cardiomyocyte-specific antigens or receptors to allow development of antibody-, peptide- or oligomer-conjugated drug delivery is a crucial step in taking these therapies toward clinical use. Increasing our understanding of the transcriptome and chromatin-state of single cardiac cells and cell-types will also help in designing epigenetic and RNA-based drugs with greater specificity and less risk of off-target effects. Improvements in vector design, delivery methods and dosage have the potential to enhance efficacy of cardiac gene therapy. With regards to enhancing NP half-life and activity beyond NEPi, it is interesting to note that the primary enzyme responsible for BNP degradation is likely yet to be discovered. Identification of this enzyme can thus lead to even more potent NP augmentation. Enhancing NP-signalling downstream of the NPR-A receptor is also an interesting therapeutic prospect for future studies. Pharmacological inhibition of the cardiac-specific cyclic phosphodiesterase PDE9A could represent a potent NP-like stimulus to treat HF-induced hypertrophy and fibrosis.

The distinct lack of clinical trials focusing on the large group of HF patients with preserved ejection fraction (HFpEF) is worth mentioning. The failure of the PARAGON-HF trial to show benefit of ARNi in patients with HFpEF is disappointing, but not entirely unsurprising given the relative heterogeneity of this patient group. Targeted approaches such as those described above would likely have a better chance of providing benefit, especially if combined with precision medicine approaches (Gori et al., 2020).

To conclude, a broad spectrum of possible therapeutic targets exists within the NP system beyond Neprilysin, all with the promise of improving HF treatment and all associated with specific challenges that must be resolved prior to clinical implementation. The research field is ripe with experimental and clinical studies addressed to meet these challenges and ultimately provide the next generation of NP augmentation therapy.

AUTHOR CONTRIBUTIONS

The author conceived the idea, performed literature search, and wrote the manuscript.

FUNDING

This author was supported by grants from the Swedish Heart- and Lungfoundation (20200532 and 20190409), the Crafoord Foundation, the Åke Wiberg Foundation, The Magnus Bergvall Foundation, and the Royal Physiographic Society and the Maggie Stephens Foundation.

ACKNOWLEDGMENTS

The author is grateful for valuable comments from J. Gustav Smith.

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frontiers in Physiology

ORIGINAL RESEARCH
published: 18 October 2021
doi: 10.3389/fphys.2021.673497

Distribution of Cardiac and Renal Corin and Proprotein Convertase Subtilisin/Kexin-6 in the Experimental Model of Cardio-Renal Syndrome of Various Severities

*Emad E. Khoury[1], Ahmad Fokra[1], Safa Kinaneh[1], Yara Knaney[1], Doron Aronson[2] and Zaid Abassi[1,3]**

[1] Department of Physiology, Bruce Rappaport Faculty of Medicine, Technion–Israel Institute of Technology, Haifa, Israel, [2] Department of Cardiology, Rambam Health Care Campus, Haifa, Israel, [3] Department of Laboratory Medicine, Rambam Health Care Campus, Haifa, Israel

OPEN ACCESS

Edited by:
Amie Moyes, Queen Mary University of London, United Kingdom

Reviewed by:
Chris J. Charles, University of Otago, New Zealand
Sergey Brodsky, Ohio State University Hospital, United States

***Correspondence:**
Zaid Abassi
abassi@tx.technion.ac.il

Specialty section:
This article was submitted to Integrative Physiology, a section of the journal Frontiers in Physiology

Received: *02 March 2021*
Accepted: *22 September 2021*
Published: *18 October 2021*

Citation:
Khoury EE, Fokra A, Kinaneh S, Knaney Y, Aronson D and Abassi Z (2021) Distribution of Cardiac and Renal Corin and Proprotein Convertase Subtilisin/Kexin-6 in the Experimental Model of Cardio-Renal Syndrome of Various Severities. Front. Physiol. 12:673497. doi: 10.3389/fphys.2021.673497

Congestive heart failure (CHF) often leads to progressive cardiac hypertrophy and salt/water retention. However, its pathogenesis remains largely unclarified. Corin, a cardiac serine protease, is responsible for converting proANP and proBNP to biologically active peptides. Although the involvement of corin in cardiac hypertrophy and heart failure was extensively studied, the alterations in corin and proprotein convertase subtilisin/kexin-6 (PCSK6), a key enzyme in the conversion of procorin to corin, has not been studied simultaneously in the cardiac and renal tissues in cardiorenal syndrome. Thus, this study aims to examine the status of PCSK6/corin in the cardiac and renal tissues of rats with CHF induced by the creation of aorto-caval fistula (ACF). We divided rats with ACF into two subgroups based on the pattern of their urinary sodium excretion, namely, compensated and decompensated. Placement of ACF led to cardiac hypertrophy, pulmonary congestion, and renal dysfunction, which were more profound in the decompensated subgroup. Corin immunoreactive peptides were detected in all heart chambers at the myocyte membranal and cytosolic localization and in the renal tissue, especially in the apical membrane of the proximal tubule, mTAL, and the collecting duct. Interestingly, the expression and abundance of corin in both the cardiac ventricles and renal tissues were significantly increased in compensated animals as compared with the decompensated state. Noteworthy, the abundance of PCSK6 in these tissues followed a similar pattern as corin. In contrast, furin expression was upregulated in the cardiac and renal tissues in correlation with CHF severity. We hypothesize that the obtained upregulation of cardiac and renal PCSK6/corin in rats with compensated CHF may represent a compensatory response aiming at maintaining normal Na^+ balance, whereas the decline in these two enzymes may contribute to the pathogenesis of avid sodium retention, cardiac hypertrophy, and blunted atrial natriuretic peptide/brain natriuretic peptide actions in decompensated CHF.

Keywords: corin, PCSK6, natriuretic peptides, furin, cardiorenal syndrome, heart, kidney

INTRODUCTION

Heart failure (HF) is a clinical syndrome characterized by inadequate peripheral blood flow, leading to the suffering of additional vital organs. In this context, the kidney is an important player, as poor renal blood supply in HF leads to a deleterious clinical setting named cardiorenal syndrome (CRS), which develops in approximately half of the HF patients (Damman and Testani, 2015; Mazurek and Jessup, 2017).

Cardiorenal syndrome involves multiple interdependent mechanisms, including the activation of neurohormonal factors, such as renin-angiotensin-aldosterone system and sympathetic nervous system, and also the increased secretion of endothelin-1, and antidiuretic hormone (ADH) (Schefold et al., 2016). Persistent activation of the neurohormonal factors results in deleterious outcomes, including sodium and water retention, along with decreased free water clearance, renal and systemic vasoconstriction, and also cardiac and renal tissue remodeling (Mazurek and Jessup, 2017). On the other hand, the natriuretic peptides (NPs) system plays a key role in opposing the abovementioned detrimental systems. *Via* its two cardiac natriuretic hormones, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), the NPs system promotes natriuresis, diuresis, decreases sodium reabsorption, and diminishes ADH secretion (Goetze et al., 2020). In addition, the activation of the NPs system results in vasodilation and lower blood pressure, reduced renal sympathetic activity, and attenuation of cardiac, renal, and vascular tissue remodeling (Goetze et al., 2020).

One of the contradictions seen in HF and CRS is the supremacy of the deleterious neurohormonal systems over the beneficial NPs, despite the remarkably elevated circulating levels of ANP and BNP (Villarreal and Freeman, 1991; Braunwald, 2008; Chaney and Shaw, 2010; Dries, 2011; McMurray et al., 2012; Yancy et al., 2013; Schefold et al., 2016). Several mechanisms have been suggested to explain this phenomenon, yet the involvement of the NPs machinery system has not been studied thoroughly (Charloux et al., 2003; Bouley, 2012; Ngo et al., 2013; Egom et al., 2015). Corin, a type-II transmembrane serine protease found mainly in cardiomyocytes, is essential for converting the proANP and proBNP hormones into their active forms (Yan et al., 1999, 2000; Wu et al., 2002; Semenov et al., 2010; Ichiki et al., 2011). To gain biological activity, corin must first undergo enzymatic cleavage by proprotein convertase subtilisin/kexin-6 (PCSK6, also named PACE4) to be activated (Seidah et al., 2013; Chen et al., 2015). In addition, corin is present in the circulation due to metalloprotease ADAM10-mediated shedding and autocleavage (Wang et al., 2015).

Although some studies have found that cardiac corin undergoes downregulation in HF, other studies reported opposite findings (Langenickel et al., 2004; Tran et al., 2004; Jiang et al., 2005; Calderone et al., 2006; Chen et al., 2010; Gladysheva et al., 2013; Ichiki et al., 2013). These conflicting observations may stem from several factors, including the application of different HF models with distinct duration and severity of the disease, inconsistency in the cardiac chamber being investigated, and the use of different analytical methodologies across these studies. Moreover, the status of renal corin has not been studied in HF so far. The kidney is an important site for the local generation of NPs, where corin is expressed at various sites of the nephron (Dong et al., 2016). The renal corin and *in situ* NPs assumedly play an essential role in the local production of ANP, thus contributing to water and salt balance under normal and disease states, including congestive heart failure (CHF). Finally, PCSK6, a recently discovered enzyme and of paramount importance for the normal activity of corin (Chen et al., 2015), has not been studied in HF before.

Therefore, the present study was designed to examine the expression of corin and PCSK6 in the cardiac and renal tissues, simultaneously, and the subsequent changes in the NPs (ANP and BNP) in an experimental model of HF of different severities, induced by the placement of aorto-caval fistula (ACF). Most recently (Khoury et al., 2018), we utilized this experimental model to study the status of PCSK6/corin in the pulmonary tissue of rats with HF. Exploring the alterations in cardiac and renal PCSK6/corin/NPs axis in HF of distinct severities is appealing, as it may provide new insights into the pathogenesis of cardiac remodeling and renal dysfunction characterizing this clinical setting.

MATERIALS AND METHODS

The samples used in the current study were collected from the same animals utilized in our animal CHF rat model as previously described (Khoury et al., 2018). While our former study focused on the pulmonary tissue, the current research analyzes the cardiac and renal specimens. Briefly, studies were performed on male Sprague–Dawley rats (Harlan Laboratories, Jerusalem, Israel), weighing 300–350 g. The rats were housed in individual metabolic cages in a temperature-controlled room and fed a standard rodent diet and tap water *ad libitum*. Urinary volume and urinary sodium concentration were measured throughout the entire study period (beginning 4 days prior to surgery). The studies involving animals were reviewed and approved by the Technion Committee for Care and Use of Laboratory Animals.

Experimental Model

An ACF was surgically created between the abdominal aorta and the inferior vena cava, as reported in detail from our laboratory (Winaver et al., 1988; Abassi et al., 1990, 2001). Briefly, under sodium pentobarbitone anesthesia (60 mg/kg BW, i.p.), the abdominal aorta and inferior vena-cava were exposed through a mid-abdominal incision. The outer wall of the vena-cava was opened, and a fistula (∼1.2 mm outer diameter) was surgically created in the common wall between the vessels. The opening in the wall of the vena cava was closed with a continuous suture. A matched group of sham-operated rats that underwent laparotomy only served as controls. The animals were allowed to recover and then returned to their metabolic cages for monitoring urine output and sodium excretion for an additional 7 days. Based on their urinary sodium excretion (UNaV), rats with ACF were divided into two subgroups: compensated (UNaV $>$ 1200 μEq/day) and decompensated

(UNaV < 200 μEq/day), accompanied by evidence of fluid retention (Winaver et al., 1988). Animals from the various groups (n = 7–8) were anesthetized, tracheotomized, and polyethylene catheters (PE50) were inserted into the carotid artery and jugular vein.

Cardiac Function

Cardiac function was monitored by inserting a Millar cardiac conductance catheter (Mikro-Tip®, Millar Instruments, TX, United States) to the left ventricle (LV) *via* the carotid artery. The system simultaneously measures high-fidelity ventricular pressure, volume, and intracardiac electrocardiography (ECG) *via* a single catheter. The LV pressure and volume signals were plotted against each other in real time, generating the characteristic left ventricular pressure-volume (PV) loops. A stable interval of 1 min was chosen for each rat. The Millar system provided the following measurements along with the raw data (pressure and volume vs. time): stroke volume, cardiac output, heart rate, end-diastolic volume, end-systolic volume, ejection fraction, maximal and minimal ventricular dP/dT.

At the end of the experiments, the heart and the kidneys were washed *via* left ventricle perfusion with 120 ml phosphate-buffered saline (0.01M PBS, pH 7.4) containing heparin (5 U/ml). Then, the heart and the kidneys were harvested and weighed. Cardiac/body weight ratio (HW/BW%), an index for cardiac hypertrophy, was calculated in the various groups of animals. Similarly, the kidney weight to body weight ratio (KW/BW%) was calculated. The collected tissues were subject to western blot and real-time qPCR analysis.

Heart and Kidney Fixation

Additional groups of rats with compensated and decompensated CHF, and also sham controls (n = 3) were anesthetized (Nembotal, 60 mg/kg, i.p.), and their heart and kidneys were fixed *via* carotid artery perfusion, first with 40 ml phosphate-buffered saline (0.02M PBS, pH 7.4) containing heparin (5 U/ml), and then with 220 ml of ice-cold 4% paraformaldehyde in 0.1 M PBS, pH 7.5 containing 4% sucrose. Hearts and kidneys from the different experimental groups were removed and embedded in 10% neutral-buffered formalin. Cardiac and renal tissues samples were then progressively dehydrated in graduated alcohol concentrations (70–100%) and embedded in paraffin.

Cardiac and Renal Fibrosis

About 5 μm-thick paraffin sections of the cardiac and renal tissues were deparaffinized and rehydrated gradually through 100–70% alcohol solution. Sections were then refixed with Bouin's solution for 1 h at 56°C followed by staining with Weigert's iron hematoxylin working solution for 10 min. Tissues were then stained in Biebrich scarlet-acid fuchsin solution followed by phosphomolybdic–phosphotungstic acid solution. Sections were then transferred to aniline blue solution and stained for 10 min. The 1% acetic acid solution was used, followed by dehydration of the sections through 95% ethyl alcohol and absolute ethyl alcohol. Finally, sections were cleared with xylene. All the images were obtained with a computerized scanner.

Immunofluorescence

Five-micrometer-thick paraffin sections of the various tissues were deparaffinized and rehydrated. Then, slides were subjected to antigen retrieval by using Proteinase K (ab64220, Abcam) for 5 min. Slides were then incubated with 5% normal donkey serum (NDS) in phosphate-buffered saline (PBS) containing 0.3% Tween-20 for 60 min to block non-specific binding and incubated overnight at 4°C with primary antibodies diluted in blocking solution and directed against Corin (1:100, rabbit, ab230311, Abcam) and PCSK6 (1:100, rabbit, ab140934, Abcam). Cy™3 Donkey Anti-Rabbit IgG was used as the secondary antibody (Jackson Laboratories, PA, United States) together with DAPI Fluoromount-G® for nuclear staining. Images were captured using a Widefield Zeiss Upright microscope and analyzed with Zen software. Representative images of the cardiac and renal tissues were obtained at ×40 magnification.

Western Blot Analysis

Cardiac, renal (both cortical and medullary), and pulmonary tissue samples from the various experimental groups were homogenized on ice. The homogenized tissue was then lysed in RIPA buffer (150 mM NaCl, 1% NP40, 50 mM Tris pH 8.0, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with a cocktail of protease inhibitors (Roche) in rotation at 4°C for 20 min and then centrifuged at 4°C for 15 min at 12,000 RPM. The cleared supernatant was collected, and the protein concentration was determined (Bradford reagent, Sigma). Equal amounts of extracted proteins (40–60 μg) were loaded and run by electrophoresis on a 9 or 15% SDS–polyacrylamide gel and were transferred to a nitrocellulose membrane. The membranes were incubated in blocking buffer, TBS-T (Tris-buffered saline, 0.1% Tween 20) containing 5% (w/v) BSA, and probed with the appropriate primary antibodies: anti-corin (1:1,000, rabbit, ab125254, Abcam), anti-PCSK6 (1:1,000, rabbit, ab151562, Abcam), anti-furin (1:1,000, rabbit, ab183495, Abcam), anti-ADAM10 (1:2,000, rabbit, ab124695, Abcam), anti-neprilysin (1:2,000, rabbit, ab126593, Abcam), anti-ANP (1:1,000, rabbit, ab209232, Abcam), anti-BNP (1:500, rabbit, ab19645, Abcam), and anti-GAPDH (1:500, mouse, sc-32233, Santa Cruz) diluted in blocking solution. After washing with TBS-T, the immunoreactive proteins were visualized with horseradish-conjugated goat anti-rabbit (1:25,000, 111-035-144, Jackson) and donkey anti-mouse (1:10,000, 715-035-151, Jackson) IgG secondary antibodies and chemiluminescent substrate.

Gene Expression Analysis by Real-Time qPCR

Total RNA was isolated from snap-frozen tissue samples using TRIzol® Reagent (Life Technologies), according to the instructions of the manufacturer, and quantified by spectrophotometry using NanoDrop2000. After oligo (dT)–primed reverse transcription of 1,000 ng total RNA, the resulting single-stranded cDNA was used for PCR. PCR conditions were as follows: an initial denaturation step at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, and hybridization at 60°C for 30 s followed by elongation at 72°C for 1 min. Finally, the

PCR reaction was terminated by incubation at 72°C for 5 min. GAPDH was used as an internal standard. The following primers were used:

Corin: F(5′-GAAGACTGTAAGGACGGGAGTGA-3′), R(5′-GTCAAGGCAACCCCGATCT-3′);
PCSK6: F(5′-GCTCACGGCTACCTCAACTT-3′), R(5′-CTGTCTCTTGACCCTGCGTT-3′);
Furin: F(5′-AGGGGTAGGCTGACATCATCT-3′), R(5′-CCAGGGCACAGTGTTAGTTTG-3′);
ADAM10: F(5′-TGGTGTTGCCGACAGTGTTA-3′), R(5′-GGATTTCCATACTGACCTCCCA-3′);
Neprilysin: F(5′-ACACATGACCAAATAAACCATTGCT-3′), R(5′-GCTCACCCCAGAGTTTGTGT-3′);
NPPA: F(5′-CCTGGACTGGGGAAGTCAAC-3′), R(5′-ATCTATCGGAGGGGTCCCAG-3′);
NPPB: F(5′-TTTCCTTAATCTGTCGCCGCT-3′), R(5′-TGGATTGTTCTGGAGACTGGC-3′);
GAPDH: F(5′-GTGCCAGCCTCGTCTCATAG-3′), R(5′-GAGAAGGCAGCCCTGGTAAC-3′).

Statistical Analysis

Data are presented as mean ± SEM. Comparison between two parametric groups was performed using the unpaired Student's *t*-test after testing for equality of variances. A value of $p < 0.05$ was considered statistically significant.

RESULTS

As previously reported, rats with ACF exhibited two distinctly different patterns of UnaV. While some of the animals displayed progressive sodium retention, severe dyspnea, and edema, characteristics of decompensated CHF, the remaining rats with ACF, termed compensated subgroup, displayed increased UNaV comparable with those measured in the control group (Khoury et al., 2018). Bodyweight decreased in the compensated (from 343 ± 10 to 328 ± 6 gr) and a more pronounced manner in the decompensated CHF rats (from 339 ± 3 to 294 ± 4 gr). The placement of ACF caused an overt increase in the heart weight due to marked left and right ventricular hypertrophy/dilation (**Supplementary Table S1**). The heart/bodyweight ratio, an index of cardiac hypertrophy, was significantly elevated after 1 week of ACF surgery in the compensated and decompensated CHF groups relative to the sham rats (0.44 ± 0.01% and 0.50 ± 0.02% vs. 0.29 ± 0.004%, $P < 0.01$, respectively). The significant increase in HW/BW in the decompensated subgroup as compared with compensated animals, but not in the absolute HW, may partially stem from the body weight loss, which was more prominent in the decompensated group (−13.1 ± 1.2%) than the compensated group (−4.15 ± 2.6%), as compared with sham-operated rats, which gained 1.3 ± 3.5% in their body weights. In contrast to the cardiac tissue, kidney weight normalized to body weight were lower in rats with compensated CHF (0.3 ± 0.01%; $P < 0.01$) and to a larger extent in decompensated CHF (0.27 ± 0.01%; $P < 0.001$) as compared with the sham group (0.33 ± 0.01%) (**Supplementary Table S1**). Cardiac and renal remodeling was accompanied by lung edema, as previously reported (**Supplementary Table S1**). Furthermore, rats with CHF showed attenuated renal hemodynamic and impaired kidney function, as was evident by reduced RBF and GFR in correlation with the severity of the disease (Khoury et al., 2018). In addition, our former study demonstrated that rats with CHF displayed significant elevation in the circulating levels of ANP, which was comparable in compensated and decompensated CHF, compared with sham controls (Khoury et al., 2018). As expected, BNP levels in the circulation were significantly elevated in rats with compensated and decompensated CHF by 6- and 14-fold compared with sham-operated animals. All these findings indicate that the applied ACF model exhibits typical manifestations of CRS and composes a reliable platform to study this clinical setting.

Cardiac Hemodynamics

Representative PV loops of compensated and decompensated subgroups and their sham controls are presented in **Figure 1**. Compensated and decompensated CHF rats exhibited significantly increased end-diastolic volumes as compared with sham operated controls (1,176 ± 104 μl; $P < 0.05$, 1,228 ± 57 μl; $P < 0.0001$ vs. 859 ± 25 μl, respectively) (**Figure 1G**). CHF rats also had increased end-systolic pressure, a parameter of LV wall stress (**Figure 1F**). As expected in the applied model, compensated but not decompensated CHF rats, displayed significantly increased stroke volume (404 ± 49 μl; $P < 0.05$) and cardiac output (138 ± 19 ml/min; $P < 0.05$) compared with controls (276 ± 3 μl and 95 ± 1.1 ml/min, respectively) (**Figures 1C,D**), but still having comparable heart rate (**Figure 1B**). Moreover, LV ejection fraction was unaltered in compensated CHF rats but significantly reduced in decompensated animals, as compared with the control group (29.7 ± 2%; P = NS, 22.4 ± 0.9%; $P < 0.0001$ vs. 31.4 ± 1.1%, respectively) (**Figure 1E**). Also, the systolic parameter of stroke work was significantly decreased in CHF rats in correlation with the severity of HF (**Figure 1H**). Finally, the maximal and minimal pressure gradients corresponding to systole and diastole, parameters of systolic and diastolic function, respectively, were reduced in rats with CHF (**Figures 1I,J**).

Cardiac and Renal Fibrosis

Figure 2 shows representative images of Masson's trichrome staining depicting interstitial fibrosis in cardiac ventricular tissue and also the cortical and medullary layer of the kidney. There was little, blue-stained fibrotic tissue in sham-operated hearts, but fibrosis was apparent in both compensated and decompensated CHF hearts, especially in the endocardium layer and perivascular areas. Interstitial fibrosis is an additional consequence of cardiac remodeling in CHF rats, where cardiac fibrosis was more prominent in decompensated rats than that observed in compensated animals. Similarly, renal fibrosis localized mainly to the medullary and perivascular regions was aggravated by decompensated CHF, but to a lesser extent in the compensated subgroup. These results suggest that besides cardiac hypertrophy and kidney dysfunction, CHF is associated with cardiac and renal fibrosis, yet it was very mild probably due to the short follow-up period (1 week) after the induction of the disease. In addition,

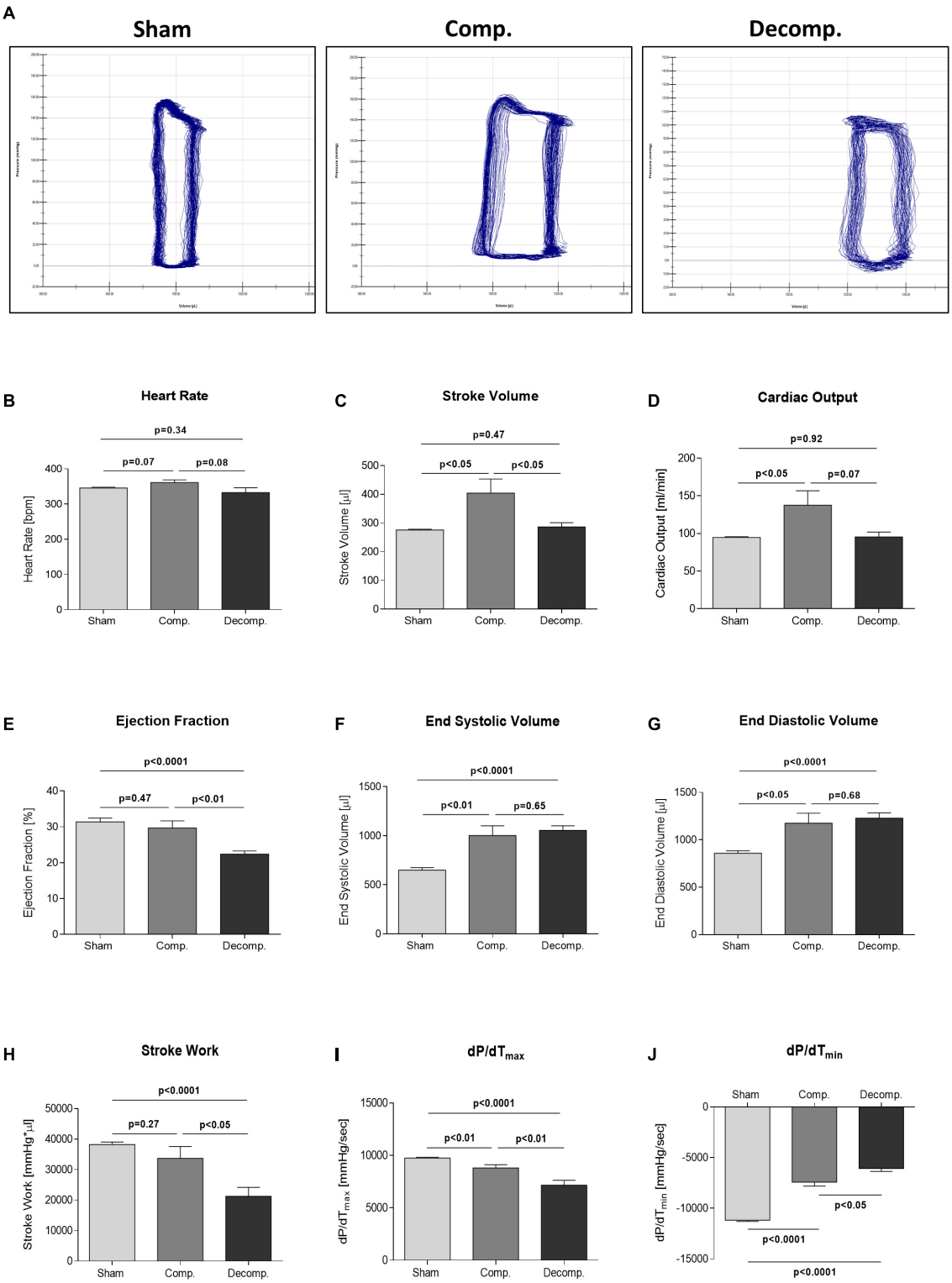

FIGURE 1 | Pressure-volume loops in compensated and decompensated CHF and their sham controls. **(A)** Representative pressure-volume loops of rats with compensated CHF, decompensated CHF, and sham controls. **(B)** Heart rate, **(C)** stroke volume, **(D)** cardiac output, **(E)** ejection fraction, **(F)** end-systolic volume, **(G)** end-diastolic volume, **(H)** stroke work, **(I)** dP/dT_{max}, **(J)** dP/dT_{min}. Ejection fraction equals (end-diastolic volume – end-systolic volume)/end diastolic volume. Stroke work is calculated as the area under the pressure-volume curve (volume*pressure product). Maximum and minimum dP/dt signify the maximal systolic pressure generation as a parameter of left ventricular contractility and the minimal diastolic pressure loss as a parameter of left ventricular relaxation capacity, respectively. Values are means ± SEM.

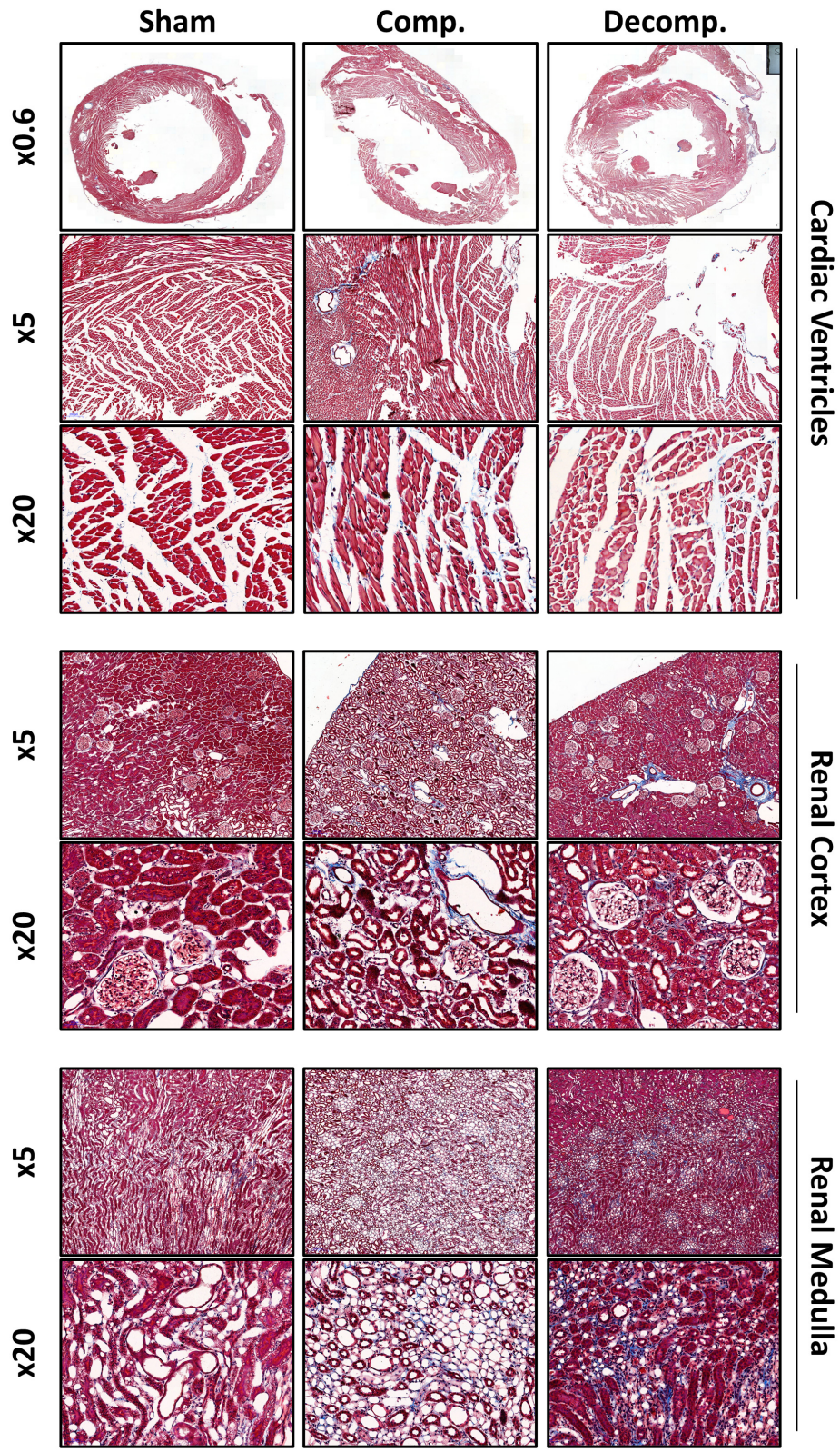

FIGURE 2 | Tissue fibrosis in ACF rats. Representative images of Masson's trichrome staining of the cardiac ventricular and renal tissues, as well as renal cortical and medullar layers, illustrating the impact of ACF placement on the development of tissue remodeling, compared with sham operated rats. Images are presented at x0.6, x5, and x20 magnification.

trichrome staining can also stain the tissue with blue color not only in the presence of fibrosis but also secondary to edema, which is a hallmark feature of HF. Finally, the renal medulla is a collagen-rich tissue, and thus, the obtained trichrome staining may represent basal rather than evolving fibrosis.

Cardiac and Renal Expression of Corin/PCSK6 in Rats With Aorto-Caval Fistula

Heart

As shown in **Figure 3**, rats with decompensated HF exhibit a severe reduction in the immunoreactive relative levels of corin in all cardiac chambers, as compared with both compensated HF and sham-operated groups (**Figures 3C,E,M,O**). In contrast, the compensated subgroup displayed an enhanced abundance of corin in both atrial and ventricular tissues. Specifically, the relative expression of corin immunoreactivity in RA, LA, RV, and LV of decompensated rats was 0.36 ± 0.08 ($P < 0.0001$), 0.16 ± 0.02 ($P < 0.0001$), 0.49 ± 0.1 ($P < 0.01$), and 0.34 ± 0.04 ($P < 0.001$), as compared with 2.61 ± 0.3, 1.74 ± 0.28, 4.01 ± 0.94, 2.33 ± 0.41, in compensated animals, respectively. While corin mRNA expression in the atria of decompensated rats followed a similar pattern, the compensated subgroup also showed downregulation of corin mRNA (**Figures 3G,I**). Interestingly, corin transcript levels in the right and left ventricular tissues were significantly elevated in decompensated HF (2.12 ± 0.36 and 2.89 ± 0.29, respectively) compared with sham controls (1.0 ± 0.04; $P < 0.05$ and 1.0 ± 0.06; $P < 0.001$, respectively) and compensated subgroup (1.38 ± 0.29; $P =$ NS, and 1.1 ± 0.13; $P < 0.0001$, respectively) (**Figures 3Q,S**).

Concerning PCSK6 abundance, both right (0.78 ± 0.04) and left (0.63 ± 0.09) atrium of decompensated HF animals displayed a significant decrease in immunoreactive levels of the enzyme, as compared with compensated HF animals (1.32 ± 0.09; $P < 0.001$ and 1.34 ± 0.13; $P < 0.001$, respectively) and sham-operated rats (1.0 ± 0.04; $P < 0.01$ and 1.0 ± 0.02; $P < 0.01$, respectively) (**Figures 3D,F**). Similarly, PCSK6 abundance was upregulated in right (2.2 ± 0.2;$P < 0.001$) and left (1.65 ± 0.22; $P < 0.05$) ventricles of compensated HF rats, but to a lesser extent in decompensated subgroup (1.55 ± 0.13; $P < 0.01$ and 1.21 ± 0.12; $P =$ NS, respectively), when compared with sham controls (1.0 ± 0.1 and 1.0 ± 0.05, respectively) (**Figures 3N,P**). While PCSK6 mRNA expression in RA (0.77 ± 0.05; $P =$ NS) and RV (0.69 ± 0.06; $P < 0.05$) of the decompensated rats was attenuated, it increased in the compensated subgroup (1.54 ± 0.29; $P =$ NS and 1.17 ± 0.07; $P =$ NS, respectively) as compared with sham controls (1.0 ± 0.17 and 1.0 ± 0.14, respectively) (**Figures 3H,R**).

Finally, the distinct patterns of myocardial PCSK6/corin behavior in rats with compensated and decompensated CHF were also confirmed by immunofluorescence analysis. Immunofluorescence staining revealed intense immunostaining of corin in the cardiac and renal tissues (**Figure 4A**). In the myocardium, corin immunofluorescence was detected mainly in the myocytes, both at the cellular membrane and cytosol. In agreement with WB analysis, immunofluorescence of cardiac corin was enhanced in compensated CHF group, but not in decompensated CHF animals. Cardiac PCSK6 immunofluorescence followed a similar pattern as corin, namely, more abundant in compensated subgroup but not decompensated animals (**Figure 4B**). It should be emphasized that PCSK6 was localized mainly to the myocytes.

Collectively, Western blot, RT-qPCR, and immunofluorescent analysis revealed upregulation of corin and PCSK6 in the myocardium of rats with compensated CHF week, but downregulation of these two enzymes in the decompensated subgroup.

Kidney

Figure 5 depicts the changes in the abundance and expression of both corin (**Figures 5B,F**) and PCSK6 (**Figures 5C,G**) in the renal tissue of compensated and decompensated CHF rats as compared with sham controls. Akin to the myocardium, decompensated rats exhibited a decline in renal corin immunoreactivity and mRNA transcript (0.47 ± 0.13; $P < 0.01$ and 0.9 ± 0.17; $P =$ NS, respectively) as compared with their controls (1.0 ± 0.07 and 1.0 ± 0.04, respectively), whereas compensated CHF displayed upregulation of this enzyme as demonstrated by both the immunoreactive and mRNA levels (1.31 ± 0.22; $P =$ NS and 1.94 ± 0.32; $P < 0.05$, respectively). In line with these changes, PCSK6 abundance was significantly increased in the compensated subgroup (1.5 ± 0.18; $P < 0.05$), but reduced in decompensated animals (0.74 ± 0.09; $P < 0.05$), as compared with sham-operated rats (1.0 ± 0.04) (**Figure 5C**), whereas the expression of PCSK6 was increased in both compensated and decompensated CHF rats (1.52 ± 0.17; $P < 0.05$ and 1.28 ± 0.23; $P =$ NS, respectively), when compared with the control group (1.0 ± 0.04) (**Figure 5G**).

Immunofluorescent analysis of the kidney unraveled the abundance of corin immunoreactivity in the proximal tubule, mTAL, and to a lesser extent in the collecting duct. In the proximal tubule, corin was localized to the apical membrane underneath the brush border and a lesser extent in the cytosol. Interestingly, immunofluorescent staining revealed a remarkable increase of corin in the renal tissue of compensated rats, but a reduction of this enzyme in decompensated animals (**Figure 5J**).

Cardiac and Renal Expression of ADAM10 in Rats With Aorto-Caval Fistula

Since corin is regulated by shedding and releasing to the blood, either by autocleavage or by ADAM10-mediated cleavage (Jiang et al., 2011), we also studied the behavior of this enzyme in the heart and kidneys of CHF rats as compared with sham-operated animals. Representative western blot analysis of cardiac ADAM10 in RA, LA, RV, and LV chambers are presented in **Figures 6A,D,G,J**, respectively. In contrast to RA (0.93 ± 0.05; $P =$ NS), ADAM10 immunoreactivity was increased in LA (1.49 ± 0.04; $P < 0.05$), RV (1.82 ± 0.29; $P < 0.05$) and LV (1.77 ± 0.19; $P < 0.05$) (**Figures 6B,E,H,K**, respectively) of compensated rats compared to their controls (1.0 ± 0.02, 1.0 ± 0.11,1.0 ± 0.06, and 1.0 ± 0.04, respectively). Noteworthy, the abundance of ADAM10 did not change in the various cardiac chambers of decompensated rats. The expression of ADAM10 followed a

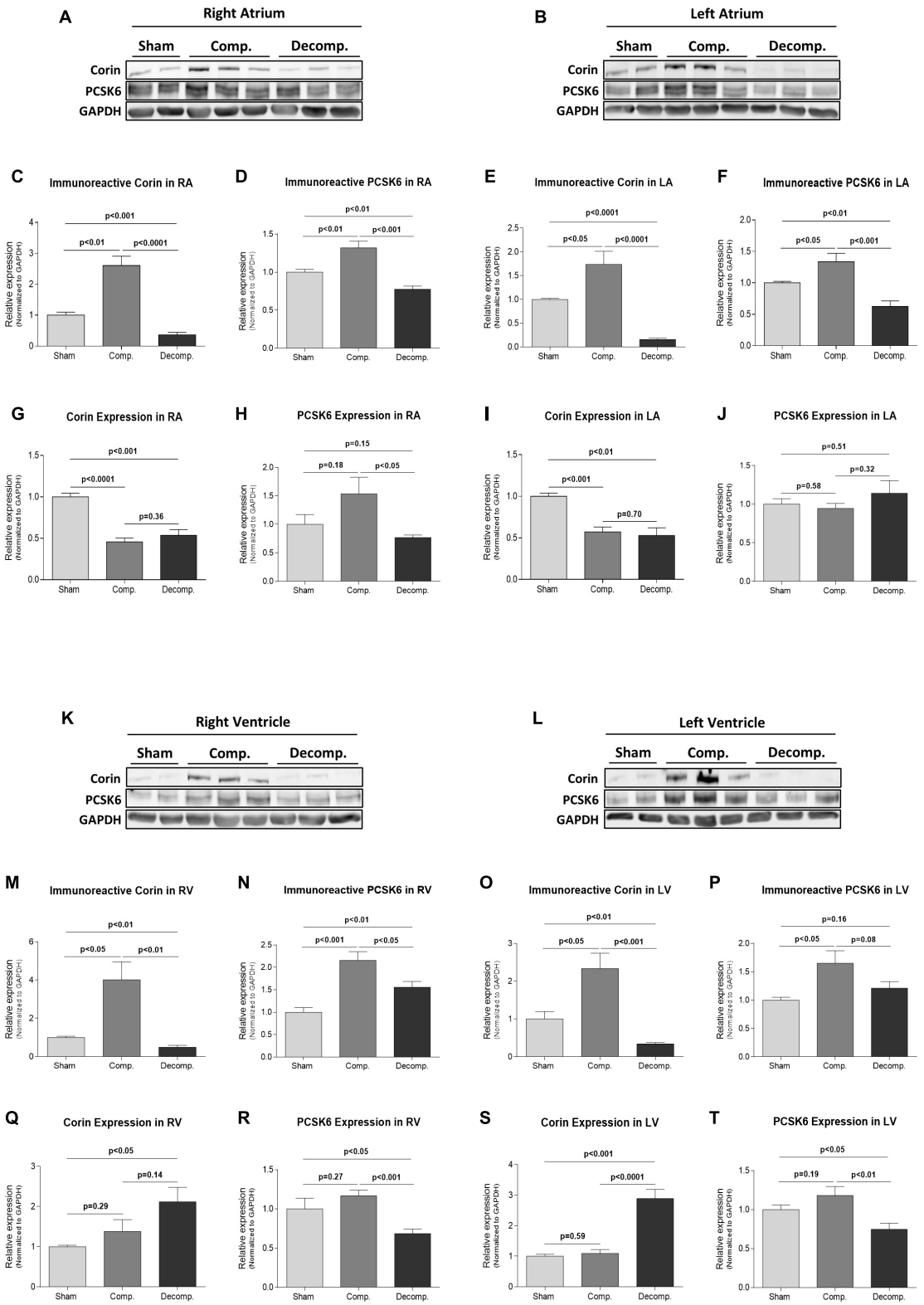

FIGURE 3 | Levels and expression of corin and PCSK6 in the cardiac tissue of compensated CHF, decompensated CHF, and sham controls. Representative western blot analysis of corin and PCSK6 in RA, LA, RV, and LV tissue lysates are shown in **(A,B,K,L)**, respectively. **(C–F,M–P)** Represent western blot analysis relative quantification of corin and PCSK6 in the different heart chambers, where GAPDH was used as a loading control. Quantification of qPCR analysis for corin and PCSK6 mRNA normalized to GAPDH are depicted in **(G–J,Q–T)**. Values are means ± SEM.

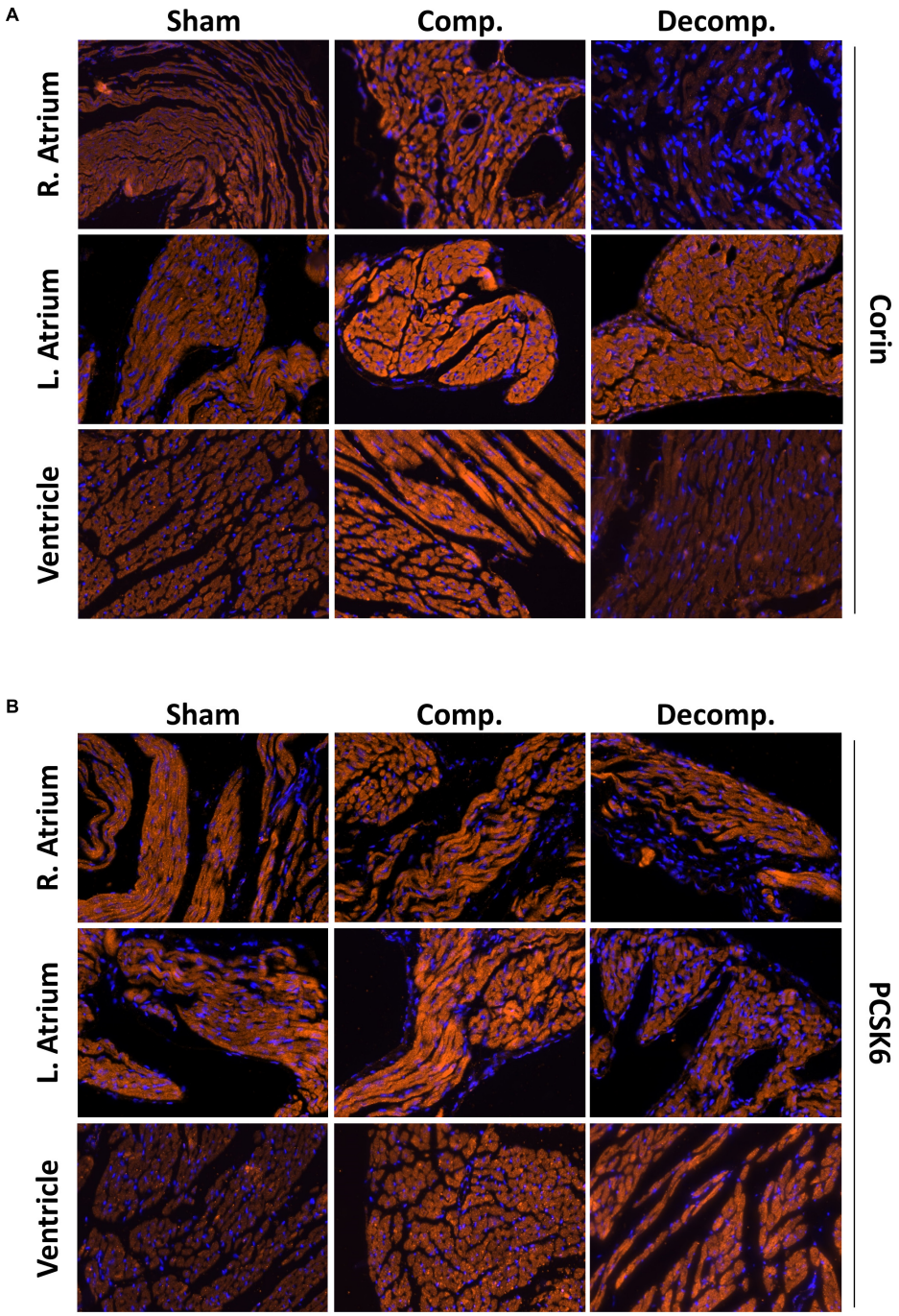

FIGURE 4 | Corin/PCSK6 staining in the heart chambers of ACF and sham-operated rats. Representative images of immunofluorescence staining for corin **(A)** and PCSK6 **(B)** in the cardiac atrial and ventricular tissues of rats with compensated CHF, decompensated CHF, and sham-operated animals. Tissues were counterstained with DAPI. All images were obtained at x40 magnification.

similar pattern as the immunoreactive levels, i.e., upregulation in RA, LA, and LV (**Figures 6C,F,L**, respectively), but not RV (**Figure 6I**). Specifically, ADAM10 mRNA was downregulated in the RV and LV of the decompensated rats, but it did not change in the atrial tissues. At the renal level, ADAM10 immunoreactivity was significantly reduced in compensated rats (0.52 ± 0.1; $P < 0.01$) but did not change in the decompensated subgroup (1.08 ± 0.19; $P =$ NS), compared with control animals (1.0 ± 0.06) (**Figure 5D**). ADAM10 expression was significantly increased in compensated CHF rats (1.47 ± 0.21; $P =$ NS) but decreased in decompensated animals (0.79 ± 0.03; $P < 0.01$), as compared with sham-operated rats (1.0 ± 0.03) (**Figure 5H**).

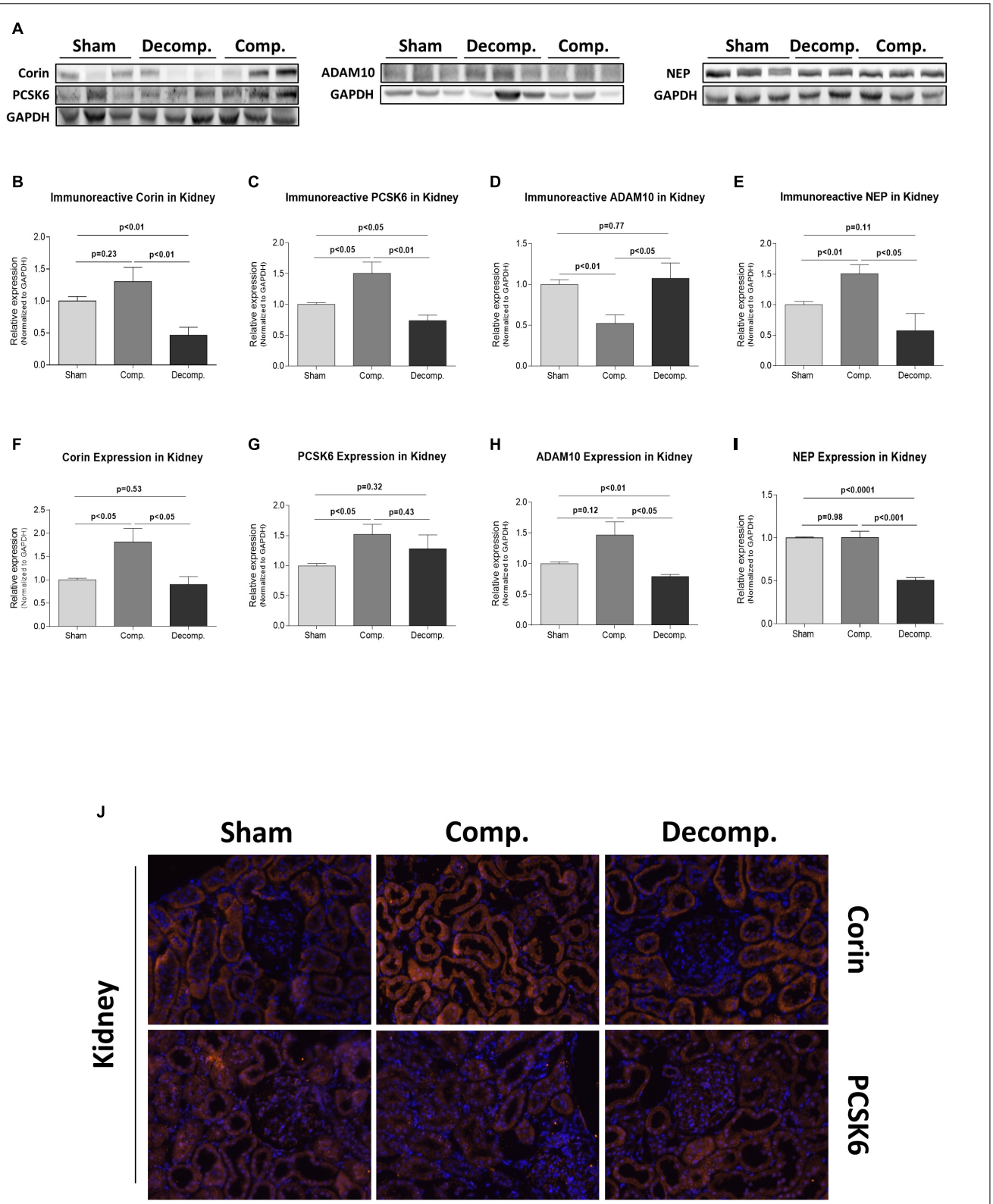

FIGURE 5 | Natriuretic peptide machinery system in the renal tissue of ACF and sham-operated rats. Representative western blot analysis **(A)** with antibodies against corin, PCSK6, ADAM10, and NEP and their quantification relative to GAPDH **(B–E)** in the kidney of ACF rats and their sham operated rats. **(F–I)** represents quantification of qPCR analysis of the mRNA of these enzymes relative to GAPDH. **(J)** Representative images of immunostaining of corin and PCSK6 in the kidney of the various animal groups, counterstained with DAPI. All images were obtained at x40 magnification. Values are presented as means ± SEM.

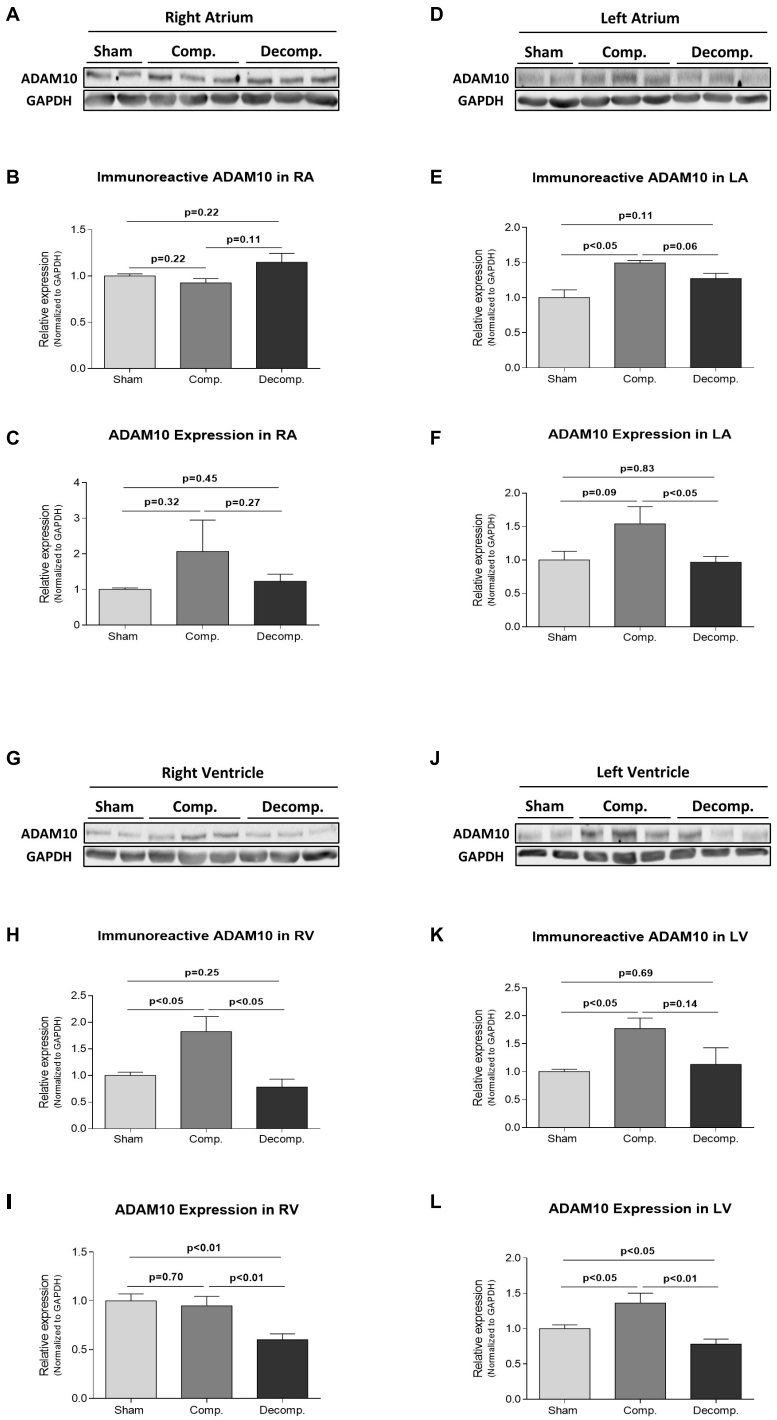

FIGURE 6 | Abundance and expression of ADAM10 in heart chambers of ACF and sham-operated rats. Representative western blot analysis of ADAM10 **(A,D,G,J)** and its quantification relative to GAPDH **(B,E,H,K)** in RA, LA, RV, and LV, respectively, of ACF and sham-operated rats. **(C,F,I,L)** Represent quantification of qPCR analysis of ADAM10 mRNA relative to GAPDH in the heart chambers of the different animal groups. Values are presented as means ± SEM.

Renal Expression of Neprilysin in Rats With Aorto-Caval Fistula

Since NEP is a key enzyme in the degradation of NPs (Kenny et al., 1993; Watanabe et al., 1997), its abundance and expression in the renal tissue were also determined. **Figures 5E,I** depict the expression and abundance of this enzyme in the renal tissue of the studied experimental groups, respectively. While NEP immunoreactivity was significantly increased in

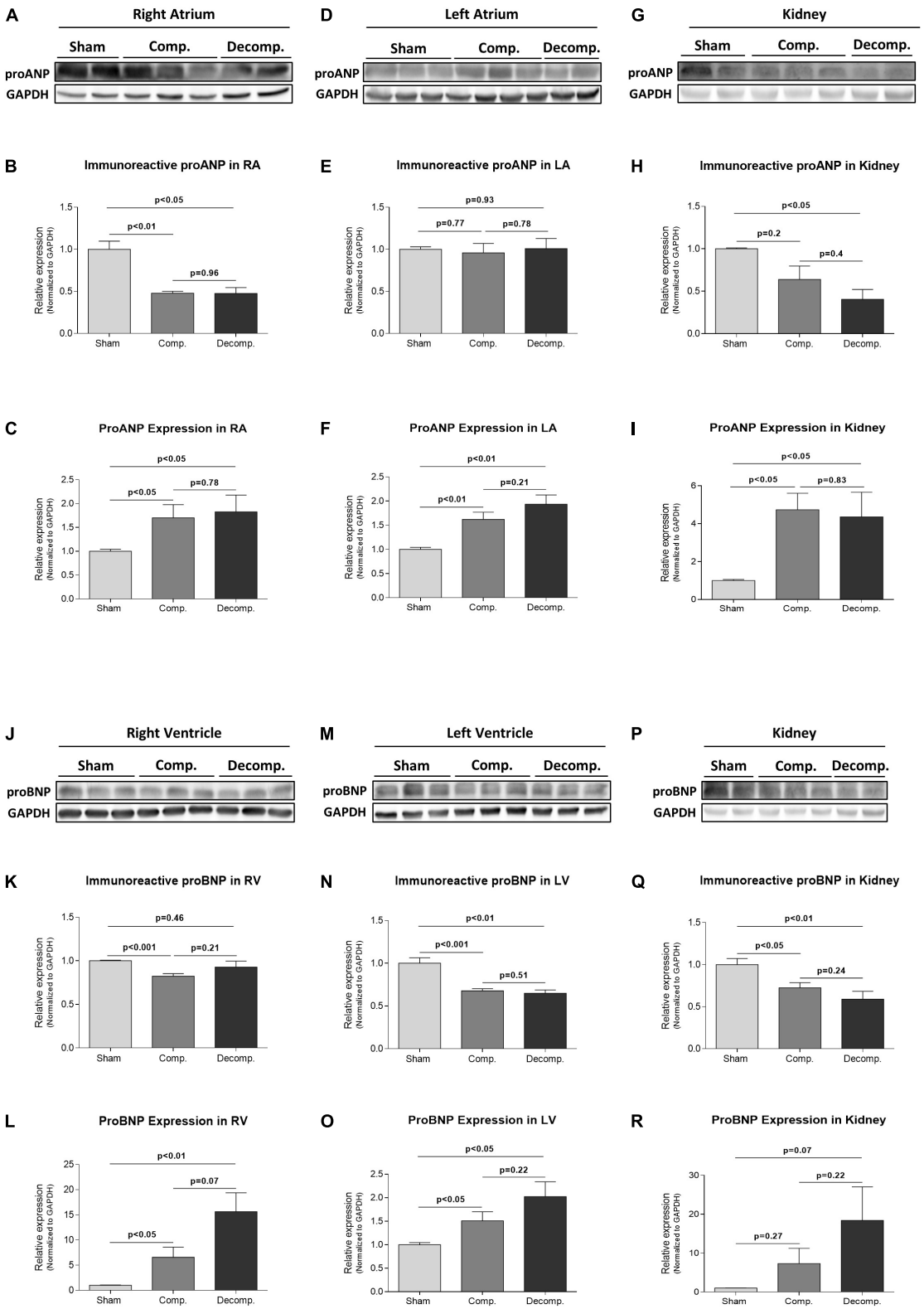

FIGURE 7 | Abundance and expression of atrial and B-type natriuretic peptides in the cardiac and renal tissues of ACF and sham-operated rats. Representative western blot analysis of proANP **(A,D,G)** and proBNP **(J,M,P)** and their quantification relative to GAPDH **(B,E,H,K,N,Q)** in heart chambers and kidneys of ACF and sham-operated rats. **(C F,I,L,O,R)** Represent quantification of qPCR analysis of proANP and proBNP mRNA relative to GAPDH in the same tissues. Values are presented as means ± SEM.

the kidney of compensated CHF animals (1.51 ± 0.51; $P < 0.01$), it slightly decreased in the decompensated animals (0.57 ± 0.28; P = NS) compared with controls (1.0 ± 0.05). NEP expression was significantly decreased in decompensated animals (0.51 ± 0.03; $P < 0.0001$) but did not change in the compensated subgroup (1.0 ± 0.07; P = NS), as compared with shams (1.0 ± 0.01).

Cardiac and Renal Expression of Atrial Natriuretic Peptide and Brain Natriuretic Peptide in Rats With Aorto-Caval Fistula

After unraveling the presence of Corin/PCSK6 transcripts, we examined the expression and abundance of proANP and proBNP hormones in the atrial and ventricular myocardial chambers, respectively, and also in the renal tissue of the various experimental groups. **Figures 7A,D,G** depict representative western blot analysis of proANP in RA, LA, and renal tissues of compensated and decompensated ACF-operated rats, as compared to control animals, respectively. While the immunoreactive levels of proANP decreased in RA (**Figure 7B**) in both compensated and decompensated CHF rats (0.48 ± 0.02; $P < 0.01$, 0.48 ± 0.07; $P < 0.01$ vs. 1.0 ± 0.01, respectively), but not in LA of rats with CHF (**Figure 7E**), the expression of proANP increased in both RA and LA as a function of CHF severity (**Figures 7C,F**). A similar pattern was obtained also in renal proANP abundance, where it declined in CHF subgroups (**Figure 7H**). The downregulation of proANP abundance in the kidney was associated with the upregulation of its renal expression in both subgroups of CHF rats (**Figure 7I**). Representative western blot analysis of proBNP in RV, LV, and renal tissues are shown in **Figures 7J,M,P**, respectively. Applying western blot analysis for proBNP in the cardiac ventricles (the major site of BNP production) revealed attenuated levels of proBNP immunoreactivity in the LV of both compensated and decompensated CHF rats (0.68 ± 0.02; $P < 0.001$, 0.65 ± 0.04; $P < 0.01$ vs. 1.0 ± 0.06, respectively), but not RV (**Figures 7K,N**). In contrast, the expression of proBNP in both RV and LV was enhanced in rats with ACF in correlation with the severity of CHF (**Figures 7L,O**). In line with the alterations in renal proANP, immunoreactive proBNP in the kidney displayed a similar pattern; namely, it declined in CHF subgroups (**Figure 7Q**), but its expression was augmented in these animals (**Figure 7R**).

DISCUSSION

The current study sheds light on the status of corin and PCSK6 in the cardiac and renal tissues of rats with CHF of various severities. As we have demonstrated in several studies, rats with ACF display cardiac hypertrophy, pulmonary congestion, and impaired kidney function, and renal hypoperfusion, which are typical manifestations of CHF (Abassi et al., 1990, 2001, 2011; Khoury et al., 2018). In this context, placement of ACF caused an overt increase in heart/body weight (BW), an index of cardiac hypertrophy, which was more evident in decompensated CHF after 1 week from surgery. In addition, rats with ACF exhibited reduced cardiac performance as a function of the severity of the disease. Moreover, absolute and normalized lung weight to BW of CHF rats was also higher than those of controls. In contrast, kidney weight normalized to BW was lower in both subgroups of CHF rats, namely compensated and decompensated, as compared with sham-operated rats. These features support the reliability of ACF as a CRS experimental model.

Along with these renal and cardiac hemodynamic alterations, the present study clearly shows that corin immunoreactive levels were significantly increased in all cardiac chambers of compensated but decreased in decompensated CHF. In line with these findings, the immunofluorescent analysis revealed upregulation and downregulation of myocardial corin immunoreactive protein in compensated and decompensated CHF, respectively. The pattern of corin staining strongly suggests cytosolic and myocyte membranal localization within cells that also contain proANP and proBNP (Hooper et al., 2000; Bialik et al., 2001; Gladysheva et al., 2008). This pattern supports the concept that corin is transported from the intracellular compartment to the cell membrane, where it converts proANP and proBNP to ANP and BNP upon secretion (Wu et al., 2009). Concomitant with the alterations in cardiac corin abundance, corin mRNA was detected in the myocardium of the various experimental groups, where its expression was downregulated in the atrial tissues of both compensated and decompensated rats. In contrast, the expression of corin was upregulated in the ventricular tissues of decompensated subgroup and to a lesser extent in the compensated one.

Concerning PCSK6, our results revealed that CHF is associated with changes in this key enzyme, corresponding to corin behavior. Specifically, PCSK6 increased in all heart chambers in compensated CHF, yet significantly decreased in the atrial tissues and RV but to a lesser extent in the LV of the decompensated subgroup. Finally, the present study unequivocally demonstrates the existence of corin and PCSK6 in the renal tissues as was evident by WB, PCR, and immunohistochemical analysis, especially in the proximal tubule and the collecting duct. Interestingly, compensated CHF was associated with a slight increase of these two enzymes, whereas decompensated CHF exhibited a decline in both the renal corin and PCSK6, suggesting a role of locally produced NPs in the regulation of salt/water excretion on one hand and that perturbations in this system may play a role in the pathogenesis of Na+ retention and edema formation during the decompensated stage.

The present study extends previously published research that examined the changes in myocardial corin and its relevance to the pathogenesis of HF. In this regard, patients with decompensated HF displayed lower levels of circulating corin along with decreased cleavage of proANP (Ibebuogu et al., 2011). In agreement with these findings, Langenickel et al. (2004) showed that corin declined in the atrium 4 weeks after CHF induction by a similar surgical maneuver applied in the present study, namely ACF (Langenickel et al., 2004). In contrast, overexpression of corin mRNA was obtained in the non-infarcted LV myocardium after 8 weeks from the induction of hypertrophied ventricle induced by left anterior

descending (LAD) artery ligation in rats (Tran et al., 2004). Unfortunately, the clinical study focused on circulating corin (Langenickel et al., 2004), whereas in the LAD model the authors measured mRNA corin, but not immunoreactive levels (Tran et al., 2004). Thus, our approach of measuring both the expression and abundance of corin simultaneously in the various heart chambers and the kidney provides a more comprehensive picture of the status of this enzyme in rats with CHF of various severities.

Our findings that corin immunoreactivity and expression in the various heart compartments increased in the compensated and declined in decompensated subgroup could be of clinical relevance. Considering that corin plays a beneficial role against the development of either dilated cardiomyopathy (DCM) or reduced ejection fraction, HF, and regulation of salt homeostasis (Tripathi et al., 2019), upregulation of cardiac corin in compensated CHF rats may play a role in maintaining normal Na^+ balance, whereas the decline in this key enzyme in decompensated subgroup likely aggravates salt retention, edema formation, and cardiac hypertrophy. Support for this concept is derived from the findings that reduced plasma corin levels have been reported in patients with HF, and the magnitude of reduction in plasma corin levels correlates with HF severity (Dong et al., 2010; Ibebuogu et al., 2011). In addition, cardiac-specific expression of catalytically active corin delays the onset of symptomatic CHF associated with lung edema and extends life in the experimental mouse model of DCM (Gladysheva et al., 2013; Ngo et al., 2013). Moreover, upregulation of cardiac corin mRNA was observed in the cardiac tissue of heart transplant recipients with preserved cardiac function in comparison with DCM and reduced ejection fraction HF (Verstreken et al., 2019). Collectively, these findings indicate that enhanced levels of locally cardiac corin levels might delay the progression of cardiac dysfunction and remodeling.

Our study also examined the changes in PCSK6, the primary activating enzyme of corin, in the various experimental groups. The current results demonstrate that the changes in myocardial corin correspond with PCSK6 alterations (Chen et al., 2015). These changes in the PCSK6/corin/ANP-BNP pathway have adverse physiological consequences which affect the production of ANP/BNP from proANP/proBNP, respectively, and eventually resulting in the disruption in sodium balance and cardiac remodeling (Ibebuogu et al., 2011). Indeed, corin deficiency in mice was associated with reduced sodium excretion and salt sensitive hypertension and cardiac hypertrophy (Chan et al., 2005; Wang et al., 2012). Likewise, PCSK6-deficient mice display salt-sensitive hypertension (Chen et al., 2015). Our findings that PCSK6/corin decreased in decompensated rats but increased in compensated ones may contribute to the enhanced cardiac hypertrophy and decline in Na^+ excretion in the former but not the latter. The sustained elevation of ANP/BNP plasma levels despite the decline in both PCSK6/corin in our rats could be attributed to assumedly sufficient levels of the remnant two enzyme or alternatively to the detached circulatory PCSK6/corin which retains proteolytic activity and is capable of maintaining enhanced production of NPs.

One of the main novel aspects of the present study is the exploration of corin/PCSK6 status in the renal tissue of compensated and decompensated CHF animals. Expression of corin has been documented in both animal (Polzin et al., 2010) and human kidneys (Langenickel et al., 2004; Tran et al., 2004) in the same localizations as those observed in our study. The status of corin in the renal tissue of rats with CHF was not studied yet. Our findings revealed upregulation and downregulation of corin in compensated and decompensated CHF, respectively. These results suggest a role of corin in kidney function, both in health and in renal dysfunction, under edematous disease states such as CHF, as this machinery may be responsible for the local conversion of proANP to ANP, which in its turn act in an autocrine manner to regulate sodium/water reabsorption. Besides CHF, Polzin et al. (2010) have demonstrated a downregulation in renal corin along with severe proteinuria and salt/water retention in an experimental model of nephrotic syndrome. Also in this study, the immunoreactive corin was localized to the proximal tubule, mTAL, and collecting duct. The latter is a well-known site for ANP action, and assumedly, ANP production. Specifically, proANP, natriuretic peptide receptor-A (NPR-A) mRNA, and their immunoreactive peptides were detected in this segment of the nephron (Zeidel, 1990; Polzin et al., 2010; Ichiki et al., 2011; Dong et al., 2016). Our discovery that proximal tubule and mTAL also expresses corin is in line with similar findings in specimens of human kidney (Dong et al., 2016) and strongly suggests that this nephron segment may be a major site of ANP production. Concerning the proximal tubule, a previous study demonstrated that proANP and NPR-A mRNA are also expressed in this segment of the tubule even more than the collecting duct (Dong et al., 2016). Interestingly, renal immunoreactive levels of both proANP and proBNP were declined in rats with compensated CHF and to a larger extent in the decompensated subgroup. The significant upregulation in the expression of these NPs in the renal tissue may represent a compensatory response to the decline in the immunoreactive peptides.

Additional enzyme that may affect the local and circulatory levels of corin is ADAM10. ADAM10 is responsible for corin shedding, thus affecting the abundance of corin on the cellular membrane, as well as its plasma levels. In this context, soluble corin was detected in the blood of normal subjects, and its levels were significantly reduced in patients with severe HF as compared with healthy subjects and even patients with mild cardiac dysfunction (Dong et al., 2010; Ibebuogu et al., 2011). These findings suggest that corin shedding from the cells by ADAM10 may be an important mechanism for regulating corin function and that altered corin shedding and/or cleavage may play a role in the pathogenesis of HF (Jiang et al., 2011). In this context, the current study demonstrated that rats with compensated, but not decompensated CHF, were characterized by upregulation of ADAM10 in most of the cardiac chambers. In contrast, renal ADAM10 was downregulated in the compensated subgroup. Considering that ADAM10 causes shedding of an active corin fragment (Jiang et al., 2011), changes in cardiac and renal expression of ADAM10 may play a role in the regulation of corin abundance and subsequently

cardiac remodeling/function and sodium balance. Moreover, corin shedding may constitute a physiological approach aimed at preventing excessive, potentially hazardous, proteolytic activities in the heart.

Since plasma levels of ANP/BNP are not only affected only by corin but also by NEP, an enzyme responsible for ANP/BNP degradation, renal abundance/expression of NEP was determined in the various experimental groups. While compensated CHF rats exhibited enhancement in NEP in the renal tissue, decompensated subgroup displayed a decline in this enzyme. The physiological significance of this phenomenon remains to be determined; however, it may represent a counterbalance response to the upregulation of corin in the compensated animals to avoid the exaggerated production of NPs.

Since furin is an additional key enzyme responsible for the activation of BNP, we previously examined the status of furin in the cardiac and renal tissues of compensated and decompensated animals and their controls (Khoury et al., 2021). Briefly, furin immunoreactivity in the LV was significantly enhanced in compensated and to a lesser extent in decompensated CHF subgroup, as compared with sham controls. A similar trend was detected in the RV of compensated, but not of decompensated CHF subgroup (Khoury et al., 2021). In line with these findings, the abundance and expression of furin were remarkably enhanced in the RA and LA of rats with CHF (**Supplementary Figures S1A–F**). Neither the abundance nor the expression of furin in the kidney has changed following the induction of CHF as was published recently (Khoury et al., 2021). Finally, similar to its behavior in cardiac tissue, both immunoreactive and expression of furin in the pulmonary tissue were significantly enhanced in correlation with CHF severity (Khoury et al., 2021).

In summary, we demonstrated similar cardiac and renal patterns of corin changes at the mRNA and protein levels as was evident by qPCR, WB, and immunohistochemistry analysis. Specifically, we found upregulation of corin and PCSK6 in the various heart chambers of compensated CHF but a decline in these two enzymes in the decompensated subgroup. Similarly, the application of the same molecular analysis to the kidney revealed comparable corin behavior as in cardiac tissue. Of note, in the kidney, the corin was abundant in the proximal tubule, mTAL, and collecting duct, suggesting that locally produced ANP may act as an autocrine mediator of salt/water homeostasis not only by acting on the CD but probably *via* proximal tubule and mTAL segments. We hypothesize that the obtained initial upregulation of cardiac and renal PCSK6/corin may represent a compensatory response to maintain Na^+ balance, which subsequently fails as the disease progresses.

Yet, our study has few limitations: (1) It does not directly address mechanisms involved in the changes in the transcription and expression of corin and PCSK6 and the molecules linked to these enzyme regulations caused by CHF. Further studies are needed to assess their status in humans with CHF, and our relevant discussion is to a large extent speculative. Yet these assumptions and suggestions should be further evaluated, since they may bear clinical relevance with plausible interventional implications in this evolving disease; (2) additional limitation of the current study is the short follow up period (1 week), which represents acute CHF, rather than a chronic condition that usually develops in humans in many years; (3) we applied an acute CHF study carried out in an experimental model induced by a volume overload method, different from what is normally seen in humans (myocardial infarction or hypertension); this makes it difficult to translate the results to patients. (4) Normalized cardiac hypertrophy (HW/BW) was more evident in the decompensated subgroup; however, body weight loss in this subset of animals may have contributed to the calculated cardiac hypertrophy; (5) trichrome staining can stain tissues with blue color not only secondary to fibrosis, but also as a result of edema.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Experiments were performed according to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) as approved by the local institutional committee for supervision of animal experiments in the Technion.

AUTHOR CONTRIBUTIONS

EK: conceptualization, data collection and analysis, methodology, writing–original draft, and writing–review and editing. AF: data collection and analysis, methodology (immunostaining and RT-PCR). SK: methodology and software. YK: methodology (immunostaining) and software. DA: conceptualization, investigation (lead), writing–review and editing. ZA: conceptualization (lead), data collection, formal analysis (lead), funding acquisition (lead), investigation (lead), methodology (equal), project administration (lead), resources (lead), software, supervision (lead), validation (lead), writing–original draft (lead), and writing–review and editing (lead). All authors: contributed to the article and approved the submitted version.

FUNDING

The authors wish to acknowledge research support from the Israel Science Foundation (Grant No. 544/18). In addition, they are grateful to Aviva Kabala for her technical assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.673497/full#supplementary-material

Supplementary Figure S1 | Abundance and expression of furin in the atria of ACF and sham-operated rats. Representative western blot analysis of furin **(A,D)** and its quantification relative to GAPDH **(B,E)** in RA and LA of ACF and sham-operated rats. **(C,F)** Represent quantification of qPCR analysis of furin mRNA relative to GAPDH in the same tissues. Values are presented as means ± SEM.

Supplementary Table S1 | Impact of aortocaval placement on heart, lung, and kidney weights as compared with sham operated controls. Cardiac, lung, and kidney weights expressed either as absolute values or relative to bodyweight of rats with compensated and decompensated CHF and their sham controls. Values are means ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ vs. sham-operated rats. $^{\dagger}P < 0.05$; $^{\dagger\dagger}P < 0.01$; $^{\dagger\dagger\dagger\dagger}P < 0.0001$ vs. compensated CHF group.

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frontiers in Physiology

ORIGINAL RESEARCH
published: 11 February 2022
doi: 10.3389/fphys.2021.815796

Natriuretic Peptides and Blood Pressure Homeostasis: Implications for MANP, a Novel Guanylyl Cyclase a Receptor Activator for Hypertension

Valentina Cannone[1,2]* *and John C. Burnett Jr.*[1,3]

[1] Cardiorenal Research Laboratory, Department of Cardiovascular Medicine, Mayo Clinic, Rochester, MN, United States, [2] Department of Medicine and Surgery, University of Parma, Parma, Italy, [3] Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN, United States

The heart serves as an endocrine organ producing the hormones atrial natriuretic peptide (ANP) and b-type natriuretic peptide (BNP) which *via* the guanylyl cyclase A (GC-A) receptor and the second messenger cGMP participate in blood pressure homeostasis under physiologic conditions. Genetic models of the ANP gene or the GCA receptor together with genomic medicine have solidified the concept that both cardiac hormones are fundamental for blood pressure homeostasis and when deficient or disrupted they may contribute to human hypertension. Advances in peptide engineering have led to novel peptide therapeutics including the ANP-analog MANP for human hypertension. Most importantly a first in human study of MANP in essential hypertension has demonstrated its unique properties of aldosterone suppression and blood pressure reduction. Physiology and pharmacology ultimately lead us to innovative peptide-based therapeutics to reduce the burden of cardiovascular disease.

Keywords: peptide, cGMP, receptors, genetic variation, blood pressure, natriuretic peptides, hypertension, MANP

OPEN ACCESS

Edited by:
Alberto Giannoni, Sant'Anna School of Advanced Studies, Italy

Reviewed by:
Thomas Langenickel, Novartis, Switzerland
Gregory Fink, Michigan State University, United States

***Correspondence:**
Valentina Cannone
valentina.cannone@unipr.it

Specialty section:
This article was submitted to Clinical and Translational Physiology, a section of the journal Frontiers in Physiology

Received: *15 November 2021*
Accepted: *24 December 2021*
Published: *11 February 2022*

Citation:
Cannone V and Burnett JC Jr (2022) Natriuretic Peptides and Blood Pressure Homeostasis: Implications for MANP, a Novel Guanylyl Cyclase a Receptor Activator for Hypertension. Front. Physiol. 12:815796. doi: 10.3389/fphys.2021.815796

INTRODUCTION

Hypertension still represents the leading cause of death worldwide. Importantly, ranking number two in cause of death is metabolic disease including diabetes and obesity both of which are predisposing and coexisting factors for hypertensive disease (Foreman et al., 2018). Much progress has been made over the last decades in developing novel therapies for hypertension both from the perspective of drugs as well as devices. Nonetheless there is increasing percentage of hypertensive patients worldwide who have uncontrolled blood pressure which cannot be attributed to non-compliance (Muntner et al., 2020). Indeed, the consensus is emerging that hypertension is a growing healthcare burden with rising morbidity and mortality. This challenge is highlighted in a report from the National Institutes of Health Working Group in Hypertension which called for new research regarding mechanisms and novel therapies especially in the setting of difficult to control hypertension such as resistant hypertension (Sigmund et al., 2020). Here, in this Review we will highlight the physiology of the heart as an endocrine organ producing the two important blood pressure lowering hormones atrial natriuretic peptide (ANP) and b-type natriuretic peptide (BNP).

Key to their biology is their common molecular target, the particulate guanylyl cyclase receptor A (GC-A), and the second messenger cyclic guanosine monophosphate (cGMP) which mediates widespread cardiovascular, renal and endocrine biological actions (Kuhn, 2016). We will also highlight the importance of human genetic population studies which built upon previous genetic animal models focused on the ANP and GC-A pathway. Indeed, these human population studies have been key in laying the foundation for innovative therapeutics targeting the GC-A/cGMP system. We also summarize key advances in designer natriuretic peptides being developed to target GC-A for the treatment of hypertension.

BLOOD PRESSURE AND THE CARDIAC HORMONES: INSIGHTS FROM THE LABORATORY AND GENETIC VARIANTS IN THE GENERAL POPULATION

The work of DeBold et al. (1981) launched the ever-expanding research area of the heart as an endocrine organ. The seminal observation of DeBold was that a substance from atrial myocardium of rats when injected into living animals reduced blood pressure and increased sodium excretion. Later studies elucidated that the substance was a peptide, which we defined as atrial natriuretic peptide (ANP), and subsequently its gene natriuretic peptide precursor A (NPPA) was identified. Further studies from the laboratory of Ferid Murad reported that the molecular target of ANP was membrane bound GC-A which activated cGMP (Waldman et al., 1984).

In two separate laboratories, mouse models were created to delete the ANP or the GC-A receptor genes (Lopez et al., 1995; Oliver et al., 1997). Both murine models validated that disruption of GC-A/cGMP pathway resulted in hypertension. Thus, it was established the concept that the heart is an endocrine organ which *via* a hormonal mechanism regulates blood pressure under physiologic conditions. Various studies involving additional genetic mice models reinforced the notion of cardiac hormones as blood pressure regulators. Further investigations also revealed that beyond blood pressure, GC-A protects the cardiomyocyte from hypertrophy (Holtwick et al., 2003). Importantly, a second cardiac hormone, BNP, which also binds to GC-A and activates cGMP, was later discovered. Like the ANP knockout mouse model, deletion of the BNP gene (NPPB) in rats resulted in hypertension and end organ damage, while BNP gene delivery chronically increased BNP in spontaneously hypertensive rats, reduced blood pressure and prevented organ dysfunction (Cataliotti et al., 2011; Holditch et al., 2015). These seminal laboratory studies cemented the important role of these two cardiac hormones in blood pressure regulation and in the pathophysiology of hypertension.

There was a seminal jump from the mouse to the human with the landmark study by Newton-Cheh et al. (2009). These investigators employed genomic medicine in large populations recruited in Northern Europe and North America and sought to identify NPPA and NPPB common genetic variants that were associated with circulating levels of natriuretic peptides and blood pressure. Importantly, the NPPA genetic variant rs5068 was shown to be associated with an increase in circulating levels of ANP and a small but significant reduction in blood pressure. Most importantly, this genetic variation, which was related to a lifelong increase in ANP levels, reduced the risk of hypertension underscoring the seminal role for this cardiac hormone in blood pressure homeostasis.

The Mayo Clinic is located in the Olmsted County, in southeastern Minnesota. Historically, Olmsted County has been inhabited mostly by Caucasians of Northern European ancestry. The county is unique from the standpoint that residents and families remain in the area for generations and are optimal for epidemiologic studies of human health. The Rochester Epidemiology Project is a longstanding National Institutes of Health supported study which takes advantage of the fact that almost all of the residents of Olmsted County take their care at the Mayo Clinic. Thus, medical histories, which are well preserved and maintained within the Mayo Clinic, are readily available with informed consent to elucidate the natural history of human disease and population phenotypes (Rocca et al., 2012). Cannone et al. (2011) sought to validate the observation of Newton-Chen that the ANP gene variant rs5068 was related with blood pressure and they further moved beyond investigating not only the cardiovascular but also the metabolic phenotype associated with rs5068 genotypes. The scientific background was provided by previous *in vitro* and *in vivo* studies together with human studies demonstrating that ANP and BNP induce lipolysis and browning of white adipocytes (Goetze et al., 2020). Importantly, Cannone et al. (2013) showed that rs5068 was associated with higher circulating levels of ANP and lower systolic blood pressure but also lower prevalence of obesity and metabolic syndrome. Other key findings included the relationship with higher HDL levels and reduced risk for myocardial infarction. Validation studies in a rural community in Sicily confirmed similar findings. In a follow up study in the Multi-Ethnic Study in Atherosclerosis (MESA) cohort, which includes African Americans without cardiovascular disease in the United States, Cannone et al. (2017) reported that rs5068 was associated with lower risk of obesity and metabolic syndrome but no association was found with hypertension. These observations might be related to the well documented lower levels of circulating ANP and BNP in African Americans, such deficiency may also explain the higher risk for cardiovascular disease in this ethnicity (Gupta et al., 2015).

The BNP gene NPPB is located adjacent to the ANP gene NPPA on chromosome one. The BNP genetic variant rs198389, which is a functional variant in the promoter region of the gene resulting in higher circulating BNP levels, and related clinical phenotype have been extensively studied. In a population study including African Americans and whites from the Atherosclerosis Risk in Communities (ARIC) study, Seidelmann et al. (2017) investigated phenotype and cardiovascular outcomes associated with rs198389 over two decades. The investigators reported that the minor allele had a frequency of approximately 40% and was associated with 41% higher levels of N-terminal–proBNP and, importantly, lower systolic and diastolic blood

pressure, use of antihypertensive medications as well as prevalence of hypertension while lifespan was increased.

Cannone and colleagues extended studies on rs198389 focusing on a population with cardiovascular risk factors. Specifically, these investigators utilized the landmark St. Vincent Screening to Prevent Heart Failure Study (Stop-Heart Failure Study), which investigated the efficacy of using BNP based screening in combination with collaborative care between primary care physicians and cardiovascular specialists in the prevention of new onset heart failure and left ventricular dysfunction (LVD) (Ledwidge et al., 2013). Importantly, the trial demonstrated that a clinical approach focused on BNP screening and cooperative care reduced the risk to develop LVD and heart failure.

Therefore, Cannone et al. (2021) genotyped the Stop Heart Failure cohort for rs198389, assessed cardiovascular phenotype along with circulating BNP levels and performed a follow up analysis to assess risk of LVD using echocardiography as well as risk for adverse multiple cardiovascular outcomes. Key findings were that among subjects at risk for heart failure (Stage A and B heart failure) the BNP genetic variant rs198389, which is associated with higher circulating levels of BNP, was also associated with lower risk of hypertension, new onset of LVD and major adverse cardiovascular events. The authors concluded that the data support the role of BNP genetic testing in such a population as well as BNP or GC-A activating therapies for the prevention of hypertension and heart failure.

Thus, this later study went beyond a simple biomarker investigation with relevance to precision medicine (Lanfear and Luzum, 2021). The study of a single genetic variant might be considered simplistic but provides proof of concept regarding the biology of the specific gene product, which, in this case, is represented by the GC-A activating hormone BNP. This study, as well as the others referenced above, support the concept that in the presence of a relative ANP or BNP deficiency, one could pursue natriuretic peptide elevating strategies to prevent adverse cardiovascular outcomes and optimally control blood pressure.

MANP–A NOVEL ANP ANALOG TARGETING GUANYLYL CYCLASE A RECEPTOR: A THERAPEUTIC OPPORTUNITY FOR HYPERTENSION

Investigations of ANP and BNP since the discovery ANP by DeBold (1981) have resulted in thousands of publications elucidating mechanisms of action supporting therapeutic potential especially in hypertension (Volpe et al., 2014; Rubattu et al., 2019). Central to the therapeutic opportunity of ANP and BNP are their physiologic properties mediated by GC-A/cGMP. These include anti-hypertrophic and anti-fibrotic actions in the heart and kidney, natriuresis and diuresis, endothelial protection, suppression of renin release and aldosterone synthesis and secretion and lipolysis and browning of adipocytes. However, as an anti-hypertensive therapeutic, ANP and BNP are limited by their relative instability in the circulation requiring that they be administered intravenously. Nonetheless, in human studies intravenous infusion of ANP in both normotensive and patients with hypertension reduces blood pressure (Biollaz et al., 1986; Janssen et al., 1989).

Advances in peptide engineering has led to novel designer natriuretic peptides for cardiovascular disease (Meems and Burnett, 2016). Employing such advances, we pursued strategic deletions, replacements or additions to the amino acid structures of native ANP, BNP and CNP, the latter the endogenous ligand to the other membrane bound GC receptor GC-B. The goals of designer peptide engineering have been to maintain or enhance safety, augment GC receptor activation, increase resistance to enzymatic degradation, individualize designs to specific clinical syndromes and develop novel delivery systems for chronic administration. MANP, developed at the Mayo Clinic, is a 40 amino acid peptide compared to 28 amino acids ANP and is highly resistant to enzymatic degradation by neprilysin and insulin degrading enzyme (Dickey et al., 2009; McKie et al., 2009; Ralat et al., 2011). It is also highly potent in activating GC-A. In the first *in vivo* study of MANP compared to ANP in normal canines, MANP demonstrated widespread superiority to ANP in reducing blood pressure, augmenting sodium excretion and inhibiting angiotensin II and aldosterone (McKie et al., 2009). In follow-up studies in an experimental model of hypertension produced by angiotensin II infusion, MANP was highly effective in blood pressure lowering and enhancing renal function while also reducing aldosterone (McKie et al., 2010). All of the above *in vivo* studies, however, utilized intravenous administration of MANP.

Recently, for the first time Chen et al. (2020) investigated the chronic actions of MANP on blood pressure, natriuresis, and cGMP activation in a model of hypertension in rats by subcutaneous (SQ) injection once daily thus following the paradigm of daily insulin injections used for the treatment of diabetes. A goal was to test the feasibility and effectiveness of SQ MANP delivery. Most importantly in hypertensive rats, SQ injection of MANP for 7 days induced cGMP elevation and long-term blood pressure reduction compared to vehicle (**Figure 1**). Thus, the study demonstrated for the first time the effectiveness of SQ administration of MANP for 7 days supporting its continued development as a novel therapeutic for hypertension. Taken together, these previous preclinical studies of MANP established MANP as a novel ANP analog which is more potent and long lasting than native ANP, and whose molecular target is GC-A resulting in enhanced production of its effector molecule cGMP. These findings support the significant therapeutic potential of MANP for the treatment of hypertension, including the challenging syndrome of resistant hypertension for which there is no FDA approved drug. Indeed, MANP possesses as a single peptide entity the pleiotropic properties of cGMP activation, natriuresis, aldosterone suppression, and BP lowering properties in preclinical studies which do not exist in any single current anti-hypertensives drug (**Figure 2**). These actions set MANP apart from conventional anti-hypertensive medications such as diuretics, aldosterone blockers, calcium channel blockers and renin angiotensin system antagonists (Volpe et al., 2021).

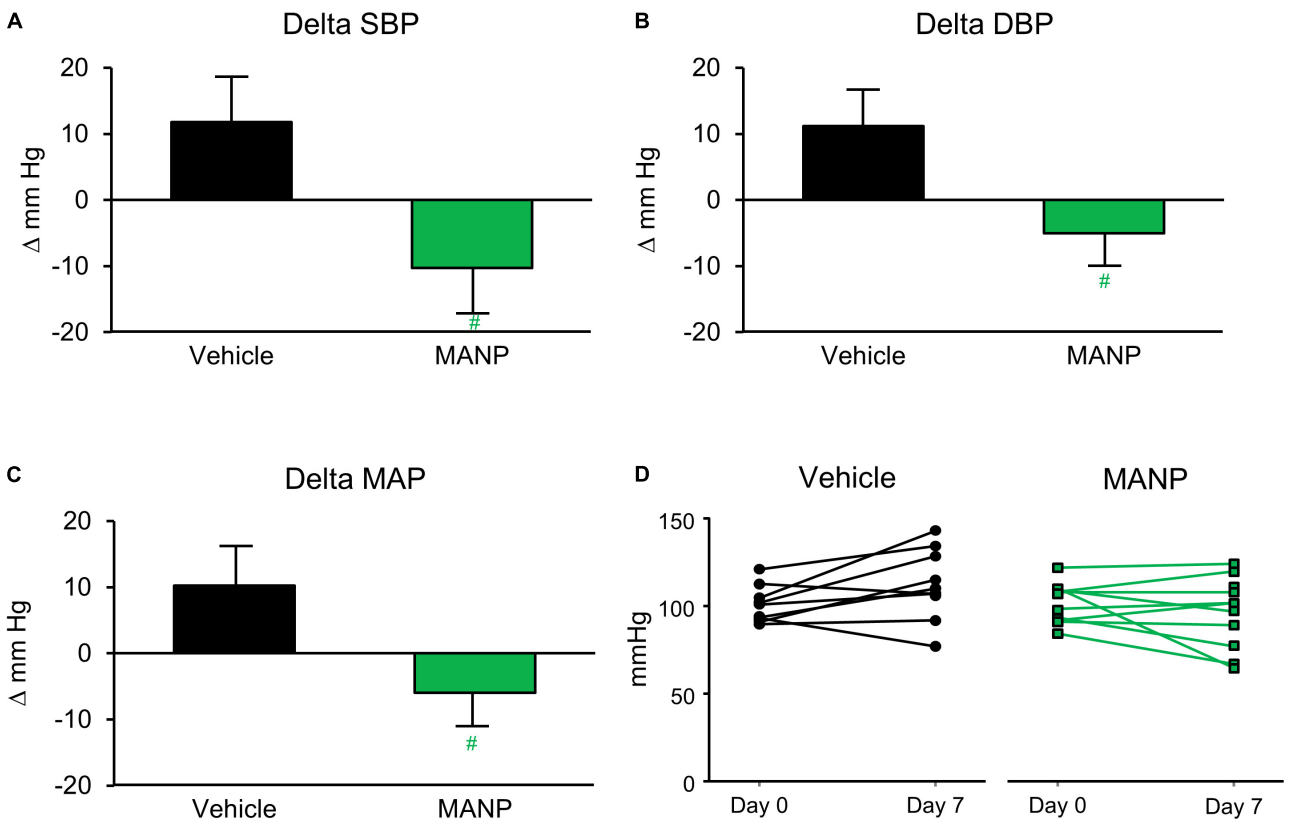

FIGURE 1 | Blood pressure (BP) changes in the chronic study in salt-depletion male rats: $n = 9$ vehicle (saline) and $n = 10$ atrial natriuretic peptide analog (MANP) (3.88 mg/kg); daily subcutaneous injection for 7 days. Conscious BP was measured *via* tail-cuff method at 20 h post-injection on day 7. Delta values were recorded as changes from baseline values on day 0. Changes in systolic BP (SBP; **A**), diastolic BP (DBP; **B**), and mean arterial pressure (MAP; **C**) and individual animal MAP values on day 7 from day 0 **(D)**. #$P > 0.05$ vs. vehicle. Data are expressed as means ± SE. Unpaired *t* test was used. Reproduced with permission from the American Physiological Society (Chen et al., 2020).

The first in human study of MANP was recently reported and performed in subjects with essential hypertension bypassing normal volunteers based upon MANP's efficacy and safety in multiple studies in normal, hypertensive and heart failure canines and rodent models (Chen et al., 2021). The goal of this first in human study was to determine MANP's overall safety, tolerability and plasma cGMP activating properties *via* SQ injection in hypertensive subjects who were off standard-of-care anti-hypertensive medications. The study was an open label sequential single ascending dose (SAD) design in multiple cohorts of hypertensive subjects. Three doses were employed. Natriuretic, aldosterone suppressing and BP lowering actions were assessed. Twelve patients were recruited. Inclusion criteria included (1) hypertensive subjects with SBP above 140 mm Hg; (2) on at least one anti-hypertensive medication; and (3) DBP equal to or in excess of 90 mm Hg. Exclusion criteria included (1) known hypersensitivity or allergy to MANP or its components, carperitide, other natriuretic peptides, or related compounds; (2) women who are pregnant or breast-feeding; (3) any disease or condition (medical or surgical) which, in the opinion of the investigator, might compromise the hematologic, cardiovascular, pulmonary, renal, gastrointestinal, hepatic, or central nervous system; or other conditions that may interfere with the absorption, distribution, metabolism, or excretion of study drug, or would place the subject at increased risk; (4) the presence of abnormal laboratory values considered clinically significant by the Investigator; (5) positive screen for Hepatitis B (HbsAg, Hepatitis B Surface Antigen), Hepatitis C (anti HCV, Hepatitis C Antibody), or HIV (anti-HIV 1/2); (6) received an investigational drug within a 30-day period of Visit 1; (7) consumption of alcohol within 48 h prior to dose administration or during any in-patient period; (8) a positive urine drug screen including ethanol, cocaine, THC, barbiturates, amphetamines, benzodiazepines, and opiates unless, in the opinion of the Investigator, a positive finding is as a result of a legitimate medical prescription for a valid medical condition; (9) a history (within the last 2 years) of alcohol abuse, illicit drug use, significant mental illness, physical dependence to any opioid, or any history of drug abuse or addiction; (10) a history of difficulty with donating blood or donated blood or blood products within

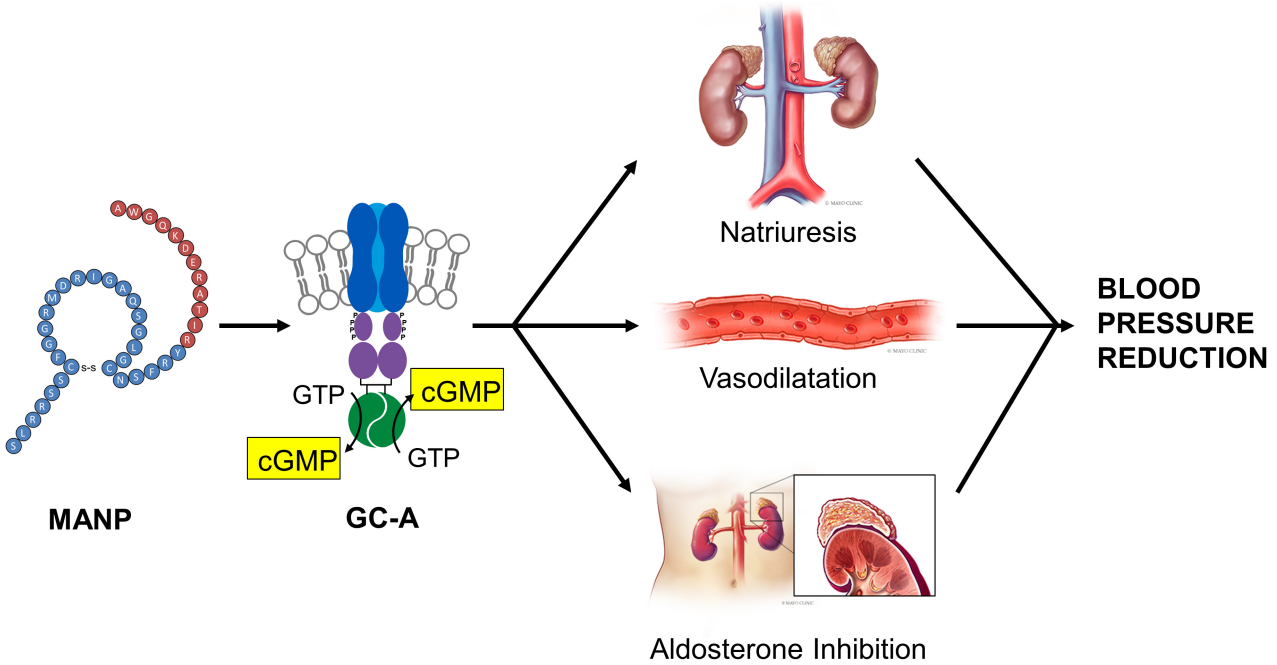

FIGURE 2 | MANP Mechanism of Action: MANP, a 40 amino acid designer natriuretic peptide, activates the GC-A receptor stimulating the generation of its second messenger cGMP. After binding to GC-A in the kidney, the vasculature and the adrenal gland, MANP mediates natriuresis, vasodilatation and aldosterone inhibition. Reproduced with permission from the American Heart Association (Chen et al., 2021).

45 days prior to enrollment; (11) clinically significant new illness in the 1 month before screening in the opinion of the Investigator; (12) history of severe allergies (e.g., anyone with a known history of anaphylaxis to medication[s] or allergens and/or asthma requiring hospitalization); (13) history of coronary artery disease; (14) history of cerebrovascular disease; (15) history of epilepsy or other seizure disorder; (16) history of syncope; (17) history of organ transplantation; and (18) malignancy within 5 years of the screening visit (with the exception of basal cell and squamous cell skin carcinoma).

Natriuresis is a signature property of MANP *via* GC-A/cGMP. In this first in human study, investigators reported a dose response to MANP in natriuresis during the 4 h after SQ MANP. While the duration of aldosterone reduction was variable among the treatment cohorts, a signal for a reduction and off-response by 24 h was clearly observed, thus, extending to the human the aldosterone suppressing actions of GC-A activation previously reported by *in vitro* and in animal studies (Atarashi et al., 1985; Ito et al., 2003; McKie et al., 2009). Most importantly, both systolic and diastolic blood pressures were reduced with a peak action at 12 h post-dose. In addition, there was target engagement as plasma cGMP was increased. Thus, MANP induces unique reno-adrenal responses in hypertensive subjects combining natriuretic actions with the favorable action of aldosterone suppression.

The seminal observation was that SQ administration of MANP at three different doses reduced BP over the 24-h period of observation (**Figure 3**). This BP effect peaked between 2 and 12 h but there was no clear dose response. Thus, SQ doses from 1 to 5 μg per kilogram may have already achieved a maximal effect requiring follow up studies to explore lower doses. Importantly, this clinical study established that intravenous administration of a natriuretic peptide may be overcome with SQ administration as is widely used for insulin and GLP-1 analogs.

A principal goal of this first in human study was safety. No major adverse events were observed. No ECG changes in any of the subjects during the 24 h of observation were reported and there were no local reactions at the injection sites.

Thus, the first in human study of MANP in essential hypertension is transformational in hypertension therapeutics and establishes that treatment with the designer ANP analog MANP engages the GC-A receptor, activates cGMP, reduces BP, enhances sodium excretion and suppresses aldosterone. Further, MANP can be administered safely and effectively SQ and is well tolerated.

The clinical development of MANP is to target resistant hypertension which remains a huge public health burden and for which there is not approved drug. A Phase 1 placebo control 3-day multiple ascending dose study in resistant hypertension has been completed and data are being analyzed from 20 patients. In this completed Phase 1 trial in resistant hypertension, MANP was administered on top of three or more antihypertensive drugs which included diuretics, angiotensin converting enzyme inhibitors (ACEi), angiotensin A1 (AT1) receptor blockers and calcium channel blockers. Thus, data will be available on the blood pressure reducing actions of MANP on top of conventional anti-hypertensive agents. A new expanded clinical trial has been initiated in 40 patients with resistant hypertension (20 Caucasians

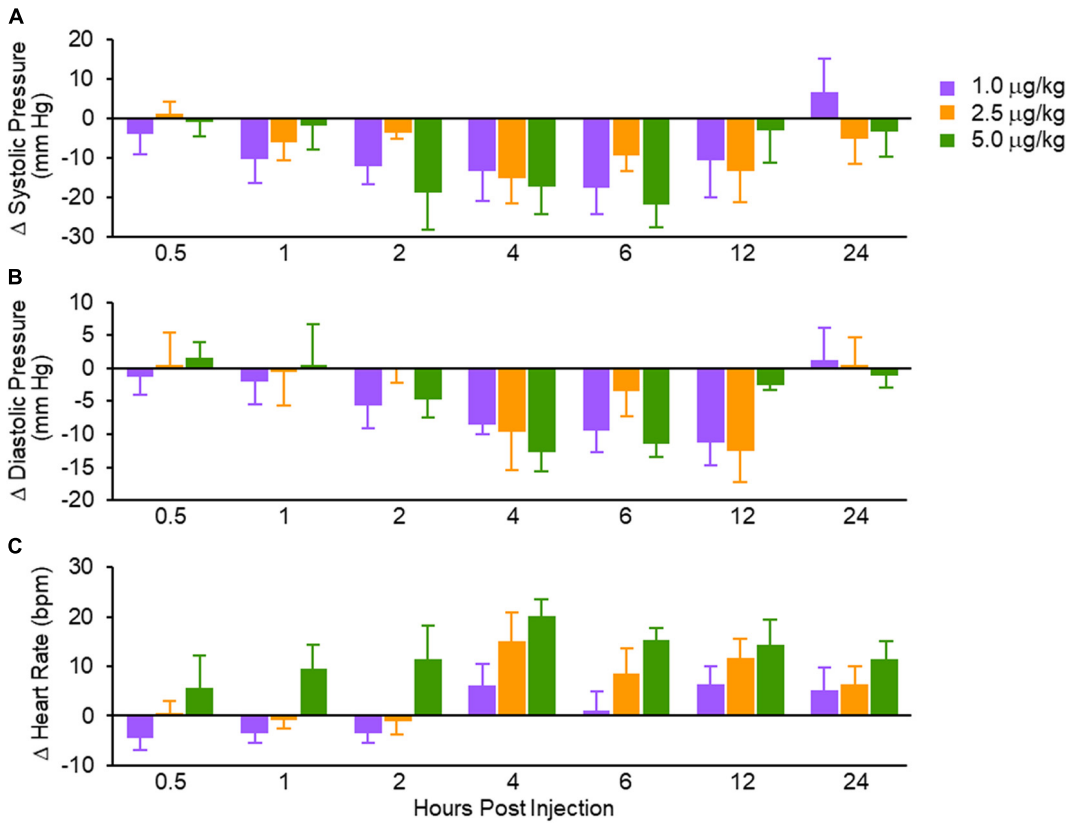

FIGURE 3 | Absolute changes in systolic blood pressure **(A)**; diastolic blood pressure **(B)** and heart rate **(C)**. Values are mean ± SEM. Reproduced with permission from the American Heart Association (Chen et al., 2021).

and 20 African Americans) which is placebo controlled and with multiple ascending doses. MANP is administered once daily for 5 days.

The current delivery method for MANP is by subcutaneous injection once daily. As advances in peptide therapeutics in cardiovascular and metabolic disease accelerate, SQ delivery is emerging as with GLP-1 agonists for diabetes and PKC9 inhibitors for hypercholesterolemia. Nonetheless, research and development of MANP also includes the development of oral delivery of MANP. Here, advances in oral delivery of peptides such as BNP and GLP-1 agonists have also been reported supporting the feasibility of this exciting delivery strategy for peptides such as MANP (Cataliotti et al., 2008; Drucker, 2020).

It should be stated that several questions remain to be addressed as MANP studies continue. These include establishing the ability of MANP to chronically reduce blood pressure and activate the cGMP pathway thus not having drug tolerance. In addition, the role of gender in responsiveness to MANP also needs to be established as sex-based differences in circulating ANP and BNP exist with plasma ANP and BNP being higher in females. Further, the potential modulating action of ANP and BNP genetic variants, which are associated with variations in circulating levels of the two hormones, should also be investigated in regard to blood pressure response to MANP. While ANP as an intravenous drug for heart failure has proved successful in Japan, the use of BNP (nesiritide) and urodilatin (ularitide) in heart failure has been disappointing. This may be related to excessively high doses which are hypotensive as well as their use only as acute drugs as opposed to chronic therapy at a lower dosage. Indeed, hypertension may be the most physiologic target for natriuretic peptide therapy based upon the evidence of a relative deficiency of ANP and BNP in human hypertension and blood pressure reduction being the signature pharmacologic action of MANP.

Another drug breakthrough that involves the natriuretic peptides system by augmenting their circulating levels is sacubitril/valsartan. Sacubitril inhibits the enzyme neprilysin (NEP) which is widely expressed in the human body and degrades ANP and BNP. Valsartan serves as an AT1 blocker. Sacubitril/valsartan was approved for heart failure based upon an improvement in mortality in the pivotal PARADIGM HF trial (McMurray et al., 2014). Ruilope et al. (2010) reported the effectiveness of sacubitril/valsartan compared to sacubitril or valsartan alone in human hypertension in which sacubitril/valsartan was superior to either NEP inhibition or AT1 blockade. Both MANP and sacubitril/valsartan may be valuable novel therapies for hypertension involving molecular mechanisms which are not activated in conventional anti-hypertensives. However, through NEP inhibition, not only is the degradation of ANP and BNP inhibited, but also vasoconstrictor peptides such as angiotensin II (ANG II), which is also degraded

by NEP, may increase in the circulation. Indeed, in heart failure, ANG II is higher in the circulation with sacubitril/valsartan than with either an AT1 blocker or ACEi (Pavo et al., 2016). Thus, MANP emerges as a selective GC-A activator devoid of non-GC-A actions and can also be administered SQ determining higher levels of ANP which exceeds that which can be achieved with NEP inhibition. A high priority is to understand populations which best respond to these two drugs in hypertension in further studies in hypertension.

Since the discovery of ANP by Adolfo DeBold that established the heart as an endocrine organ, the field of natriuretic peptides is again accelerating. It is not unusual that it has taken four decades to also establish the therapeutic potential natriuretic peptides and testing in humans. That may be the usual cycle of drug development. This includes designer peptides such as MANP and novel small molecules as sacubitril/valsartan which may inhibit the breakdown of endogenous ANP and BNP and boost their circulating levels (Ruilope et al., 2010; Chen et al., 2021). Perhaps also employing precision medicine to identify patients with ANP and/or BNP deficiency along with cardiac hormone replacement therapeutics might be the future successful strategy to reduce the burden of cardiovascular disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Both authors conceived the outline of the manuscript, wrote the first and final draft, contributed to the article, and approved the submitted version. JB designed the figures. VC finalized the references.

FUNDING

This work was supported by National Institute of Health (R01 HL136340 and R01 HL134668), American Heart Association (16SDG29930003), and Mayo Clinic Marie Ingalls Cardiovascular Research Career Development Fund in honor of Alexander Schirger.

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Conflict of Interest: Mayo Clinic has licensed MANP to E-STAR BIO and JB is the inventor of MANP.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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