BIOMARKERS OF OXIDATIVE STRESS

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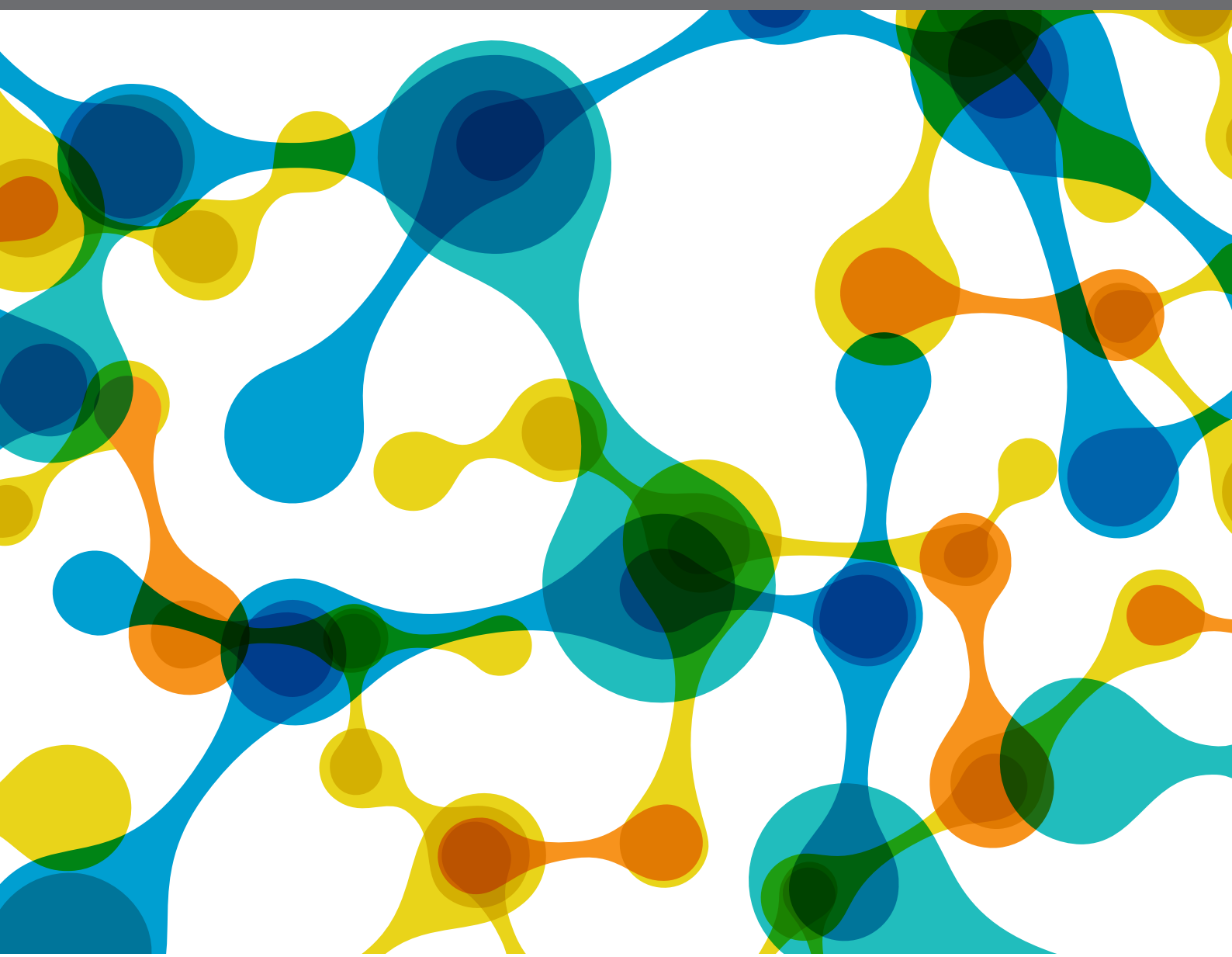

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BIOMARKERS OF OXIDATIVE STRESS

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EDITORIAL
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Editorial: Biomarkers of Oxidative Stress

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Keywords: reactive oxygen species, pathological mechanisms, mitochondria, hemoglobin-/heme scavengers, endoplasmatic reticulum, metabolic dysfunction

Editorial on the Research Topic

Biomarkers of Oxidative Stress

INTRODUCTION

The term "oxidative stress" is often used to describe physiological conditions where there is an imbalance between oxidants and antioxidants. This can lead to a disruption of redox signaling and induction of molecular damage in cells and tissues (Halliwell and Gutteridge, 2015). Important groups of oxidant molecules are reactive oxygen species (ROS) and free radicals, molecules that are highly reactive due to the presence of unpaired electron(s). The human body is constantly exposed to ROS, free radicals and other oxidants, both exogenously via the environment (food, air, smoke, irradiation, etc.) and endogenously as by-products of normal metabolism. Oxidative stress is involved in many pathological processes, such as inflammation, ischemia/reperfusion and infection, and may lead to development of several pathologic conditions, including neurodegenerative disease, cancer, renal disease, diabetes, cardiovascular diseases and inflammatory bowel disease (Olsson et al., 2012).

The scope of the present Research Topic was to reach out to an audience of scientists working in the field of oxidative stress. We encouraged the submission of papers describing biomarkers of oxidative stress and to discuss their possible usefulness in improving our understanding of disease pathogenesis, aiding in the diagnosis of diseases, developing new therapeutic strategies, and monitoring treatment outcome. A collection of original research and review articles with different approaches, ranging from basic science to evaluation of patient material, contributed to this Research Topic highlighting interesting aspects of biomarkers in oxidative stress. In this editorial, we have summarized the contributions under the following headings: new biomarkers, new pathological mechanisms, hemoglobin/heme-scavengers, and mitochondria, endoplasmic reticulum stress, and metabolic dysfunctions.

Proposing New Biomarkers

As expected from the Research Topic title, several of the contributions have proposed new biomarkers of oxidative stress. Prasad et al. adapted electron spin resonance spectroscopy and two-dimensional ultra-weak photon emission to detect UV light-induced triplet excited carbonyls and singlet oxygen formation in skin biopsies. This may be a new tool to investigate the effect of UV-induced oxidative stress of human skin. Larsson et al. suggest that levels of the human antioxidant α_1-microglobulin (A1M) in synovial fluid may be used as a biomarker to predict long-term risk of

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development of arthrosis after knee injury. Sharma et al. suggest that transthyretin may be a new biomarker with prognostic value for a wide range of oxidative stress-related medical conditions, including Alzheimer's disease and Parkinson. Kalapotharakos et al., finally, show that there are increased plasma levels of the heme-scavenger A1M in high-risk pregnancies and therefore plasma A1M could be a potential biomarker of pregnancy-related diseases.

Proposing New Pathological Mechanisms

Preeclampsia, a disease of pregnancy which is associated with oxidative stress, was the focus of two contributions each investigating alternative and independent pathological mechanisms. Sánchez-Aranguren et al. could show that soluble Fms-like tyrosine kinase-1 (sFlt-1, also known as vascular endothelial growth factor receptor-1, VEGFR-1), which is elevated in plasma from preeclamptic pregnant women, may contribute to metabolic disturbance and to the development of the disease by triggering mitochondrial ROS-formation in endothelial cells and trophoblasts. Kalapotharakos et al., on the other hand, studied the involvement of extracellular fetal hemoglobin (HbF) originating from placental hemolysis, in the development of preeclampsia. Proposals of new interesting pathological mechanisms of action of the plasma protein transthyretin, commonly known as a transporter of thyroxin and retinol, are reviewed by Sharma et al. Novel oxidative stress-associated pathological mechanisms, related to the release of heme from hemoglobin (Hb), are proposed for knee arthropathies and atherosclerosis, by Larsson et al. and Gáll et al., respectively. Vrbanovic et al. propose an association between salivary oxidative stress and pain in temporomandibular disorders, and Prasad et al. propose novel pathways in ROS-formation during UV-light exposure of skin. Valacchi et al., finally, introduce the novel concept of "OxInflammation" to describe the long-term cross-talk between pro-oxidants, free radicals and inflammatory mediators during inflammation.

Hemoglobin/Heme-Scavengers

As described above, the contributions by Kalapotharakos et al., Larsson et al. and Gáll et al. discuss the importance of pathological mechanisms related to extracellular Hb and release of heme. In addition, these contributions hypothesize that, when the increased Hb-levels are unmet by the Hb- and heme-scavenger proteins haptoglobin (Hp), hemopexin (Hpx) and/or A1M, excessive hemolysis in placenta, knee-joints or atherosclerotic plaques generate locally increased levels of ROS by reactions between the heme-chelated iron atom and oxygen. The resulting oxidative stress is thought to contribute to preeclampsia (Kalapotharakos et al.), arthrosis of the knee (Larsson et al.) and atherosclerosis (Gáll et al.). The contribution by Maamoun et al. describe the cytoprotective role of the inducible heme-degrading enzyme heme oxygenase-1 (HO-1) as an antioxidant and regulator of ROS-induced toxicity, thereby protecting against endoplasmic reticulum (ER)-stress in diabetic endothelial cells.

Mitochondria, Endoplasmic Reticulum Stress, and Metabolic Dysfunctions

Chen et al. review the growing knowledge of mitochondrial involvement in modulating oxidative stress and the innate immune response in cardiovascular diseases, autoimmunity, and metabolic syndrome. Mitochondria constitute a "convergent signaling hub" that regulates the cellular redox balance and homeostasis, they argue, and highlight that mitochondria are centrally involved in the driving pathogenic responses upon injury or damage to cells and tissues. As outlined above, Sánchez-Aranguren et al. give an example of this, showing that sFlt-1 targets mitochondria of placental endothelial cells and trophoblasts, disturbs the mitochondrial redox balance, and as a result contributes to the development of preeclampsia. In addition to the mitochondria, two papers in this Research Topic have focused on the ER and the dysfunctional state of this organelle, ER-stress. Maamoun et al. review current knowledge on how the interplay between oxidative stress and ER-stress contributes to aberrant angiogenesis and endothelial dysfunction in diabetes. Gáll et al. show that ROS-induced ER-stress is involved in development of atherosclerosis.

CONCLUSION

The above referenced articles show that the Research Topic presents an exciting scientific arena, attracting interest from different aspects and at different levels, from basic to translational research. We hope these articles can contribute to the development of new ideas and advancements in the field.

AUTHOR CONTRIBUTIONS

MG and BÅ wrote, revised, and approved the manuscript.

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ORIGINAL RESEARCH
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Soluble Fms-Like Tyrosine Kinase-1 Alters Cellular Metabolism and Mitochondrial Bioenergetics in Preeclampsia

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Preeclampsia is a maternal hypertensive disorder that affects up to 1 out of 12 pregnancies worldwide. It is characterized by proteinuria, endothelial dysfunction, and elevated levels of the soluble form of the vascular endothelial growth factor receptor-1 (VEGFR-1, known as sFlt-1). sFlt-1 effects are mediated in part by decreasing VEGF signaling. The direct effects of sFlt-1 on cellular metabolism and bioenergetics in preeclampsia, have not been established. The goal of this study was to evaluate whether sFlt-1 causes mitochondrial dysfunction leading to disruption of normal functioning in endothelial and placental cells in preeclampsia. Endothelial cells (ECs) and first-trimester trophoblast (HTR-8/SVneo) were treated with serum from preeclamptic women rich in sFlt-1 or with the recombinant protein. sFlt-1, dose-dependently inhibited ECs respiration and acidification rates indicating a metabolic phenotype switch enhancing glycolytic flux. HTR-8/SVneo displayed a strong basal glycolytic metabolism, remaining less sensitive to sFlt-1-induced mitochondrial impairment. Moreover, results obtained in ECs exposed to serum from preeclamptic subjects demonstrated that increased sFlt-1 leads to metabolic perturbations accountable for mitochondrial dysfunction observed in preeclampsia. sFlt-1 exacerbated mitochondrial reactive oxygen species (ROS) formation and mitochondrial membrane potential dissipation in ECs and trophoblasts exposed to serum from preeclamptic women. Forcing oxidative metabolism by culturing cells in galactose media, further sensitized cells to sFlt-1. This approach let us establish that sFlt-1 targets mitochondrial function in ECs. Effects of sFlt-1 on HTR-8/SVneo cells metabolism were amplified in galactose, demonstrating that sFlt-1 only target cells that rely mainly on oxidative metabolism. Together, our results establish the early metabolic perturbations induced by sFlt-1 and the resulting endothelial and mitochondrial dysfunction in preeclampsia.

Keywords: preeclampsia, sFlt-1, mitochondrial dysfunction, endothelial dysfunction, oxidative stress and metabolic perturbations, metabolism

INTRODUCTION

Preeclampsia (PE) is an often-fatal cardiovascular complication related to pregnancy, that affects almost 8% of all pregnancies worldwide. Globally, it is associated with approximately 80,000 maternal and over 500,000 infant deaths annually, impacting the lives of over 4 million women worldwide(Duley, 2009). PE is characterized by maternal endothelial dysfunction (ED) (Powe et al., 2011; Sánchez-Aranguren et al., 2014), hypertension (LaMarca et al., 2008) and proteinuria (Maynard and Karumanchi, 2011). Alterations in circulating anti-angiogenic factors levels, such as the soluble form of the vascular endothelial growth factor (VEGF) receptor-1 (sVEGF-R1), commonly known as sFlt-1, have been associated to the onset of PE (Maynard et al., 2003; Levine et al., 2004, 2006). Nonetheless, the pathophysiology of PE remains unknown (Kanasaki and Kalluri, 2009).

In normal pregnancies, uterine blood flow increases to allow adequate perfusion of the placental intervillous space and physiological oxidative stress (Chaiworapongsa et al., 2014). In PE, a prolonged hypoxic placental microenvironment, due to a reduction in placental perfusion and oxygen availability, results in exacerbated oxidative stress (Chaiworapongsa et al., 2014). Hypoxia triggers several cellular responses, including increased placental angiogenesis (Zamudio, 2003), cell survival and metabolic adaptations (Illsley et al., 1984), established in developmental biology as "placental metabolic reprogramming" (Illsley et al., 2010; Jose et al., 2011).

Mitochondrial activity is essential in pregnancy because it sustains the metabolic activity of the placenta throughout gestation (LaMarca et al., 2008). Recently, a potential association between increased soluble anti-angiogenic factors levels and mitochondrial dysfunction has been suggested (Jiang et al., 2015). Exogenous administration of sFlt-1 in pregnant mice have shown to induce placental mitochondrial swelling, oxidative stress and apoptosis in trophoblasts (Jiang et al., 2015). In addition, preeclamptic plasma mediators induced deleterious effects on mitochondrial function of human umbilical vein endothelial cells (HUVEC) (McCarthy and Kenny, 2016). Together, findings suggest that mitochondrial function plays an important role in the onset of PE. Nevertheless, the role of early dysregulated sFlt-1 levels in PE, to induce perturbations in the mitochondrial oxygen consumption and bioenergetics in endothelium and placenta, has not been established.

Here, we report the effects of PE serum on mitochondrial oxygen consumption and metabolism in ECs and first-trimester extravillous trophoblasts (HTR-8/SVneo). As early elevated circulating levels of sFlt-1 are known to be implicated in the development of the disease (Maynard et al., 2003; Levine et al., 2004, 2006), we also established the effects of increasing levels of exogenous sFlt-1 on mitochondrial function. We demonstrate that PE serum significantly affects mitochondrial maximal respiration and spare respiratory capacity of ECs and trophoblast, enhancing a metabolic glycolytic phenotype. In addition, PE serum-induced mitochondrial reactive oxygen species (mtROS) formation. These effects were partially abrogated by exogenous VEGF. Finally, sFlt-1 treatment caused a dose-dependent loss of mitochondrial oxygen consumption in ECs and trophoblasts, affecting mitochondrial maximal respirations and spare respiratory capacities, and, inducing a metabolic phenotype switch to glycolysis, only in ECs. Our results provide novel insights on the differential metabolic perturbations exerted by sFlt-1 in the endothelium and placenta and their overall role in the development of PE.

MATERIALS AND METHODS

Subjects

Antecubital blood samples were collected from preeclamptic (PE) ($n = 23$) and normotensive women (NOR) ($n = 23$), before cesarean delivery. Subjects were recruited from the Maternal Fetal Units of Fundación Cardiovascular de Colombia (FCV), Floridablanca, Colombia and Clínica Materno Infantil San Luis (CMISL), Bucaramanga, Colombia, using protocols approved by hospital respective Ethics Committees. Ten non-pregnant subjects were recruited as controls (CTL). Preeclampsia diagnosis was established according to the American College of Obstetricians and Gynecologists (American College Obstetricians Gynecologist Task Force on Hypertension in Pregnancy, 2013) criteria (**Supplementary Table 1**). Subjects gave informed consent for their inclusion in the study.

Cell Culture

Bovine aortic endothelial cells (ECs) (passages 5–8) were obtained from Cell Applications, Inc. (San Diego, CA, USA). ECs were cultured in DMEM supplemented with 10% FBS (CellGro), 5.5 mM glucose, 4 mM L-glutamine, 1 mM sodium pyruvate and 100 U/ml penicillin/streptomycin at 37°C and 5% CO_2. Prior to experiments, ECs were cultured in DMEM containing 2% FBS for 24 h. Extravillous trophoblast cells (HTR-8/SVneo, passages 75–82), are a well characterized, authenticated and immortalized first-trimester extravillous trophoblast (EVT) cell line(Graham et al., 1993). HTR-8/SVneo were cultured in RPMI-1640 (Gibco, Grand Island, NY, USA) supplemented with 5% FBS and 100 U/ml penicillin/streptomycin at 37°C and 5% CO_2. To force mitochondrial metabolism, ECs and HTR-8/SVneo cells were grown in galactose supplemented media, matching the same glucose concentrations used previously, for two passages prior the analysis. Human recombinant sFlt-1 (VEGF-R1/Flt-1), and VEGF were obtained from R&D Systems, (Minneapolis, MN, USA). For *in vitro* correlation studies, cells were exposed to 2% serum from recruited women (Rodgers et al., 1988; Maynard et al., 2003).

Cell Viability Assays

MTT assay was performed as cell viability assays. Cells were seeded at a density of 1×10^4 cells/well on 96-well plates and cultured for 24 h. Then, cells were treated with 50 ng/mL of sFlt-1 recombinant protein (Novoprotein Scientific, Summit, NJ) for another 24 h. Cells were cultured in glucose and galactose supplemented media. After treatments, cells were exposed to 100 μL of MTT solution (5 mg/mL). Two hours later, formazan crystals were solubilized in dimethyl sulfoxide (DMSO). Absorbance was measured in a plate reader at 570 nm

using a Varioskan Flash multimodal plate reader (Thermo Fisher Scientific, Vantaa, Finland).

Mitochondrial Oxygen Consumption

Mitochondrial bioenergetics was assessed using an XFe24 Extracellular Flux Analyzer (Agilent Seahorse, Billerica, MA, USA). ECs and HTR-8/SVneo were seeded in V7 Seahorse micro-well plates at 3.5–4.0 $\times$ 10^4 cells/well in 100 μL standard growth media. Cells were treated with sFlt-1 recombinant protein (Novoprotein Scientific) and 2% serum from recruited women, respectively, and incubated at 37°C and 5% CO_2 for 24 h. Following treatments, culture media was changed to a non-buffered DMEM media, to allow temperature and pH equilibrium. Initially, oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were measured simultaneously three times to establish a baseline rate. Then, to evaluate mitochondrial function, oligomycin (1 mM) (Sigma Aldrich), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (0.5 mM) (Sigma Aldrich) and a mixture of rotenone and antimycin A (Rot/AntA) (1 mM) (Cayman Chemicals) were injected into each well sequentially, with intervals of 3–5 min of mixing between the injections, to respectively inhibit the ATP synthase, uncouple oxidative phosphorylation, and estimate non-mitochondrial respiration. Cellular respiratory control ratio (RCR) was obtained as the ratio of basal and oligomycin-inhibited (basal RCR) and FCCP-stimulated and oligomycin-inhibited mitochondrial respiratory rates (maximal RCR) (Brand and Nicholls, 2011; Dranka et al., 2011). OCR and ECAR measurements were normalized to protein content by the Bradford method (Supplementary Methods and **Supplementary Figure 1**).

Aerobic Glycolysis

Treated cells were washed and subjected to glucose (5.5 mM) (Sigma Aldrich), oligomycin (1 mM) (Sigma Aldrich) and 2-deoxi-glucose (2-DG) (100 mM) (Sigma Aldrich), subsequently, to respectively induce glycolysis, inhibit ATP synthase and estimate non-glycolytic acidification. Measurements were normalized to protein content by the Bradford method (Supplementary Methods and **Supplementary Figure 1**).

Mitochondrial ROS Formation

Mitochondrial ROS production (mtROS) was evaluated by fluorescence microscopy, using the fluorescent probe MitoSOX Red (Invitrogen, Oregon, USA). Briefly, ECs and trophoblast cells were cultured in 24-well plates, exposed to serum from recruited women and exogenous sFlt-1 (50 ng/mL), respectively, for 24 h. Cells were washed twice with HBSS and incubated with 5 μM MitoSOX Red probe in HBSS for 15 min at 37°C in 5% CO_2, protected from light. Then, cells were washed again with HBSS and the red fluorescence emitted at 580 nm was analyzed, using a Nikon Eclipse Ti-S inverted microscope and NiS Elements software.

Mitochondrial Membrane Potential

Changes in mitochondrial membrane potential ($\Delta\Psi m$) were performed using the JC-1 fluorescent dye by fluorescence microscopy (Invitrogen, Oregon, USA). JC-1 accumulates within the mitochondria and forms aggregates that emit red-orange fluorescence (Ex: 550/Em: 600 nm). When the Ψm is dissipated, cells emit green fluorescence (Ex: 485/Em: 535 nm). Briefly, ECs and HTR-8/SVneo cells were cultured in 24-well plates at densities of 5.0 $\times$ 10^4 cells/well. Treatments with sFlt-1 or serum were applied for 24 h. Then, cells were washed twice with sterile Phosphate Buffered Saline (PBS) at 37°C, followed by incubation with 5 μM of JC-1 in culture media for 30 min at 37°C, protected from light. The red/green fluorescence was evaluated using a Nikon Eclipse Ti-S inverted microscope and NiS Elements software.

Statistical Analyses

Each test was performed in three independent experiments. Data were plotted as means ± SEM. Statistical analysis was performed using Student *T*-test and ANOVA with Bonferroni's *post hoc* test, using Stata 8 statistical software (StataCorp, TX, USA). Values of $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.

RESULTS

In Vitro Correlation Model of PE: Role of VEGF in Mitochondrial Bioenergetics

Early metabolic perturbations are considered the hallmark of common human diseases (DeBerardinis and Thompson, 2012). To understand the metabolic basis that would accompany the onset of PE, we assessed the cellular bioenergetics profile of ECs and HTR-8/SVneo cells, exposed to serum from pregnant and not pregnant women. For these purposes, we established a case-control study in where pregnant preeclamptic (PE), normotensive (NOR), and non-pregnant women (CTL), were recruited (**Supplementary Table 1**). Circulating sFlt-1 levels were determined in recruited women, to corroborate preeclamptic conditions. As reported previously, sFlt-1 levels were increased in PE women (Maynard et al., 2003; Levine et al., 2004) by several orders of magnitude in comparison with NOR and CTL patients (**Supplementary Figure 2**). Then, serum from all groups was used to replace FBS at 2% in culture media, to establish an *in vitro* correlation model (Rodgers et al., 1988; Maynard et al., 2003) of PE. Next, the effects in mitochondrial metabolism and bioenergetics were assessed, using Agilent-Seahorse technology (Dranka et al., 2011) as described in methods sections.

As evidenced in **Figure 1A** respiratory traces show that treatment of ECs with serum from PE women induced a profound change in mitochondrial respiration rates. Maximal respiration and spare respiratory capacity rates were calculated after FCCP administration. FCCP induces the collapse of the mitochondrial membrane potential, leading to the determination of maximum OCR (Brand and Nicholls, 2011; Dranka et al., 2011). Administration of CTL and NOR serum did not affect respiration rates (**Figure 1A**). As observed in cell respiratory control ratios (RCR) (State 3/State 4), PE serum significantly affected mitochondrial respiration profile,

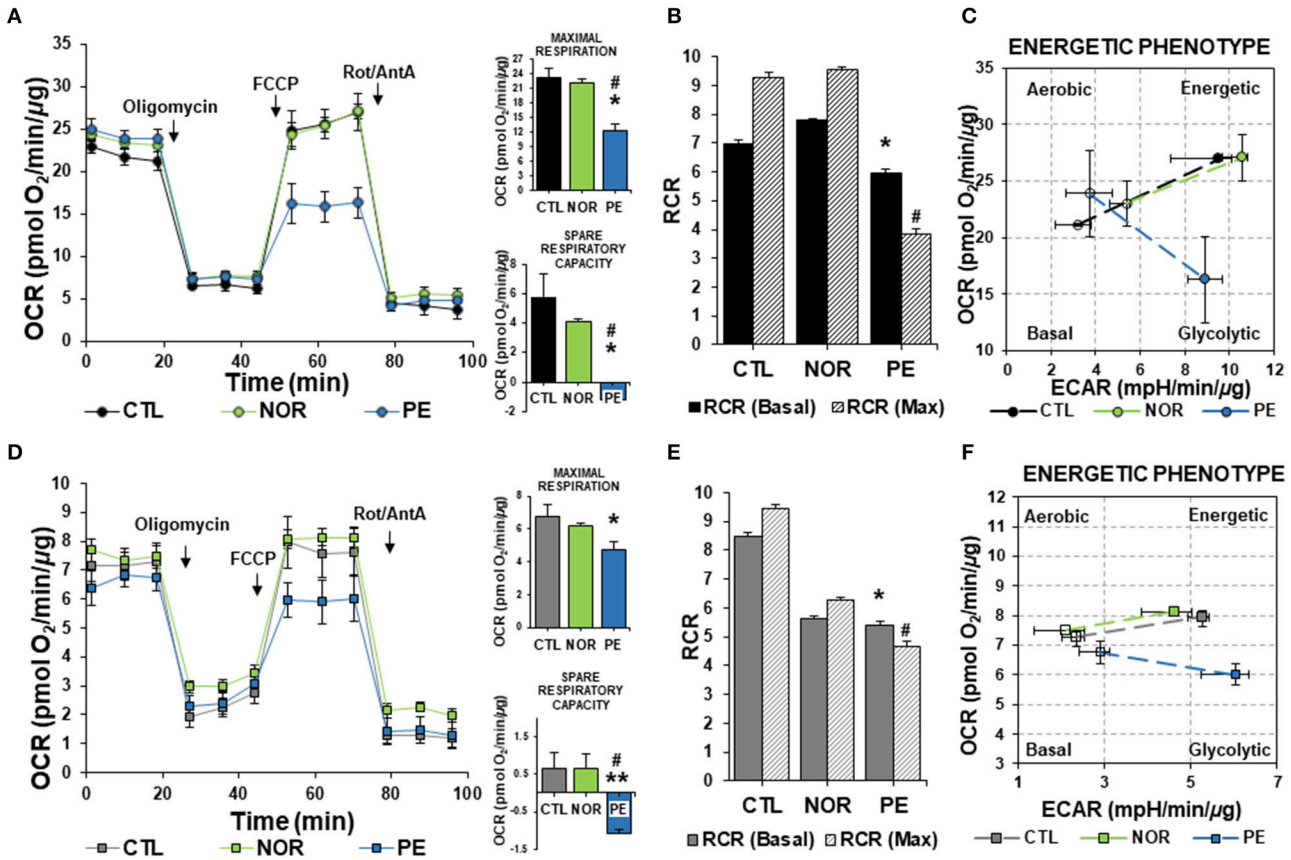

FIGURE 1 | Bioenergetics profile of cells treated with serum from PE women. ECs and HTR-8/SVneo cells were treated with 2% serum from NOR, CTL, and PE women for 24 h. **(A)** Basal oxygen consumption rates (OCR), maximal respiration (OCR after FCCP administration) and spare respiratory capacity (Difference between basal and maximal OCR), **(B)** Ratios of basal and maximal (Max) respiratory control (RCR) (State 3/state 4), **(C)** Energy phenotype map of basal and maximal OCR and extracellular acidification rates (ECAR), measured in ECs. **(D–F)**, are the same experiments as in **(A–C)**, respectively, but evaluated in HTR-8/SVneo cells. Data are presented as means ± SEM. ($n = 5$). In **(A,D)**: $^{*}P < 0.05$, $^{**}P < 0.01$ vs. CTL, $^{\#}P < 0.05$, vs. NOR. In **(B,E)**: $^{*}P < 0.05$, vs. basal CTL RCR, $^{\#}P < 0.05$, vs. CTL maximal RCR. ANOVA (*Bonferroni's post-hoc test*).

suggesting mitochondrial dysfunction, associated with a low substrate oxidation capacity (**Figure 1B**).

Interestingly, when we assessed the cell energy phenotype of ECs, we found a slight increase in basal OCR in ECs treated with NOR and PE serum. However, when cells were challenged with FCCP to uncouple mitochondria, only CTL and NOR treated cells showed an increase in both OCR and ECAR. ECs treated with PE serum exhibited a drastic drop in OCR below basal levels, with a non-significant increase in glycolytic function (**Figure 1C**). This suggested that ECs under PE conditions (modeled with serum from women with PE), are not going to be able to cope with physiological challenges that will require an increase in energy utilization via mitochondria.

To establish the effects of PE serum in placenta, we used HTR-8/SVneo cells, which is an immortalized first trimester EVT cell line, as our model. Since early perturbations of sFlt-1 will be developing before the 20th-week of gestation, a trophoblast cell line that resembles the metabolic profile from those early stages was used. Results obtained after treatment with serum from PE women showed a reduction in maximal respiration in comparison with NOR and CTL serum of about 30% (**Figure 1D**). As in ECs, PE serum depleted the spare respiratory capacity rates. RCR determinations identified reduced oxidative phosphorylation in NOR serum-treated trophoblasts, along with an enhanced glycolytic response (**Figure 1E**). As observed in ECs, PE serum induced a significant decrease in OCR parameters, consistent with a weak coupling of respiration for ATP synthesis and enhanced ability to activate glycolytic pathways (**Figures 1D–F**).

Together, these results suggest that under full manifestation of PE conditions, the combination of several PE-key players, along with upregulated sFlt-1 (Maynard et al., 2003; Levine et al., 2004, 2006; Tosun et al., 2010; Vitoratos et al., 2010), will induce an insightful effect in the energetic phenotype of both, ECs and trophoblast, that will not allow them to metabolically respond to harsh conditions.

Role of VEGF in Mitochondrial Function in PE

Once the impact of PE serum was established in ECs and trophoblasts, it was imperative to verify the implications of

sFlt-1 in these observations. Although sFlt-1 levels in PE serum were higher than in NOR and CTL, other factors like pro-inflammatory molecules (i.e., TNF-α and IL-6), are known to be present in serum as described extensively in the literature (Tosun et al., 2010; Vitoratos et al., 2010), contributing with sFlt-1 to the effects observed.

To address the role of sFlt-1 and VEGF bioavailabilities in the early metabolic perturbations in PE, VEGF (20 ng/mL) was administered to cells exposed to serum. VEGF administration should discriminate the antagonizing effects of sFlt-1 in serum from other possible factors. ECs and trophoblasts were treated with serum alone, and serum containing VEGF. As shown in (**Figure 2A**), VEGF administration to ECs treated with NOR serum did not affect maximal respiration or spare respiratory capacity. However, PE serum impaired the maximal respiration and spare respiratory capacity of ECs (**Figure 1A**) and these effects were partially reverted by VEGF (**Figure 2A**). This suggests that reduced VEGF bioavailability, due to sFlt-1 up-regulation, affects mitochondrial function (**Figures 2A,B**). VEGF co-treatment significantly improved maximal respiration rates, resulting in increased RCR when compared to ECs treated with PE serum (**Figures 2A,B**). Still, this improvement was not enough to reach the levels detected in cells treated with NOR serum. This scenario was similar in trophoblasts. PE serum caused a significant decrease in the maximal respiration rate and the addition of VEGF was not able to restore these values (**Figure 2C**). Then, the dramatic decrease in the spare respiratory capacity and RCR of cells treated with PE serum was partially restored by VEGF (**Figures 2C,D**). These observations suggested that sFlt-1 might be responsible for antagonizing the homeostatic activity of VEGF, having a direct impact on mitochondrial function and bioenergetics. However, it is expected that other factors in PE serum (and to a lesser extent in NOR and CTL women) are also contributing to the effects observed.

Next, we examined the effects on ECs and trophoblasts of PE serum alone and with VEGF, in mitochondrial function, by assessing the production of mtROS with Mito-SOX fluorescence and $\Delta\Psi m$ with JC-1 dye. In ECs, treatment with PE serum caused a drastic increase in mtROS in comparison with CTL and NOR serum. VEGF was only able to reduce mtROS generation by approximately 30% in comparison with ECs treated with PE serum (**Figure 3A**). In contrast, treatment in trophoblasts caused a significant increase as compared to CTL serum of 33% with NOR serum treatment and 71% increase in mtROS with PE serum (**Figure 3B**). VEGF decreased mtROS production about 30%, as in trophoblasts treated with NOR serum. PE

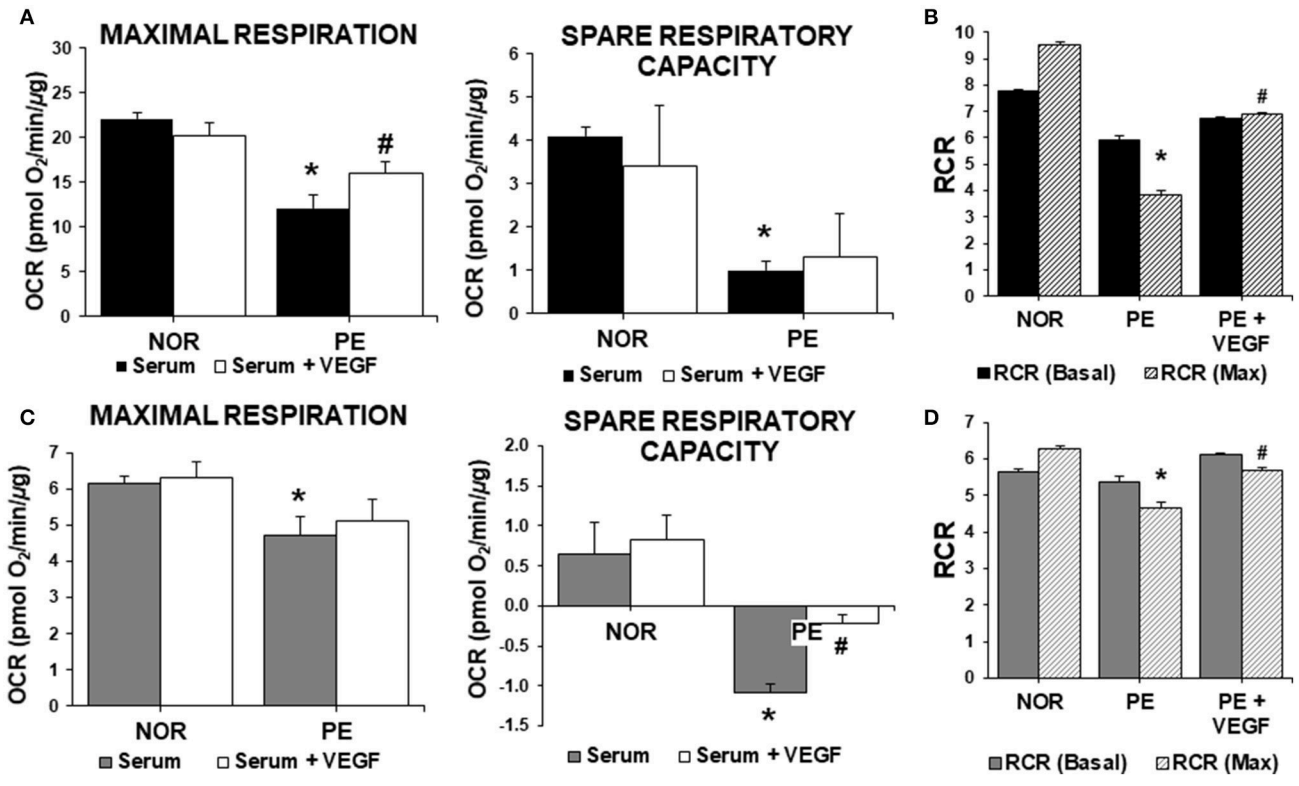

FIGURE 2 | Role of VEGF in mitochondrial bioenergetics in preeclampsia. ECs and HTR-8/SVneo cells were treated with 2% serum from NOR, CTL, and PE women with and without exogenous VEGF (20 ng/mL) for 24 h. **(A)** Maximal respiration (OCR after FCCP administration) and spare respiratory capacity (Difference between basal and maximal OCR) in endothelial cells, **(B)** Basal and maximal (Max) RCR, **(C,D)** are the same experiments as in **(A,B)**, respectively, but evaluated in HTR-8/SVneo cells. Data are presented as means ± SEM. ($n = 5$). In **(A,C)**: $^{*}P < 0.05$, vs. NOR, $^{\#}P < 0.05$, vs. cells exposed to PE serum alone. In **(B,D)**: $^{*}P < 0.05$, vs. NOR maximal RCR, $^{\#}P < 0.05$, vs. PE maximal RCR. ANOVA (*Bonferroni's post hoc test*).

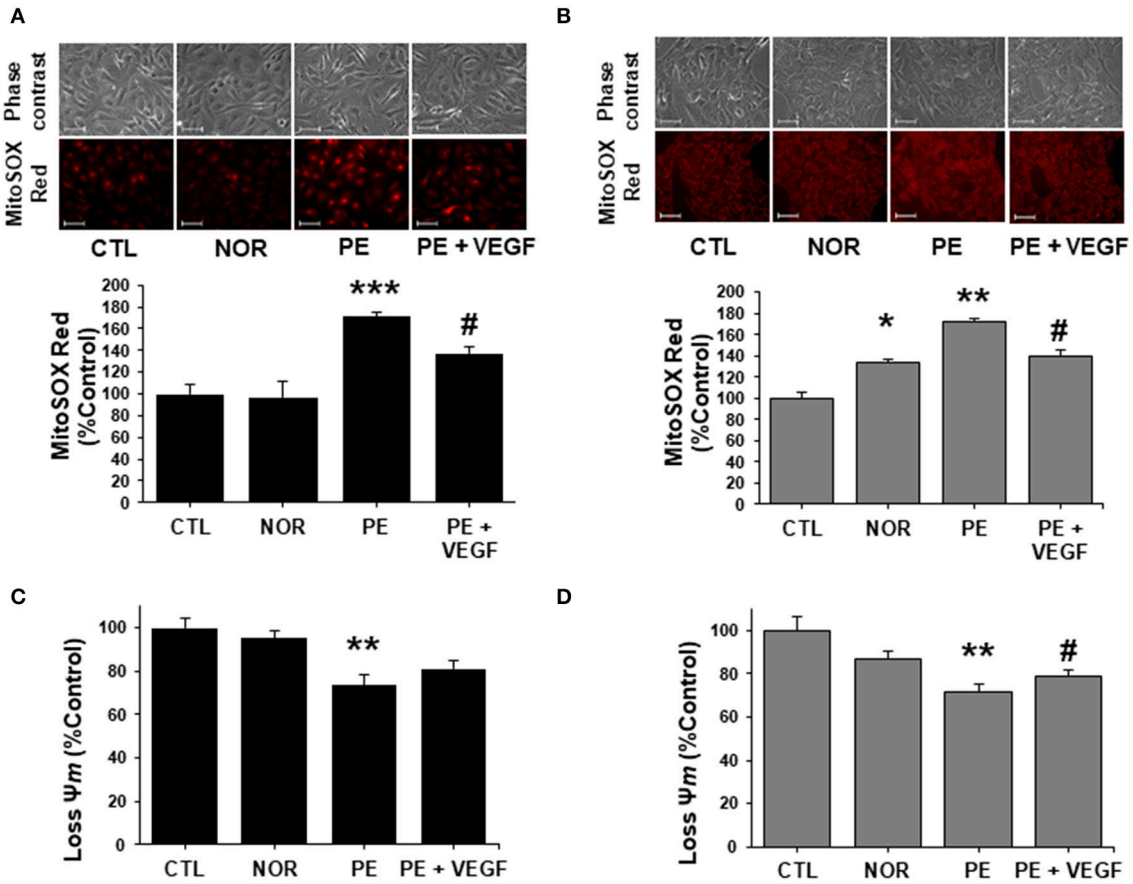

FIGURE 3 | VEGF restores mitochondrial function and abrogates ROS in preeclampsia. ECs and HTR-8/SVneo cells were treated with 2% serum from NOR, CTL, and PE women with and without exogenous VEGF (20 ng/mL) for 24 h. **(A)** Mitochondrial ROS (mtROS) determinations by fluorescent microscopy using MitoSOX Red fluorescent probe in ECs. **(B)** As in **(A)** but with trophoblasts cells. **(C)** PE serum dissipated the mitochondrial membrane potential (Ψm) measured by fluorescent microscopy using JC-1 fluorescent dye in ECs and **(D)** trophoblasts. VEGF (20 ng/mL) restored the mitochondrial membrane potential to CTL levels in trophoblasts. Scale: 100 μm. Data are presented as means ± SEM. ($n = 3$), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, vs. CTL exposed cells. $\#P < 0.05$, vs. PE serum exposed cells, ANOVA (*Bonferroni's post hoc test*).

serum also caused changes in the Ψm in both ECs and HTR-8/SVneo cells. Treatment with PE serum caused a drop in Ψm of about 27% in comparison to ECs treated with CTL serum (**Figure 3C**). Administration of VEGF was not able to restore the Ψm as evidenced in the 8% increase in Ψm in treated ECs. Similar results were obtained in treated trophoblasts (**Figure 3D**). Cells treated with PE serum experienced a drop in Ψm of 28% in comparison with CTL serum. Treatment with PE serum and VEGF was only able to restore the drop in mitochondrial membrane potential with a 7% increase. In contrast, the effects of VEGF almost recover the changes in Ψm to similar levels of cells treated with NOR serum.

sFlt-1 Induces a Metabolic Phenotype Switch to Glycolysis in ECs, but Not in Trophoblasts

Circulating levels of sFlt-1 are known to increase drastically prior the onset of clinical signs of PE (Maynard et al., 2003; Levine et al., 2004, 2006). To assess the effects of sFlt-1 on the mitochondrial bioenergetics of ECs and HTR-8/SVneo, cells were treated with sFlt-1 (0–50 ng/mL) for 24 h and subjected to OCR and ECAR measurements. As shown in **Figure 4A**, sFlt-1 decreased basal and ATP-dependent OCR in a dose-dependent manner in ECs. Also, significant changes in FCCP-stimulated OCR were shown in sFlt-1-treated cells, indicating that mitochondrial spare respiratory capacity was also affected (**Figure 4A**). These evidence demonstrate that sFlt-1-induced angiogenic imbalance, reduce the capacity of the endothelium to respond to changes in energy demand coupled to mitochondrial respiration (**Supplementary Figure 3A**). The analysis of OCR versus ECAR in sFlt-1-treated ECs showed an inhibition of the mitochondrial respiration, with a dose-dependent ability to switch to glycolysis (**Figure 4B**). In addition, the glycolytic response increased more than 5-fold in 50 ng/mL sFlt-1-exposed cells, suggesting a metabolic phenotype switch to glycolysis in ECs, after treatment with sFlt-1 (**Figure 4C** and **Supplementary Figure 3B**). Regarding HTR-8/SVneo cells,

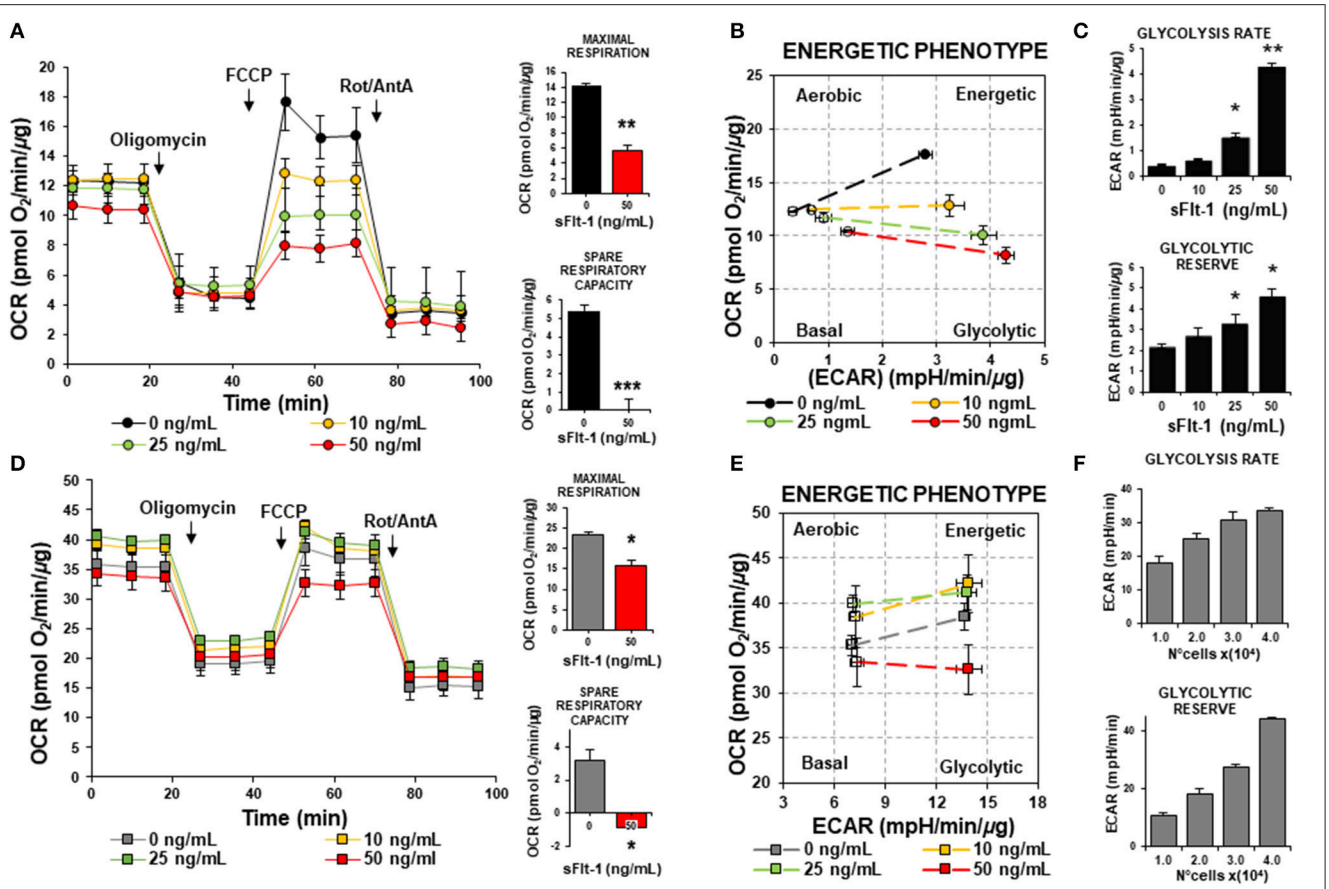

FIGURE 4 | sFlt-1 induces a metabolic phenotype switch to glycolysis in ECs but not in trophoblasts. ECs and HTR-8/SVneo cells were exposed to 0, 10, 25, and 50 ng/mL of rh-sFlt-1 for 24 h. **(A)** Oxygen consumption rates (OCR), maximal respiration (OCR after FCCP administration) and spare respiratory capacity (Difference between basal and maximal OCR), **(B)** Energetic phenotype map, basal and maximal OCR and extracellular acidification rates (ECAR), **(C)** ECAR determinations of glycolysis rate and glycolytic reserve, evaluated in endothelial cells. **(D-F)**, are the same experiments as in **(A–C)**, respectively, but evaluated in HTR-8/SVneo cells. Data is presented as means ± SEM. ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. untreated controls, In **(A,D)**: Student *T*-test. In **(C,F)**: ANOVA (*Bonferroni's post hoc test*).

cells demonstrated a higher basal OCR with an overall reduced FCCP-dependent OCR, in comparison to ECs, displaying high glycolytic rates and glycolytic reserves even at basal conditions (**Figures 4E,F**). Response to sFlt-1 treatment showed a reduction in mitochondrial respiration without increases in the glycolytic flux exerted by 50 ng/mL of sFlt-1 (**Figures 4D–F**).

Results obtained clearly state that in both, ECs and HTR-8/SVneo, sFlt-1 is acting as a disruptor directly into mitochondria. Nonetheless, based on the differential cell energy phenotypes of ECs and HTR-8/SVneo, the effects are interestingly dissimilar in both cell lines.

sFlt-1 Acts as a Mitochondrial Bioenergetics Disruptor in ECs and HTR-8/SVneo

To further confirm the effects of sFlt-1 as a mitochondrial bioenergetics disruptor, mitochondrial metabolism (OXPHOS) was forced by growing cells in galactose media (Robinson et al., 1992; Marroquin et al., 2007). This approach forces cells to exclusively rely on mitochondrial metabolism of glutamine for their ATP energy requirements (Reitzer et al., 1979). Reliance on OXPHOS for ATP production, sensitize cells to mitochondrial disruptors(Marroquin et al., 2007; Dott et al., 2014).

ECs and HTR-8/SVneo exposed to galactose presented slower growth rates in comparison to cells cultured in glucose media (**Figure 5A**). ECs, cultured in galactose, did not evidence changes in their viability or proliferations rates in comparison to cells exposed to glucose (**Figure 5B** and **Supplementary Figure 4A**). Nonetheless, when ECs were exposed to sFlt-1 in galactose media, they had significant reduction in the proliferation rate and viability of about 20% in comparison to non-treated cells (**Figure 5B** and **Supplementary Figure 4A**).

Divergently, the culture of HTR-8/SVneo cells in galactose, evidenced their dependence on glycolysis as a main source of energy, as described previously (**Figures 4E,F**). By forcing OXPHOS in HTR-8/SVneo cells, we confirmed diminished proliferation and viability rates of about 50% in comparison to cells grown in glucose (**Figure 5C** and

Supplementary Figure 4B). These observations are consistent with HTR-8/SVneo evolutionary metabolism of a first-trimester trophoblast, previously defined to be contingent mainly on non-oxidative pathways to support its energetic demands (Illsley et al., 2010). Thus, treatment of HTR-8/SVneo cells with sFlt-1 in galactose media evidenced a reduced viability of about 40% in comparison to controls in galactose (**Figure 5C** and **Supplementary Figure 4B**). Together, these results evidenced that sFlt-1, as a mitochondrial disruptor, affects more dramatically cells that rely on mitochondria machinery for energy proposes, rather than those with a basal glycolytic dependence for their metabolism.

To further corroborate the impact of sFlt-1 over the mitochondrial function and ROS formation, we evaluated the production of mtROS and $\Delta\Psi m$ in ECs and trophoblasts, as measured by fluorescence microscopy. As shown in **Figure 6A**, using fluorescent probe MitoSOX Red, we evidenced that sFlt-1 (50 ng/mL) significantly increased mtROS formation in ECs 1.5-fold, but these effects were not observed in trophoblasts (**Figure 6B**). Then, the evaluation of the $\Delta\Psi m$ using JC-1 fluorescent dye, established that doses of 50 ng/mL of exogenous sFlt-1, reduced Ψm in ECs by 20% (**Figure 6C**). Again, no significant changes were found in trophoblast cells (**Figure 6D**). These results confirm our previous observations

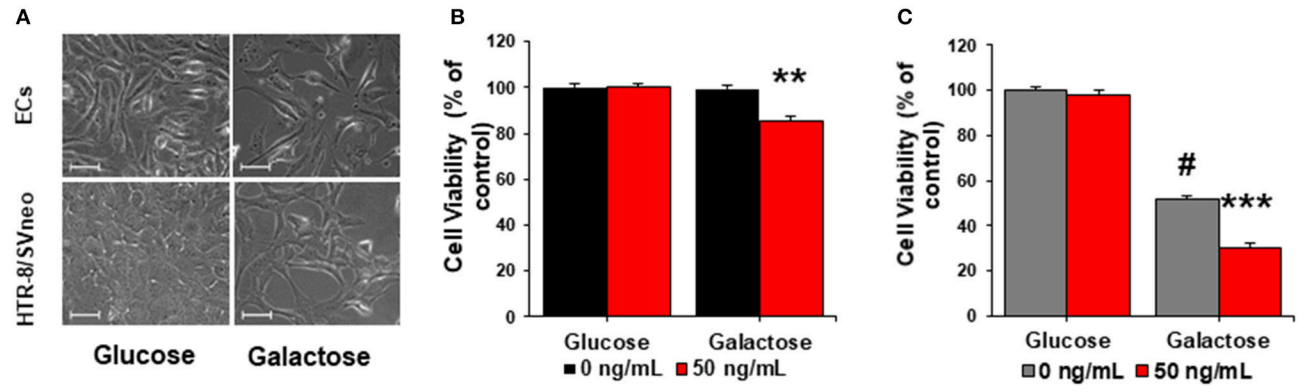

FIGURE 5 | sFlt-1 acts as a mitochondrial bioenergetics disruptor in preeclampsia. **(A)** Morphological changes in endothelial cells (ECs) and trophoblasts cultured in glucose and galactose media (40X). **(B)** Cell viability of ECs and **(C)** trophoblasts cultured in glucose and galactose media and exposed to exogenous 50 ng/mL of sFlt-1 during 24 h. Scale: 100 μm. Data are presented as means ± SEM. ($n = 3$), $^{**}P < 0.01$, $^{***}P < 0.001$, vs. galactose exposed cells. $^{\#}P < 0.05$, vs. glucose-exposed cells. Student *T*-test.

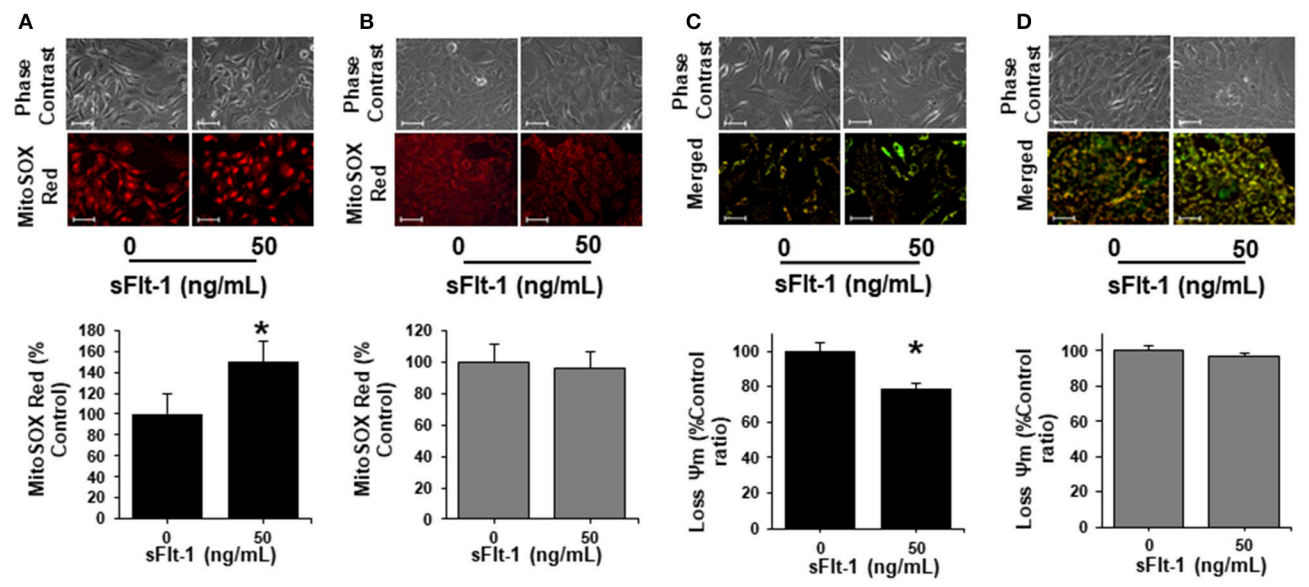

FIGURE 6 | sFlt-1 induces mitochondrial dysfunction *in vitro*. **(A)** Mitochondrial ROS (mtROS) determinations by fluorescent microscopy using MitoSOX Red fluorescent probe demonstrate that sFlt-1 significantly induced mtROS formation in endothelial cells (ECs), while these effects were not observed in **(B)** trophoblasts. **(C)** sFlt-1 dissipated the mitochondrial membrane potential (Ψm) measured by fluorescent microscopy using JC-1 fluorescent probe in ECs, but not in **(D)** trophoblasts. Cells were exposed to sFlt-1 (50 ng/mL) for 24 h. Data is presented as means ± SEM. ($n = 3$), $^{*}P < 0.05$ vs. untreated controls. Student *T*-test.

regarding the role of sFlt-1 to induce alterations in mitochondrial bioenergetics in ECs and its impact on the overall mitochondrial function, as previously demonstrated *in vivo* (Jiang et al., 2015).

DISCUSSION

PE remains as the leading cause of maternal and neonatal deaths worldwide (Duley, 2009). Within the last decade, several reports opened a novel window for understanding the pathogenesis of the disease, by describing the role of circulating anti-angiogenic factors like sFlt-1, in the development and early detection of the disease (Maynard et al., 2003; Levine et al., 2004; Young et al., 2010; Perni et al., 2012; Verlohren et al., 2012). sFlt-1 has been found to provoke endothelial dysfunction (Powe et al., 2011; Sánchez-Aranguren et al., 2014), hypertension (LaMarca et al., 2008) and proteinuria (Maynard and Karumanchi, 2011), demonstrating its culprit role in the onset of PE (Roberts and Rajakumar, 2009). The present study demonstrates the striking effects of sFlt-1 and serum from PE women in the overall cell energy metabolism, mitochondrial bioenergetics and mitochondrial dysfunction, linked to oxidative stress in ECs and first trimester EVT (HTR-8/SVneo cells). Early metabolic perturbations are known to be the hallmark of a range of human pathologies (DeBerardinis and Thompson, 2012). Then, the study of cellular energy metabolism arrives a novel strategy for understanding the etiology of diseases and potentially to develop novel treatments targeting these alterations in energy metabolism (Gohil et al., 2010).

sFlt-1, Increased in PE Serum, Induce Metabolic Perturbations in Preeclampsia

By the aid of the Seahorse-Agilent Extracellular Flux Analyzer, we evaluated the metabolic perturbations preceding the onset of PE. In order to establish a model that resemble PE conditions, ECs and HTR-8/Svneo cells were treated with serum from pregnant women, normotensive and preeclamptic, based on current diagnostic criteria (American College Obstetricians Gynecologist Task Force on Hypertension in Pregnancy, 2013). ELISA analyses showed increased sFlt-1 levels in serum from PE women by several orders of magnitude, in comparison with NOR and CTL serum levels, as reported previously (Maynard et al., 2003; Levine et al., 2004, 2006).

In a normal pregnancy, circulating sFlt-1 levels increase with gestation age (Maynard et al., 2005). However, in PE, due to unknown mechanisms, sFlt-1 is dramatically upregulated (Fan et al., 2014), resulting in increased circulating levels of sFlt-1 prior the onset on the clinical signs of PE (Maynard et al., 2003; Levine et al., 2004, 2006). Here, we have demonstrated and described the alterations in the mitochondrial bioenergetics profiles induced by sFlt-1 increased levels, in both cell lines tested. Our results are in accordance with recent work showing that incubation of HUVEC with 3% plasma from PE women, significantly reduced the overall OCR, measured by a fluorescence-based approach, when compared to cells treated with serum from uncomplicated and non-pregnant women (McCarthy and Kenny, 2016). In addition, our results demonstrate the culprit role of sFlt-1 in the onset of metabolic alterations in PE. sFlt-1 might be responsible for antagonizing the homeostatic activity of VEGF, having an impact at the mitochondrial level. However, it is likely that other molecules, existing in the maternal serum of PE women, could be potentiating the effects observed.

We also showed that mtROS are increased in trophoblasts exposed to NOR serum in comparison to cells exposed to CTL serum. This fact is consistent with the increased oxidative stress status at the intrauterine level, observed by others in normal pregnancies (Chaiworapongsa et al., 2014). PE serum, in ECs and trophoblasts, led to a significant increase in mtROS production in comparison to cells exposed to NOR serum. The augmented mitochondrial oxidative stress, evidenced an alteration in mitochondrial function induced by elevated sFlt-1 levels, present in PE serum. It is likely that increased mtROS production would be directly related to the effects of sFlt-1 on mitochondrial bioenergetics. As it has been reported previously *in vivo*, sFlt-1 induces ROS formation in placental vessels and trophoblasts (Jiang et al., 2015). Potential mechanisms of increased mtROS formation have been associated to a reverse electron transport through complex I, in the mitochondrial electron transport chain, in response to an elevated NADH/NAD^+ ratio (Murphy, 2009). Our observations suggest that dysregulated sFlt-1 levels during pregnancy, induce metabolic perturbations and oxidative stress that might contribute to vascular endothelial dysfunction in PE.

sFlt-1 Induce a Metabolic Phenotype Switch to Glycolysis in Endothelial Cells

To better understand the metabolic bases that would accompany the onset of PE, we studied the role of increasing concentrations of sFlt-1, as a potential early metabolic disturber. We showed a dose-dependent loss of mitochondrial function in ECs treated with sFlt-1. Treatment with increasing concentrations of sFlt-1, evidenced a metabolic phenotype switch from OXPHOS to an enhanced glycolytic cellular response, not been establish before. Our data suggest that in PE, increasing sFlt-1 levels could result in loss of mitochondrial function early in gestation, leading to impaired bioenergetics profiles. Cell energy metabolism varies in tissue of different origins (Benard et al., 2006). Therefore, cell metabolism can be adapted according to the microenvironment surrounding cells. In evidence, other authors have reported that various growth conditions may alter metabolism, contributing to a greater cell dependence on glycolysis (Jose et al., 2011). Our observations of an enhanced glycolytic metabolism, along with an impaired oxygen consumption, suggested a metabolic reprogramming process, as described in tumor biology studies (Jose et al., 2011). These observations illustrate how ECs can alternate OXPHOS to glycolytic pathways under sFlt-1-induced stressful conditions. Nonetheless, the potential effects derived from the prolongation of a aerobic glycolytic metabolism (Warburg effect) in ECs, remains to be determined. Enhanced glycolysis linked to a reduced OXPHOS could markedly affect ECs capabilities to switch from a quiescent metabolic state to an energetic phenotype during angiogenesis.

Regarding HTR-8/SVneo cells, we found, as reported before (Illsley et al., 2010), that they have a functional mitochondrial machinery. In contrast to ECs, sFlt-1 treatment in trophoblasts, causes a non-significant mild drop in OCR. Since the cell basal energetic profile is glycolytic, the overall energetic phenotype after sFlt-1 treatment was not impaired. Studies performed on term isolated trophoblasts have shown that both, syncytium and cytotrophoblast cells, exhibit high reserve respiratory capacity (Maloyan et al., 2012) when compared with our results. This suggests that trophoblast cells, in early stages of gestation, are metabolically programmed to overlap and compensate for the effects of metabolic disruptors. Based on these facts, we presumed that in later stages of pregnancy, when placental function reaches its inevitable end, the metabolic profile of trophoblasts changes as their biological functions terminate. These suggest that cells isolated from full-term placentas may not be the most appropriate approach to study the effects of early metabolic perturbations.

sFlt-1 Acts a Mitochondrial Disruptor

Our results demonstrate a dose-dependent alteration in the mitochondrial bioenergetics, suggesting that sFlt-1 is acting as mitochondrial disruptor. To demonstrate the ability of sFlt-1 to act as a mitochondrial disruptor that drives energy metabolism from OXPHOS to glycolysis, we forced ATP dependence on OXPHOS, by culturing cells in galactose media. Since oxidation of galactose to pyruvate through glycolysis yields no net production of ATP, cells are more sensitive to mitochondrial toxicants, than when grown in glucose (Marroquin et al., 2007). First, culture of ECs in galactose did not involve changes in proliferation rates or viabilities, consistent with their metabolic flexibility to switch from ATP predominantly generated by OXPHOS, to glycolysis as their main energy source (Vallerie and Bornfeldt, 2015). sFlt-1 impaired ECs viability and proliferation rates under OXPHOS dependence, demonstrating that cells that rely mainly in mitochondrial metabolism are highly sensitive to sFlt-1. Results were markedly drastic in trophoblasts. Galactose media enhanced the effects of sFlt-1, decreasing cell viability and proliferation rates of about 40%. In both cell lines tested, culture in galactose media overblown sFlt-1 effects, demonstrating its role as a mitochondrial disrupter, effects that are not basally appreciated in glucose media. Previously, culture in galactose has been employed to identify mitochondrial toxicants (Dott et al., 2014) and molecules that target cellular metabolic

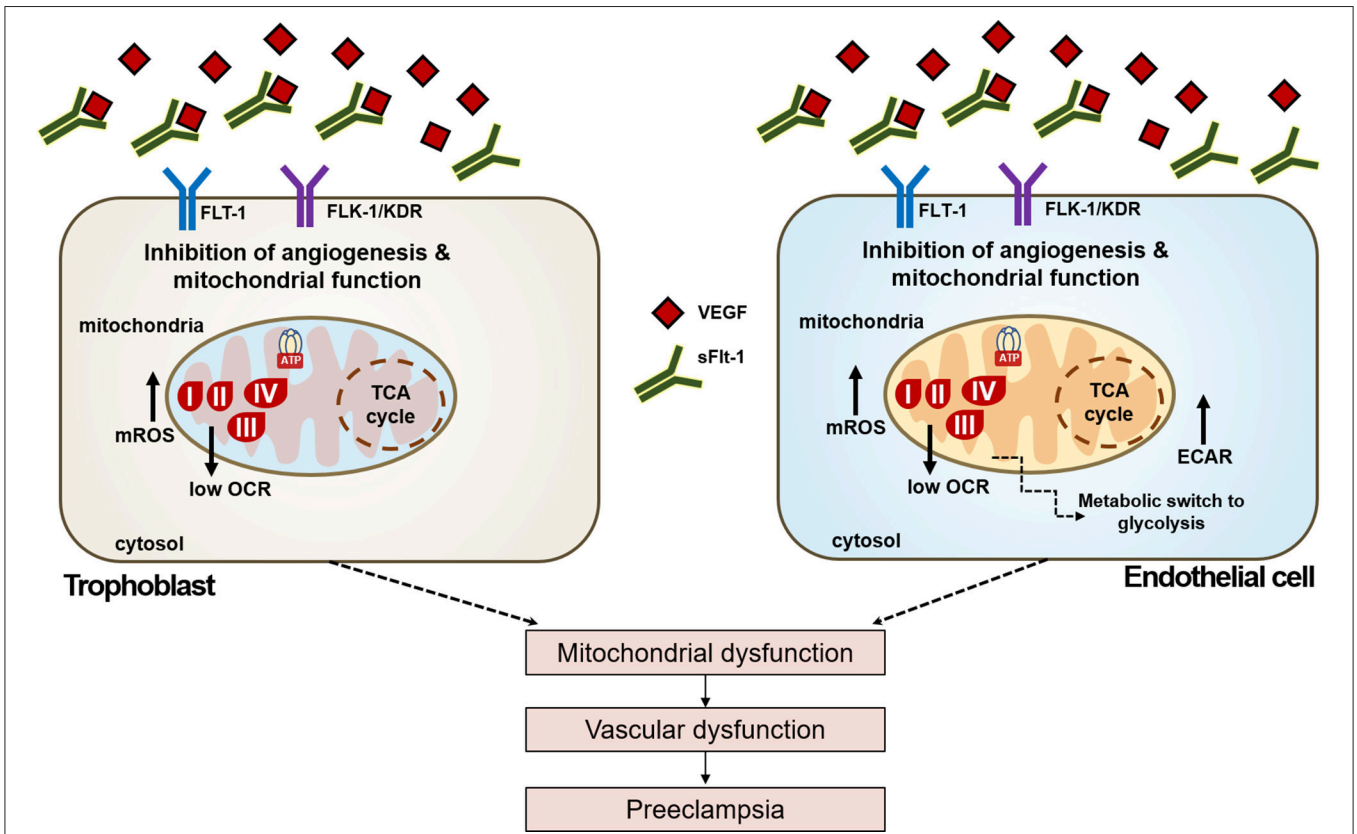

FIGURE 7 | Schematic view of the effects of sFlt-1 dysregulation over cellular metabolism and bioenergetics in PE. Dysregulated VEGF signaling due to up-regulation of sFlt-1 levels in preeclampsia leads to reduced activation of VEGF receptors Flt-1 and Flk-1/KDR, respectively. Effects of dysregulated VEGF bioavailability affect mitochondrial oxygen consumption (OCR), inducing a metabolic phenotype switch enhancing glycolytic response (ECAR) in endothelial cells, but not in trophoblasts. sFlt-1 due to loss of mitochondrial bioenergetics increase oxidative stress in mitochondria (mtROS). Together, these events lead to mitochondrial dysfunction, that would result in vascular dysfunction and the onset of preeclampsia.

shifts as potential therapeutics for pathologies associated with ischemia/reperfusion damage (Gohil et al., 2010).

The increased mtROS production and decreased mitochondrial respiration, coupled with a higher glycolytic capacity of ECs exposed to sFlt-1, evidenced oxidative stress and a metabolic phenotype switch to compensate the detrimental effects of sFlt-1 and mediators present in serum from PE women, over the endothelium. Our results demonstrated that exogenous sFlt-1 induce mtROS formation and a drop in the mitochondrial membrane potential in ECs, but not in trophoblasts. This suggested that sFlt-1 plays a key role in metabolic modulation and reprogramming in endothelium during pregnancy. Whereas, when sFlt-1 levels increase drastically, their role is balanced toward an anti-angiogenic state that leads to metabolic impairment, vascular dysfunction, and PE. sFlt-1 seems to be an important linker between mitochondrial dysfunction, oxidative stress and endothelial dysfunction (**Figure 7**).

Here we have demonstrated that in trophoblast and endothelial cells, disruption of the finely-tuned VEGF signaling by sFlt-1 affects mitochondrial function and metabolism in preeclampsia. As **Figure 7** reviews, sFlt-1 strongly impairs mitochondrial metabolism and bioenergetics, increasing mROS and inducing a metabolic switch to glycolysis in ECs. These findings are very important because they confirm the differential responses of sFlt-1 in both ECs and trophoblasts that are directly related in the development of the disease, from the maternal circulation and placenta, respectively. In addition, results obtained in ECs have strong implications in the maternal hypertension events that are the hallmark of PE. Various reports have revealed the direct implication of VEGF signaling in the regulation of mitochondrial function and angiogenesis (Wang et al., 2011; Guo et al., 2017; Kim et al., 2017). However, the exact mechanisms on how VEGF and downstream events regulate mitochondrial function are still unknown. Nevertheless, the role of the PI3k/Akt/mTOR pathways and eNOS and NO production in relation to high levels of sFlt-1, VEGF signaling, and mitochondrial function is currently under study. Establishing the clear role of sFlt-1/VEGF signaling in PE is key for developing novel strategies for preventing or treating this multifactorial disease.

AUTHOR CONTRIBUTIONS

LS-A and ML: conceived and designed the experiments with the help from CE-G, LG-O, and SS-B; CR-M and AN provided serum samples and clinical expertise; LS-A: drafted the manuscript. Analysis was conducted by LS-A and ML; LS-A, AA, JV-V, and ML: read/revised and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.00083/full#supplementary-material

Supplementary Figure 1 | Parameters of mitochondrial bioenergetics and cellular metabolism in intact cells. **(A)** Oxygen consumption rates (OCR) determinations and parameters of mitochondrial function. **(B)** Energetic phenotype map shows the metabolic state of cells and **(C)** extracellular acidification rates (ECAR) determination and parameters of the glycolytic function.

Supplementary Figure 2 | sFlt-1 levels measured by ELISA. Serum levels were measure in non-pregnant controls (CTL), normotensive (NOR) and preeclamptic (PE) women by ELISA. Data is presented as means ± SEM. ($n = 6$), $^{*}P < 0.05$ vs. CTL, $^{\#}P < 0.05$ vs. NOR. ANOVA (Bonferroni's *post hoc* test).

Supplementary Figure 3 | sFlt-1 induced mitochondrial bioenergetics dysfunction *in vitro*. **(A)** Ratios of basal and maximal (Max) respiratory control (RCR) (State 3/state 4) and **(B)** Extracellular acidification rates (ECAR) yield by time, demonstrate a metabolic phenotype switch from mitochondrial phosphorylation to glycolysis, in endothelial cells exposed to 0, 10, 25, and 50 ng/mL of exogenous sFlt-1 for 24 h. Data is presented as means ± SEM ($n = 5$), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. maximal RCR measured in untreated controls. ANOVA (Bonferroni's *post-hoc* test).

Supplementary Figure 4 | sFlt-1 acts as a mitochondrial bioenergetics disruptor. **(A)** Cell proliferation counts measured in endothelial cells and **(B)** trophoblasts cultured in glucose and galactose media and also exposed to 50 ng/mL of exogenous sFlt-1 for 24, 48, and 72 h. Data is presented as means ± SEM. ($n = 3$), $^{*}P < 0.05$, vs. galactose exposed cells. $^{\#}P < 0.05$, vs. glucose exposed cells. ANOVA (Bonferroni's *post-hoc* test).

Supplementary Table 1 | Clinical characteristics of the recruited women. Data is presented as means ± SEM. Primiparous variable values are presented in %. $^{*}P = <0.05$, vs. non pregnant controls (CTL), $^{\#}P < 0.01$ vs. NOR group. N.A. Data not available.

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REVIEW
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OxInflammation: From Subclinical Condition to Pathological Biomarker

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Inflammation is a complex systemic response evolved to cope with cellular injury, either due to infectious agents or, in general, with sporadic events challenging tissue integrity and function. Researchers involved in different fields have the tendency to look at the inflammatory response with different angles, according to their specific interest. Established its complexity, one of the most evident features of the inflammatory response is the generation of a pro-oxidative environment due to the production of high fluxes of pro-oxidant species. This production begins locally, close to the sites of tissue damage or infection, but eventually becomes a chronic challenge for the organism, if the inflammatory response is not properly controlled. In this review, we focus on this specific aspect of chronic, low-level sub-clinical inflammatory response. We propose the term "OxInflammation" as a novel operative term describing a permanent pro-oxidative feature that interact, in a positive feed-back manner, to a not yet clinically detectable inflammatory process, leading in a long run (chronically) to a systemic/local damage, as a consequence of the cross talk between inflammatory, and oxidative stress mediators. Therefore, it could be useful to analyze inflammatory markers in pathologies where there is an alteration of the redox homeostasis, although an inflammatory status is not clinically evident.

Keywords: biomarkers, neurological disorders, oxidative stress, inflammation mediators, NFκB

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INFLAMMATION AS A COMMON FEATURE OF DYSFUNCTION AND DISEASE

Inflammation is a tissue response to damage characterized by a fine regulated set of events in which several cell type are sequentially activated and able to secrete key mediators (Calder et al., 2013).

With the term inflammation it is usually indicated a confined response to injury or infection characterized by phenomena such as: increased blood flow, capillary dilatation, leucocyte infiltration, and the release of chemical mediators, involved in initiating the damaged tissue repair, and the elimination of noxious agents (Calder et al., 2013).

The main cells involved in the inflammatory responses are neutrophils, macrophages, and other immune cells, controlled by chemical mediators generically called cytokines and chemokines (Mantovani et al., 2011). Indeed, the first step of an inflammatory response is the up-regulation of genes encoding for cytokines, chemokines, and other mediators, through activation of transcription factors such as nuclear factor κ B (NF-κB), activator protein-1 (AP-1), nuclear factor of activated T cells (NFAT), and signal transducer and activator of transcription-3 (STAT3) (Smale, 2010).

The activation of the inflammasome, a multiprotein complex, that serves as a platform for caspase-1 activation and resulting in the proteolytic maturation and secretion of interleukin (IL)-1β and IL-18, is another alternative pathway triggering an inflammatory response (Guo et al., 2015).

The production of specific cytokines or chemokines immediately results in leukocytes recruitment to the site of damage or infection, where they express adhesion properties and produce bactericidal chemicals (including reactive oxygen and nitrogen species, ROS and RNS) and further inflammatory mediators to increase local blood flow and activate phagocytic cells, including macrophages, to eliminate dead cells and tissue debris.

In general, the acute inflammatory episodes are terminated by the removal of pathogens and exogenous agents, (resolution of inflammation), that lead to the inflammatory cells clearance. Several mechanisms work in a coordinated way to consent the proper inflammatory resolution. This very fine and coordinated process aimed to re-establish tissue integrity and physiology. Overall, the block of chemokine signal prevents further neutrophil infiltration, while immune cells such as monocytes and macrophages are attracted by apoptotic neutrophils. Then, the uptake of apoptotic neutrophils by macrophages switch those cells to the resolving phenotype, crucial event to restore tissue homeostasis. Successively, endothelial cells and fibroblasts are then involved tissue repair thanks also to the anti-inflammatory molecules involved in the feedback mechanism responsible for the "switches off" the inflammatory event (Serhan and Savill, 2005). In other words, inflammation is (or should be) a rapid, intense response aimed to tissue protection, and recovery, promptly controlled to avoid possible detrimental consequences arising from an "over-response."

Very often two distinct types of inflammation are considered: the acute and the chronic inflammatory response, according not only with the length of occurrence, but also with the intensity of the cellular response, low grade and high grade, respectively. In general, when the tissue offense is removed, the infection has been cleared, and the damaged tissue repaired we have the so called "inflammation resolution."

As mentioned above, the inflammation is switched off when feedback mechanisms trigger the expression of anti-inflammatory molecules, inducing lipoxins, bioactive autacoid metabolites of arachidonic acid that modulate the transition from neutrophil to monocyte recruitment, the clearance of cells debris and the beginning of tissue repair at the related site (Serhan and Savill, 2005). In some cases, when the trigger(s) that activated the acute inflammatory response persist or, for different causes, the resolution phase is not efficient, a chronic inflammatory state may occur.

Typically, this condition takes place in the presence of chronic infections, unrepaired tissue damage, persistent allergens, but also in the presence of foreign particles, such as environmental nanoparticles or cigarette smoking (Nathan and Ding, 2010; Magnani et al., 2016). In these cases, chronic inflammation is localized and somehow confined to the site of original inflammatory inducer. This condition often results in different types of local tissue remodeling, such as the formation of granulomas and the generation of tertiary lymphoid organs at the site of infection. In the airways, persistent chronic inflammation induced by allergens or pollutant nanoparticles associates to lung epithelial tissue remodeling, inducing in turn asthma and respiratory dysfunction (Murdoch and Lloyd, 2010).

In the recent years, a growing number of chronic inflammatory conditions have been recognized where the initiating trigger is not well defined but does not seem to involve infection or tissue damage. These inflammatory conditions are of particular interest because they accompany many diseases of industrialized countries, including obesity, and type 2 diabetes, atherosclerosis, neurodegenerative diseases, cancer, and aging (Monti et al., 2016).

INFLAMMATION IN HEREDITABLE DISEASES

Under this perspective, chronic inflammation plays an important role in determining a vicious cycle with the associated pathological process, either associated with inherited genetic diseases. For instance, children with the trisomy 21 have a differential expression of genes related to inflammation (BDKRB1 and LTA4H) not located on chromosome 21 and higher levels of pro-inflammatory cytokines compared with normal children, contributing to the systemic aspects of the disease (Broers et al., 2012). In adults with Down's syndrome, the immunophenotypic alterations have been attributed to an early onset of immunosenescence associated to a dysregulation of NF-κB transcription factor (Kim et al., 2006; Trotta et al., 2011).

Similarly, the Rett syndrome (RTT), an orphan progressive neurodevelopmental disorder due to a mutation in the methyl-CpG binding protein 2 gene (*MECP2*), is characterized by an altered cytokine profile. More specifically, T helper Type 2-(Th2) cytokine response, and TH2 cytokines IL-9, and IL-13 levels have been found up regulated in peripheral blood mononuclear cells (PBMC) of RTT patients compared to controls (Pecorelli et al., 2016a). In addition, Sickle cell disease, a monogenic globin disorder characterized by the production of structurally abnormal hemoglobin, is associated with a dysregulation of the inflammatory response resulting in chronic inflammation. Systemic inflammation is evident in Sickle cell patients that present elevated steady-state concentrations of several plasma inflammatory markers, such as IL-1, IL-6, and IL-8, and TNF-α, which are elevated further during acute episodes of illness (Owusu-Ansah et al., 2016).

INFLAMMATION IN NON-HEREDITABLE METABOLIC DISEASES

Beside "genetic disease," also other degenerative diseases due to the combination of genetic risk and life style factors such as the obesity and obesity-associated metabolic syndrome have a significant inflammatory component. For instance, there is solid

evidence that obesity is associated with inflammation, and that chronic inflammation can promote obesity-associated diabetes, in part by inducing insulin resistance (Hotamisligil, 2006). Similar positive feedback loops are present in atherosclerosis, obesity, cancer, and other chronic inflammatory diseases (Pawelec et al., 2014; Kolb et al., 2016; Soeki and Sata, 2016). Indeed, this type of reciprocal relationship may be responsible, at least in part, for the chronic nature of these inflammatory conditions and distinguishes them from the first type of chronic inflammation, which is caused by persistence of the inflammatory inducer, e.g., a microbial/viral invasion or a physical injury.

Obesity is an emblematic example of a negative loop between a pathological (or pre-pathological) phenotype and chronic inflammation. Actually, inflammation has been proposed to be one a major factor involved in the progression of the large spectrum of dysfunctions, finally resulting in an overt disease associated to a positive energy balance, overweight, and eventually to obesity (Hotamisligil, 2006). Obesity-associated inflammation is first triggered by excess nutrients that allows to activate metabolic signaling pathways such as c-Jun N-terminal kinase (JNK), NF-κB, and protein kinase R (PKR) (Solinas and Karin, 2010). Activation of these pathways leads to the release of inflammatory cytokines resulting in a low-grade inflammatory response (Gregor and Hotamisligil, 2011). In addition, obesity is also linked to the increased endoplasmic reticulum stress and the activation of unfolded protein machinery, able to activate NF-κB, JNK, and induce a redox perturbation defined oxidative stress, that will further up-regulate inflammatory cytokines release (Maher Cnop et al., 2012). Altogether, these pathways initiate and sustain obesity-associated inflammation, which is initially confined in white adipose tissue, but eventually spread to other tissues including the liver, pancreas, and brain (Cildir et al., 2013). This condition determines a "systemic low-grade inflammatory response" that affect immune cell infiltration and polarization toward adipose tissue (Hotamisligil, 2006). Indeed, macrophages are recruited in the white adipose tissue in obesity condition, being the main source of cytokines release in this tissue (Weisberg et al., 2003).

Overweight and obesity are major drivers of a complex and undefined pre-pathological condition referred as "metabolic syndrome." In fact, several pathologies such as type 2 diabetes, atherosclerosis and cardiovascular diseases are all often a consequence of the development of metabolic syndrome which is characterized by high blood pressure, atherogenic dyslipidemia, insulin resistance, and increased glucose levels, a pro-thrombotic state and, according to the matter of this commentary, to a mild, persistent pro-inflammatory state (Esser et al., 2014).

OXIDATIVE STRESS: A FAILED PHYSIOLOGICAL RESPONSE TO A CHALLENGE

Living in an aerobic environment, the generation of potentially noxious oxidants is, of course, an inevitable phenomenon. Ever since oxygen became an important component of the atmosphere, aerobic organisms evolved to utilize it for a much better energetic exploitation of organic substrates and also evolved strategies to embody oxygen and oxygen-derived molecules in intracellular signaling and within defense strategies from potentially harmful events.

In order to get to this goal, efficient machineries had to evolve too, able to overcome the consequences of an "oxidative challenge," due to the presence of oxygen or oxygen derived reactive species (Maher and Yamamoto, 2010). The consequences of oxidative challenges are frequently referred as "oxidative stress," which is probably one of the less defined and abused term in biochemistry and cellular physiology. According to one of the most comprehensive and acceptable definition, oxidative stress can be considered "the consequence of the failure to maintain the physiological redox steady state, which is the self-correcting physiological response to different challenges" (Ursini et al., 2016). The important and "dual" role of oxygen and ROS in aerobic organism implies that a moderate and finely tuned production of reactive species is crucial in cell biology within a certain range and the possibly excess of ROS is physiologically prevented by a cellular specific defensive system. The perturbation of this physiological adaptive dynamic equilibrium, induced by a persistent challenge or inappropriate feedback response, leads to the inflammatory state (Forman et al., 2014; Ursini et al., 2016).

MAIN PLAYERS IN REDOX HOMEOSTASIS

Thus, ROS and RNS, main cell electrophiles, are involved in an extremely delicate and easily corruptible balance between harm (i.e., biological damage) and benefit (i.e., redox signaling). The need of a perpetual flux of these oxidants is primarily satisfied by mitochondria, since the physiological electron leakage from respiratory chain (complex I, II, and III) results in a generation of anion superoxide (Rimessi et al., 2016). Interestingly, the extent of mitochondria contribution in cellular ROS has been recently questioned (Fridovich, 2004), on the basis of novel findings suggesting that this source is much less quantitatively relevant in respect to the original estimation by Chance et al. (1979). Remaining in mitochondrial environment, there are other enzymes capable to participate in ROS production, such as dihydrolipamide dehydrogenase, monoamine oxidase etc. (Rimessi et al., 2016).

To preserve within a physiologically tolerable range the level of superoxide, mitochondria has been geared with the most efficient ROS scavenger, the manganese superoxide dismutase (MnSOD) which, as the other cytosolic isoenzyme (i.e., copper zinc SOD), is the only biological agent able to convert superoxide into hydrogen peroxide (Zou et al., 2017). Owing its relatively mild reactivity hydrogen peroxide can serve as the prominent component of intracellular signal transduction. However, when the concentration of this ROS exceeds the homeostatic level, it can give rise, especially in presence of free copper or iron, to the most potent free radical, hydroxyl radical, i.e., the main responsible of oxidative stress-related damage to proteins, lipids

and DNA. One of the few reactive species with a comparable cytotoxic potentials is peroxynitrite, which stems from the combination between nitrogen monoxide and superoxide ion (Pacher et al., 2007). Glutathione peroxidase (Gpx) and catalase (CAT) play a prominent role in preventing from hydroxyl radical formation, because they can definitely covert hydrogen peroxide into water, although via different catalytic mechanisms. Gpx is greatly important in maintaining within a suitable concentration range the most abundant redox couple in a cell, reduced, and oxidized glutathione (GSH and GSSG, respectively) (Cadenas and Davies, 2000). The other enzymatic and non-enzymatic factors involved in glutathione cycle and other defensive agents (e.g., thioredoxin) have been already described in-depth in some fascinating previous reviews (Chance et al., 1979; Cadenas and Davies, 2000; Fridovich, 2004; Pacher et al., 2007; Forman et al., 2014; Rimessi et al., 2016; Ursini et al., 2016; Zou et al., 2017).

Besides mitochondria, there are other cellular constituents that directly participate in redox processes; for example, peroxisomes are rich in CAT, allowing them to cope with the elevated propensity of these organelles for the generation of superoxide anion and hydrogen peroxide (Davies et al., 2017). The endoplasmic reticulum also contributes to cellular levels of ROS, derived by protein folding processes and membrane bound NADPH oxidase (NOX) enzymes. Different location but similar catalysis products characterize other pro-oxidant enzymes such as lipoxygenases, cyclooxygenases, and xanthine oxidase (Cho et al., 2011).

It is undoubted that the complex network of defensive agents, including SOD, CAT, Gpx etc. acting, essentially, to prevent the formation of the highly cytotoxic hydroxyl radical (·OH), is the primary, in terms of both time, and importance, defensive layer against ROS/RNS induced damage. Possible leaks from this outpost can be then countered by further repair systems such as proteinases, lipases, DNA repair enzymes (Davies et al., 2017). Important defensive contribution is also afforded by proteasome, the ubiquitous multicatalytic protease responsible for degradation of intracellular target proteins, including oxidized proteins (Aiken et al., 2011). The proteasome is composed by a 20S particle harboring the proteolytic active sites, which is capped to one or both sides by the 19S regulatory particles, which assist the processing of poly-ubiquitinated substrates. The 20S exists also in the uncapped form which is proposed to cope with the degradation of oxidized and unfolded/aggregating-prone substrates (Schmidt and Finley, 2014). It has been shown that an increase in oxidant species leads to disassembly of 26S, into the catalytic cores 20S and the regulatory caps 19S. 20S, but not the 26S, is able to remove oxidized proteins thereby preventing their aggregation and cross-linking (Aiken et al., 2011; Demasi et al., 2014). Interestingly, it has been hypothesized that the effect of proteasome cell redox balance perturbation is biphasic: low levels of reactive species induces activation of 20S activity while high levels (and thus full-blown oxidative stress) an inhibition of its activity (mostly by posttranslational modification) (Aiken et al., 2011; Demasi et al., 2014).

MUTUAL ACTIVATION BETWEEN INFLAMMATORY AND OXIDATIVE STRESS MEDIATORS

Exogenous stressors (pollution, smoking, fat diet, etc.) or endogen factors (diseases and inflammation itself) are potentially able to disrupt this finely tuned homeostasis, inducing the formation of oxidants, so called electrophiles, such as ROS and RNS, that take one or two electrons from a nucleophile, from various sources (Cadenas and Davies, 2000; Forman et al., 2014).

Inflammation is indeed an important source of oxidative stress. In the course of an inflammatory event, oxidants molecules can originate from the activity of lipoxygenases, cyclooxygenases, xanthine oxidase, phagocytic, and non-phagocytic nicotinamide adenine dinucleotide phosphate oxidases (NOXs) and also from Fenton/Haber—Weiss reactions catalyzed by transition metals, possibly locally freed in the milieu of a physical injury and eventually released systemically (Forman and Torres, 2002; Bergamini et al., 2004; Valko et al., 2005; Sies, 2015). In particular, NOX2 and mitochondria-derived ROS are required for respiratory burst occurring in activated leukocyte. During this semi-physiological event, the levels of superoxide increase from picomolar to micromolar concentrations inside the cells within a few minutes (Sethi and Tabel, 1990; Wenzel et al., 2017). This makes systemic acute (e.g., sepsis), but also low-grade inflammation, a terrific source of ROS, as demonstrated by the several epidemiological studies reporting a strong and positive association between peripheral makers of inflammation and oxidative stress in individuals affected by various pathologies (Nonaka-Sarukawa et al., 2003; Abramson et al., 2005; Bougoulia et al., 2006; Cottone et al., 2006).

NOXs function and regulation are a clear-cut example of the mutual connection between redox processes and inflammation. Notably, the members of this membrane-bound multi-enzyme complex are the only endogenous source of ROS with the specific function to produce signaling free radicals molecules whereas all other aforementioned sources produce ROS either upon redox modifications (e.g., thiol oxidation in xanthine dehydrogenase generates the oxidase form) or by mitochondria (Wenzel et al., 2017). ROS (O_2 and H_2O_2) generated by NOX_S essentially serve for microbial killing at sites of inflammation (NOX2) and redox signaling. Pro-inflammatory mediators, in primis TNFα and IL-1β, represent the major inducers of activation of both phagocytic and non-phagocytic NOX (Lee and Yang, 2012). In turn, the increase in ROS production can activate cells of immune system (but also of endothelium, and different types of epithelium) to induce protein kinase cascade (PKC, MAPKs etc.) and redox-sensitive transcription factors, in primis AP-1, and NF-kB. This concatenation of events ultimately brings about the expression of a number of pro-inflammatory mediators, including those involved in the stimulation of NOX. It is this ability to translate an inflammatory stimulus into pro-oxidant impulse that accounts for the widely postulated central role of NOXs in the pathogenesis of various diseases, such as cardiovascular diseases, lung diseases, cancer and Alzheimer's disease (AD) (Yang et al., 2007; Grammas,

2011; Lee and Yang, 2012; Heneka et al., 2015; Dias et al., 2016; Wenzel et al., 2017).

In conclusion, not only inflammatory mediators such as the release of cytokines are able to implement the production of ROS via the activation of specific enzyme present in the cells (NOX, XO, etc.) but also ROS can themselves modulate inflammation (Mittal et al., 2014). For instance, it has been well demonstrated that ROS can activate transcription factors involved in the inflammatory process such as NFAT-1, AP-1, HIF-1a, and NF-κB (Morgan and Liu, 2011). In addition, ROS can act as second messengers and induce a cascade of events by MAPK activation (p38, JNK, and ERK1/2) which can also lead to migration of inflammatory cells and therefore augment the inflammatory response (Son et al., 2013; Mittal et al., 2014). It is worth mentioning that, based on the ROS concentration, it is possible to have opposite results. Several authors have shown that an acute and intense oxidative stress can lead to the oxidation of NF-κB, affecting its ability to translocate to the nucleus and bind to the DNA (Morgan and Liu, 2011).

OXINFLAMMATION AS SPECIFIC FEATURE IN THE PATHOGENESIS OF COMMON AND RARE DISEASES

The central role of the reciprocal interplay between oxidative stress and inflammation is a general notion. In this review we want to point out that a specific chronic failure in the control of oxidative events, resulting in a mild, long-term pro-oxidative cellular environment, is a specific feature in the genesis of diseases.

As mentioned above, there are abundant lines of evidence indicating that a low grade/chronic inflammatory response, escaping for different reason to a resolving feedback regulation, and the presence of reactive species in the inflammatory processes are hallmark feature of several pathologies, ranging from genetically determined diseases, such as Rett, and Down syndrome, to the most common metabolic dysfunctions, including diabetes, cardiovascular disease, cancer, and neurodegenerative disorders, such as AD and Parkinson's disease (Kaneto et al., 2007; Pou et al., 2007; Yang et al., 2007; Perluigi and Butterfield, 2012; Heneka et al., 2015; Cervellati et al., 2016; Dias et al., 2016; Pecorelli et al., 2016b; Valacchi et al., 2017; Wenzel et al., 2017).

DIABETES

Oxidative stress and inflammation are considered critical factors for the pathogenesis of diabetes mellitus (DM) type I and II and both plays a crucial role in the development of the frequent micro vascular and cardiovascular complications of this chronic disease (Kaneto et al., 2007). The most direct body evidence in favor of this biological link has been generated from the several population-based studies showing association between DM and systemic levels of inflammation or oxidative stress (Pradhana et al., 2001; Hu et al., 2004; Wu et al., 2004; Dalle-Donne et al., 2006). At least partially, the mechanistic explanation of how the mediators of redox/inflammation could act as downstream/upstream player in the impairment of endocrine activity of pancreas, insulin resistance, and diabetes-specific pathology have been illustrated in recent reviews (Baynes and Thorpe, 1999; Ceriello and Motz, 2004).

Type I DM is caused by the autoimmune destruction, with collateral and consequent inflammatory process of β cells of the endocrine pancreas. ROS/RNS, along with pro-inflammatory cytokines generated by islet-infiltrating immune cells, may contribute to the impairment of β-cell function, targeting cell metabolism and potassium (adenosine-5'-triphosphate) channels (Drews et al., 2010) and inducing mitochondrial dysfunction (Rachek et al., 2006) [further worsened by hyperglycemia (Rolo and Palmeira, 2006)], thus exacerbating oxidative challenge against islet cells.

The implication of Oxidative stress and inflammation-related phenomena have been mostly studied in type II DM.

Growing lines of evidence suggest that oxidative stress may be the primary triggers of tissue damage due to hyperglycemia by several mechanisms. As recently described by Giacco et al. (Giacco and Brownlee, 2010) and others (Rolo and Palmeira, 2006), the proposed mechanisms consist in alteration of polyol and hexosamine pathway, increase in intracellular formation of advanced glycation end-products (AGEs) and their receptors (RAGEs), and activation of protein kinase C (PKC) isoforms.

One of the best known processes consists in the enhanced intracellular formation of advanced glycation end-products (AGEs), resulted from the non-enzymatic reaction of glucose and other reactive sugars with amino groups of amino acids (as well as lipid and DNA). This multi-step reaction is accelerated by ROS (Ahmed, 2005).

It has been suggested that the interaction with RAGEs might result in an increased production of reactive species (Giacco and Brownlee, 2010) (from mitochondria, auto-oxidation of glucose, and NOXs) and activation of various redox sensitive transcription factors such as NF-κB (Haslbeck et al., 2005), with subsequent upregulation of pro-inflammatory and thrombogenic genes. By fueling this pathways, the dichotomy AGE/RAGE might contribute to the development of typical diabetes complications, including polyneuropathy, retinopathy, and atherosclerosis (Haslbeck et al., 2005; Giacco and Brownlee, 2010). In support with this claim, transgenic mice lacking RAGE showed, less propensity to develop atheroma, possible effect of decreased expression of proinflammatory mediators, and ROS (Soro-Paavonen et al., 2008). Noteworthy, the vicious cycle involving oxidative stress and inflammation in diabetes find confirmation in the recent evidence that some pro-inflammatory agents might be better ligand for RAGEs than AGEs their self (Giacco and Brownlee, 2010).

Insulin resistance occurs in most of patients with type II DM, and this adverse metabolic condition is closely associated with overall and, mostly, central obesity (Esser et al., 2014). As already underpinned, obesity can be almost regarded as a synonymous of (systemic) inflammation (Gregor and Hotamisligil, 2011), although infrequent cases of metabolically healthy obesity exist. Elevated levels of the typical markers of obesity-induced low grade inflammation, hs-CRP and TNF-α are strongly predictors

of type 2 DM in adults (Dandona et al., 2004). In turn, increased of these and other downstream- or upstream- linked cytokines promote enzymatic (in primis NOX) and non-enzymatic pro-oxidative processes.

CARDIOVASCULAR DISEASE

As discussed earlier, DM is a major risk factor for atherosclerosis, the leading cause of CVD. Like the disease of carbohydrate metabolism, atherosclerosis is widely referred as to chronic inflammatory pathology, where oxidative stress plays a crucial pathogenic role. Vascular sources of ROS/RNS are multiple and include mitochondria, the uncoupling nitric oxide synthase, and various enzymes, in particular xanthine oxidase, lipoxygenase, and NOX (Manea et al., 2015). The function of NOX as a "bridge" between inflammation and oxidative stress is essential in the development and progression of atherosclerosis. Various NOXs are constitutively expressed in endothelial cells, smooth muscle cells, adventitial fibroblasts, and circulating and tissue-resident immune cells participating in atherosclerotic processes [comprehensive review on the topic here (Manea et al., 2015; Kattoor et al., 2017)]. NOX isoforms involved in vascular pathologies appear to affect the activation of transcription factors (NF-kB, AP-1, and signal transducer and activator of transcription, STAT) found within atherosclerotic lesions and in the vascular wall of animals and human diabetic and hypertensive patients (Manea et al., 2015). It is well known that these factors are master regulators of genes associated with differentiation, proliferation, and migration of immune cells and resident vascular cells, and modulate the expression of a plethora of pro-inflammatory and immune factors (Celada et al., 1996). Relevant to the concept of OxInflammation as a self-perpetuating vicious cycle of cytotoxic substances coming from different sources, these redox-sensitive transcription factors, along with others involved in vascular remodeling, have been implicated in the regulation of vascular NOX (Madamanchi et al., 2005; Singh and Jialal, 2006). This regulation can occur directly, through direct transcription factor- NOX gene promoter interaction mechanisms, and/or indirectly, by upregulating the expression of pro-inflammatory cytokines which are agonist of NOX activity (Manea et al., 2015). The resulting, still not completely deciphered, redundant signaling pathways have been deemed to be intimately implicated in atherosclerosis onset.

Immune cells play a crucial role in the initiation, propagation, and both acute and chronic complications of CVD. Activated leukocytes participate in vascular inflammation response and secrete the content of their azurophilic granules in close vicinity to inflamed tissues (Huang et al., 2013). One of the main component of these granules is the heme protein Myeloperoxidase (MPO) (Huang et al., 2013). This enzyme, which is abundant in human atheroma and in patients affected by CVD (Podrez et al., 2000), is able to amplify the oxidative potential of its co-substrate H_2O_2 (derived from NOXs, xanthine oxidase etc.) forming potent oxidants (e.g., hypochlorous acid) capable of chlorinating and nitrating phenolic compounds (Carr et al., 2000). It has been shown that MPO, released within both the circulation and within atherosclerotic plaque, binds to high density lipoprotein (HDL), leading to oxidative modification and inactivation of proteins responsible of the antioxidant and anti-inflammatory proprieties of the lipoprotein (Huang et al., 2013). The major targets of MPO deleterious effect are the functionally couple of proteins, apoliprotein A1, and paraoxonase-1 (PON-1) (Cervellati et al., 2015a).

It has been suggested that the most important physiological function of PON1 is to contrast the oxidation of low density lipoproteins (LDLs) (Huang et al., 2013; Cervellati et al., 2018). This process, yielding to the formation of the highly pro-atherogenic oxidized LDL (oxLDL), is caused by the altered local and systemic production of ROS (Madamanchi et al., 2005). Ox-LDLs induce endothelial cell activation, dysfunction, death, and contribute causally to atherosclerosis, onset and progression, through (again) the activation of NF-κB and AP-1 pathways (Valente et al., 2014). Endothelial injury induces the expression of adhesion molecules and chemiotactic cytokines, thereby promoting activation and migration of immune cells vascular smooth muscle cell (Madamanchi et al., 2005). Subsequently, oxLDLs (but also not-modified LDLs) are transported into and across the endothelium, likely at the site of endothelial damage provoked by oxLDLs their-self (Madamanchi et al., 2005; Kraehling et al., 2016). Within the intima layer, these particles are further oxidized by ROS produced by resident macrophages, that take up oxLDL and become foam cells. These deadly cells exacerbates the inflammatory processes that terminate with atheroma formation (Madamanchi et al., 2005). Therefore, also oxLDL represent a player able to translate the oxidative challenge in an inflammatory event, which in turn can induce endogenous ROS formation and further feed the OxInflammation vicious cycle.

Both central and peripheral redox homeostasis dysregulation seem to occur in a very large spectrum of pathologies, if not all, regardless of the pronounced systemic involvement that characterizes any specific disease. Some tissues and organs are known be highly vulnerable to reactive species challenge. For instance, cells composing the brain have a long life, relatively low levels of endogenous antioxidants (particularly glutathione), high levels of peroxidizable polyunsaturated fatty acids, and high levels of pro-oxidant metals. Moreover, the high ratio of oxygen consumption further supports the "normal" physiological extent of ROS leakage from mitochondrial activity (Golden et al., 2002; Cervellati et al., 2014a, 2016). This organ-specific susceptibility to oxidative insults accounts, at least partially, to the well-known involvement of oxidative stress in several neurological pathologies such as Down's syndrome, AD, Parkinson's, etc. (Abramov et al., 2004; Olivieri et al., 2011; Cervellati et al., 2013, 2014a, 2016; Thanan et al., 2014). In the next session, we will mainly focus on the involvement of OxInflammation in brain related diseases.

ALZHEIMER'S DISEASE

The "amyloid cascade hypothesis" is currently the most widely accepted model explaining AD aetiopathogenesis. β-amyloid

(Aβ) deposition and aggregation would be the main and the first neuropathological event leading to the formation of senile plaques and then favoring the neurofibrillary tangles (NFT) formation (the other AD neuropathological hallmark), neuronal cell death, and dementia (Hardy and Higgins, 1992). Nevertheless, important concerns on the centrality of Aβ on AD onset and other hypotheses has been advanced in the years (Nunomura et al., 2006; Crowley, 2014; Cervellati et al., 2016). Noteworthy, in spite of the variety of proposals in this framework, inflammation, and oxidative stress always emerged as possible interconnected fundamental components of AD pathogenesis and pathophysiology (Zhao and Zhao, 2013; Cervellati et al., 2016).

One of the central principle of the proposed concept of "OxInflmmation" is that the dynamic and self-perpetuating connection between the two short-circuits (biochemical and immune) is not locally (CNS) confined but reverberates at systemic level. AD properly fits this definition, since systemic manifestations, including inflammation and oxidative stress (as well as diabetes and other metabolic dysfunctions) are not merely risk factors of the diseases, but coexist and drive (and are driven by) neurodegeneration (Leuner et al., 2012; Metti and Cauley, 2012; Cervellati et al., 2014a, 2016; Morris et al., 2014). Consistent with this scenario, peripheral inflammatory markers such as C-reactive protein (CRP), IL-6 and TNF-α, have been repeatedly, although not always, found to be cross-sectionally and longitudinally associated with an increased risk of AD (Metti and Cauley, 2012). These findings are not surprising since the hypothesis of a communication between the systemic immune system and the CNS is gaining wide acceptance (Minihane et al., 2015). According to this, it has been shown that a systemic and chronic presence of proinflammatory cytokines can lead to the brain's activation of the innate immune system, prelude of neuroinflammation, an important component of AD pathogenesis and physiopathology (Heneka et al., 2015). Microglia, brain resident macrophages, provide the most significant innate and adaptive immune responses and function as CNS "sensor" of alteration the peripheral immune homeostasis (Heneka et al., 2015). Notably, animal experiments have shown that in the aging CNS, microglia exhibit enhanced sensitivity to inflammatory stimuli, so highly susceptible of further stimulation by inflammatory mediators (in primis, IL-6, TNF-α and IL-1β) that can easily cross the blood brain barrier (BBB) (Banks et al., 1995; Metti and Cauley, 2012; Perry and Teeling, 2013). In turn, this detrimental cross-talk entails the production of neurotoxic cytokines, chemokines, prostanoids as well as reactive oxygen species, which has been suggested to contribute to the formation and/or toxicity of Aβ and neurofibrillary tangles (Patel et al., 2005; Heneka et al., 2015).

NOX (mostly NOX2), localized not only in microglia but also in astrocytes and neurons, plays a central role in the aforementioned detrimental processes occurring since the early stage of AD. It has been shown that exposition of Aβ to microglia results in respiratory burst due to NOX activation and release of superoxide anion, and pro-inflammatory cytokines and chemokines (Mander and Brown, 2005; Wilkinson and Landreth, 2006). In turn, the soluble mediators derived from NOXs of these brain resident cells but also, as discussed in the previous paragraph, of peripheral immune cells, can further activate NOX and exacerbate OxInflammation. Furthermore, the cytokines can also stimulate inducible nitric oxide synthase (iNOS) in microglia and astroglia, producing high concentrations of nitric oxide (Mander and Brown, 2005). As clearly shown by Mender et al. (Mander and Brown, 2005), when iNOS and microglial NOX are simultaneously activated can lead to neurons death. Indeed, as mentioned earlier, nitric oxide becomes (neuro) cytotoxic only in presence of high concentration of superoxide anion, leading to the formation of peroxynitrite. This RNS, in turn, can mediate the post-translational modification of Aβ peptide and the nitration of these peptides has been shown to increase the propensity of Aβ to aggregate and to initiate plaque formation (Heneka et al., 2015).

Oxidative stress due to NOX over-activation or mitochondrial dysfunction has been also envisaged to be, not a mere effect, but the primary trigger of Aβ and NFT formation in AD brain (Moreira et al., 2010; Cai et al., 2011; Cervellati et al., 2016). The generation of Aβ occurs through two sequential cleavages of amyloid precursor protein (APP), elicited through β-secretase and γ-secretase. Experiments on animal models showed that oxidative stress significantly increases the catalytic activity of these two enzymes, which in turn augments Aβ production (Praticò et al., 2001). The deposition of Aβ in the neuronal tissue could reflect a compromised blood–brain barrier (BBB) and oxidative stress could contribute to damaging BBB either directly or through the activation of metalloproteinases (Cai et al., 2011; Aliev et al., 2014; Cervellati et al., 2016). As a consequence of the loss in BBB physical integrity, influx of Aβ from cerebrospinal fluid (CSF) and noxious substances (such as oxidants and pro-inflammatory agents) form systemic circulation, may increase and exacerbate AD pathological alterations.

In a similar fashion, oxidative stress can be both a downstream and upstream factor for NFT formation (Chauhan and Chauhan, 2006). These oligomers and toxic filaments are the result of aberrant hyperphosphorylation of microtubule-associated protein tau, which, in turn, is due to the dysregulated activities of kinases and phosphatases (Chauhan and Chauhan, 2006). It has been reported that abnormal polymerization of tau might be promoted by lipid peroxidation process, which is indirectly catalyzed by excess of iron present in intraneuronal NFT (Cristóvão et al., 2016). Accordingly, treatment of primary rat cortical neuron cultures with iron plus hydrogen peroxide enhanced tau hyperphosphorylation (Lovell et al., 2004). This aberrant process was also shown to be primed by antioxidant deficiency in AD transgenic mice, most likely because oxidative stress can activate one of the kinases (p38 MAPK) putatively involved in tau hyperphosphorylation (Giraldo et al., 2014). As observed for Aβ, also the neurotoxic effect of tau might be at least partially mediated by oxidative damage. Consistently, in a drosophila model of human tauopathies, the induced decrease of antioxidant enzymes worsened tau-related neurodegeneration, while upregulation attenuated this detrimental process (Dias-Santagata et al., 2007). The link between tau and oxidative stress was further demonstrated by studies on transgenic mice overexpressing tau gene and developed NFT; extensive mitochondrial dysfunctions associated

with increased production of ROS and protein carbonyl levels were found to chiefly characterize the brain of these animals (Dumont et al., 2011).

To close the here proposed OxInflammation loop, it has been hypothesized that also neuro-inflammation could participate in the formation of NFT. The most compelling proof in support of this link was obtained by Kitazawa et al, who showed that administration of a known inducer of brain inflammation increased tau hyperphosphorylation, but not Aβ accumulation, in AD transgenic mice that develop both pathological hallmarks in an age-dependent manner in disease-relevant brain regions (Kitazawa, 2005). It has been also shown that tau dysfunction (characterizing animal models of tauopathies) is associated with a dysregulation of cytokine secretion by microglia, eventually enhancing tau p38 MAPK-dependent hyperphosphorylation (Bolós et al., 2017).

RETT SYNDROME

The concept of OxInflammation has been firstly proposed in the pathogenic processes of RTT (Pecorelli et al., 2016b; Valacchi et al., 2017). This genetic disease is characterized by a parallel alteration of systemic inflammation and severely compromised redox balance, with the latter aberration repeatedly found in several models including mouse brain, non-neuronal tissues (e.g., fibroblasts), CSF and serum/plasma (Sierra et al., 2001; Pecorelli et al., 2011; De Felice et al., 2014; Cervellati et al., 2015b; Hayek et al., 2017).

Redox homeostasis dysregulation in RTT appeared to be the result of concentric alterations in antioxidant defensive mechanisms, mitochondria dysfunction and abnormal constitutive activation of NOX2 (Cervellati et al., 2015b; Pecorelli et al., 2016b). This last phenomenon has been explained as possible result of the chronic stimuli of marked and chronic immune dysregulation characterizing the typical RTT phenotype. A mechanism leading to self-perpetuating the inflammation-oxidative stress detrimental cycle in RTT might also be the chronic intermittent hypoxia (De Felice et al., 2009). This phenomenon is associated with some typical clinical manifestations of the genetic disorder, such as central apneas, significant obstructive apneas and hyperventilation (Hagberg, 2002). It is well-recognized that hypoxia can, in cooperation with oxidative stress, induce pro-inflammatory cytokines production via NF-κB, AP-1 and HIF-1-α activation (Cervellati et al., 2014b; Pecorelli et al., 2016b).

The most direct evidence in support of a role of oxidative stress in RTT development stem from the multiple findings of higher levels of lipoperoxidation byproducts, in particular 4-hydroxynonenal (4HNE), in peripheral cells and plasma of affected individuals compared to controls (Signorini et al., 2013). Under redox imbalance, 4HNE can indiscriminately react with proteins and nucleic acids, leading to cellular and tissue damage (Uchida, 2003). In particular, the formation of 4HNE-protein adducts (4HNE-PA), as a consequence of the covalent link between 4HNE and proteins, can irreversibly affect their structure/function, as reported in several diseases (Valacchi et al., 2017). In addition, the generation of these protein adducts can induce humoral immune responses (Kurien et al., 2006). The increase in 4HNE-PA levels leads, when it is accompanied by a unequate proteosome degrading activity, to intracellular and extracellular deposition of self-aggregating misfolded proteins. As shown by Kurien et al., this aberrant process results in the generation of neo-antigens that, once recognized by different immune receptors, are able to trig both innate and adaptive immune responses (Kurien et al., 2006). The impairment in proteosome machinery, and likely autophagy, observed in RTT cells, represents the ideal scenario for the accumulation of these immunogenic misfolded proteins and may contribute to the release of inflammatory mediators, which in turn contribute to the disease progression and severity (Cervellati et al., 2015b; Pecorelli et al., 2016c; Valacchi et al., 2017).

OXINFLAMMATION AS FRUITFUL SOURCE OF PATHOLOGICAL BIOMARKERS

An ideal biomarker should be non-invasive (blood and urine are the most accessible specimens), enabling broader clinical access or eventually efficient population screening and reflect disease pathophysiology and be informative of the disease process, even in the early phase (Dalle-Donne et al., 2006). OxInflammation is a potential fruitful source of biomarkers that fit the above definition. This statement is true mainly for the most clinically and epidemiologically validated markers of (even subclinical) systemic inflammation such as Hs-CRP, IL6, IL8, IL1, and TNFα. In particular, Hs-CRP, although gap in knowledge of its function still remains, is the marker of choice for the evaluation of systemic inflammation and the most assessed in clinical studies, due to the high sensitivity/specificity (Danesh et al., 2000). The assessment of this marker allows the prompt and accurate quantification of the risk of most of the diseases now ascribed as inflammatory based, such as CVD, type II diabetes, metabolic syndrome etc. (Danesh et al., 2000; Pradhana et al., 2001; Reuben et al., 2002; Ansar and Ghosh, 2013). Besides, as mentioned in the previous paragraphs, elevated levels of hs-CRP, and its principal downstream inducers, including IL6, has been repeatedly found to be cross-sectionally associated with classically described CNS disorders such AD, vascular dementia, and RTT (Zuliani et al., 2007; Koyama et al., 2013; Cortelazzo et al., 2014).

The proposed concept of OxInflammation is further supported by the wealth of epidemiological/clinical evidence showing intercorrelation between peripheral (serum/plasma) markers of inflammation and those of oxidative stress (Pou et al., 2007; Il'yasova et al., 2008; Ouyang et al., 2009; Paltoglou et al., 2017).

In contrast with the aforementioned inflammation markers, which are measured by standardized and high-throughput methods, no validated peripheral index of oxidative stress is still available (Frijhoff et al., 2015; Cervellati and Bergamini, 2016). Indeed, despite years of intense research effort, there is a lack of consensus regarding the validation, standardization, and reproducibility of methods for the measurement (Dalle-Donne et al., 2006; Frijhoff et al., 2015). The biomarker discovery

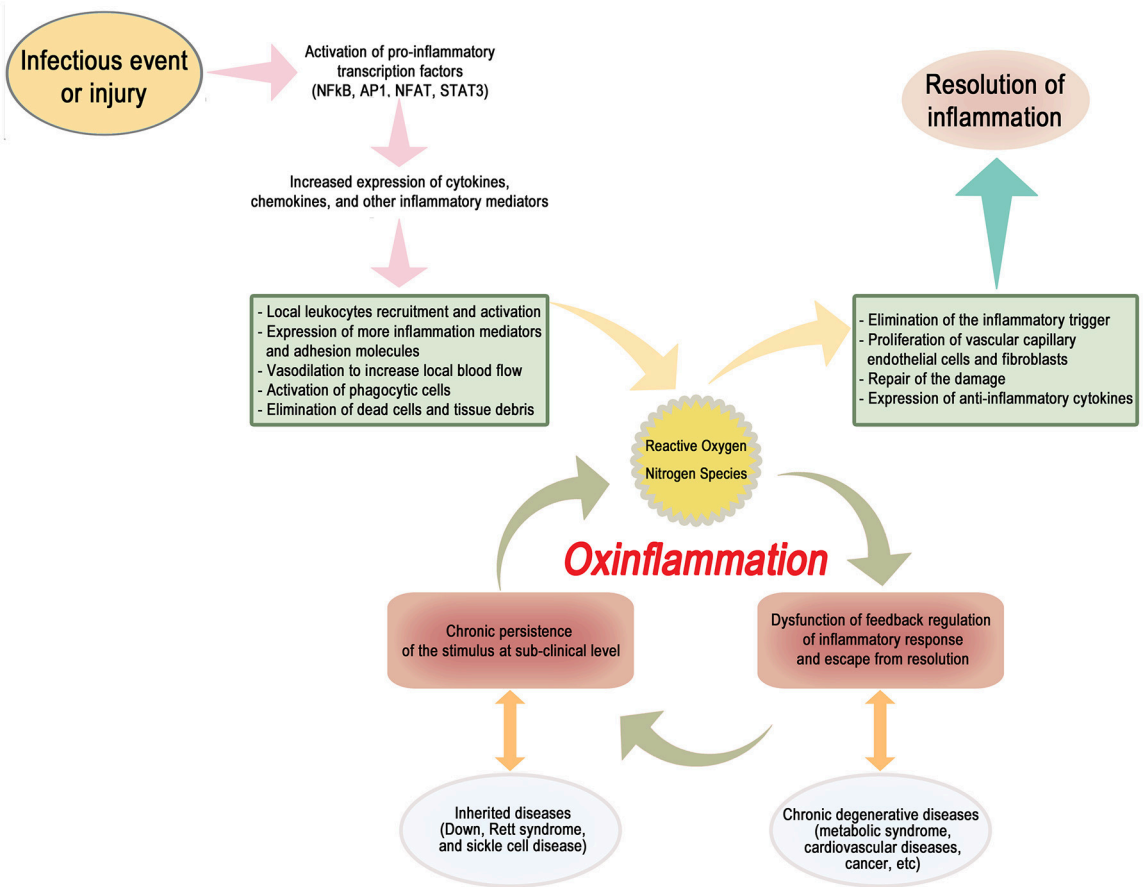
TABLE 1 | Main biomarkers of oxidative stress-induced damage.

Damaged biomolecules	Markers (common abbreviation)	Biological sample	Methods	Distinctive features and sources
LIPIDS (POLYUNSATURATED FATTY ACIDS)				
	F_2-isoprostanes (F_2-iso)	urine/serum/plasma/cells/ breath/CSF	GC-MS/MS LC-MS/MS ELISA	- Specifically derived from oxidation of arachidonic acid - Widely regarded as the best marker of lipid-peroxidation (Dalle-Donne et al., 2006)
	Malondialdehyde (MDA)	serum/plasma/urine/cells	GC-MS/MS LC-MS/MS HPLC-FD Spectrophotometric assay Spectrofluorimetric assay	- Physiologic ketoaldehyde produced by lipid-peroxidation - The most assessed marker of lipid-peroxidation - Spectrophotometric/spectrofluorimetric assays lack of specificity and sensitivity - Can easily form stable protein adducts (Dalle-Donne et al., 2006)
	4-hydroxynonenal (4-HNE)	serum/plasma/cells	GC/MS ELISA	- Aldehyde produced by lipid-peroxidation - Can easily form stable protein adducts (Valacchi et al., 2017)
	Lipid hydroperoxides (LOOH)	serum/plasma	GC/MS Spectrophotometric assay Spectrofluorimetric assay	- Relatively stable byproducts of lipid peroxidation - Specificity/sensitivity problem of spectrophotometric/spectrofluorimetric methods
	2-propenal (acrolein)	serum/plasma/cells	LC–MS–MS LC/GC–MS Immunoblot ELISA	- The most abundant aldehydes produced by lipid-peroxidation - Reacts with DNA, phospholipids and protein (Tully et al., 2014)
	Oxidized low density lipoprotein (oxLDL)	serum/plasma	HPLC Spectrophotometric assay ELISA	- Derived from oxidation of lipid component of LDL - Well-established biomarker of cardiovascular disease risk - The validity of oxLDL a marker of oxidative stress has been questioned (Frijhoff et al., 2015)
PROTEINS				
	Protein carbonyls	serum/plasma/CSF/cells	HPLC ELISA Immunoblot Spectrophotometric assays	- Aldehydes and ketones produced from nonspecific oxidation of protein side chains - Spectrophotometric and ELISA, although very unspecific, are the most frequently used assay methods (Dalle-Donne et al., 2006)
	Advanced Oxidation protein products (AOPP)	serum/plasma/urine	Spectrophotometric assays	- Class of dityrosine-containing protein products - Available high-throughput methods - Analytical specificity problem (Cervellati et al., 2016)
	Nitrotyrosine (Tyr-NO_2)	serum/plasma /urine	GC-MS/MS HPLC-MS/MS HPLC-ED ELISA	- Stable byproduct of oxidation mediated peroxynitrite anion and nitrogen dioxide - Specificity/sensitivity problem of immunological methods (Dalle-Donne et al., 2006)
NUCLEIC ACIDS				
	8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG)	urine/serum/plasma/CSF/cells	GC-MS/MS HPLC-MS/MS ELISA	- Major product of oxidative DNA damage (Goto et al., 2008)
	8-hydroxyguanosine (8-OHG)	urine/serum/CSF/cells	HPLC-MS/MS HPLC ELISA	- Major product of oxidative RNA damage (Henriksen et al., 2009)
CARBOHYDRATES				
	Advanced glycationend products (AGEs)	urine/plasma/serum/CSF/cells	LC-MS/MS HPLC ELISA Spectrophotometric assays	- Byproducts of nonenzymatic reaction of reducing sugars with amino groups of lipids, DNA, and proteins - Analytical methods are limited by high heterogeneity of AGEs (Frijhoff et al., 2015)

CSF, cerebrospinal fluid; ELISA, enzyme linked immunosorbent assay; GC, gas chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; LC, liquid chromatography; HPLC, high pressure liquid chromatography; FD, fluorimetric detection; ED, electrochemical detection.

process in the oxidative stress field has been hindered by several methodological challenges, in particular the great complexity of the direct measurement of ROS/RNS in biological systems (Murphy et al., 2011). A number of analytical approaches can be used, such as electron spin resonance and mass spectrometry (MS) techniques, but none of them with application in clinical practice. As consequence of this intrinsic limitation, the most suitable approach is to assess these reactive species by evaluating the levels of their fingerprints, i.e., by-products of oxidatively damaged biomolecules (Frijhoff et al., 2015). Even for these more chemically stable molecules, problems regarding analytical specificity/sensitivity and other technical challenges still make difficult to translate these into clinical use. In the following paragraph (and **Table 1**), we briefly described the most important oxidative stress biomarkers, more details have been comprehensively described in recent reviews (Dalle-Donne et al., 2006; Frijhoff et al., 2015).

By-products derived by peroxidation of polyunsaturated fatty acids (PUFAs) are the most assessed in epidemiological studies. Among these markers are "primary" products such as hydroperoxides (LOOH), or "secondary" products such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), acrolein, and F_2-isoprostanes (IsoPs). The reliability of these markers chiefly depends on the assay used to measure them. An emblematic example in this frame is MDA. The concertation of aldehyde can be measured in various biological specimens by using a fast and easy spectrophometric assay known as thiobarbituric acid reacting substances (TBARS); this method is one of the most commonly used in oxidative stress quantification, even if it is highly unspecific (Frijhoff et al., 2015). For an accurate detection of MDA serve more labor- and time-consuming methods such as high pressure liquid chromatography (HPLC) plus fluorimetric detection and gas chromatography (GC)—tandem MS (MS/MS) (Dalle-Donne et al., 2006). Similarly, the most reliable marker of lipid peroxidation, F_2-isoprostane, by-products of free-radical mediated oxidation of arachidonic acid, is properly measured but GC-MS/MS and LC-MS, while the widespread commercial



SCHEME 1 | A dysfunction of a proper negative feedback regulation of the inflammatory response may result in the stabilization of a low-grade chronic activation of a vicious circle, characterized by a constant generation of a pro-oxidant environment, triggering in turn a sub-clinical systemic pro-inflammatory status. This condition, to be defined as "OxInflammation," can induce a derangement from adaptive homeostatic capacities of the organism and eventually lead to overt pathological metabolic dysfunctions such as type II diabetes and related degenerative disease. OxInflammation plays an important role both in determining the clinical outcomes associated to several "genetic diseases," such as Down syndrome, Rett Syndrome and sickle cells disease and also to other complex diseases determined by complex gene/environment interaction, such as metabolic syndrome and related degenerative complications, including cardiovascular diseases and cancer.

ELISAs are poorly accurate (Frijhoff et al., 2015; Cervellati and Bergamini, 2016).

Other widely used marker of oxidative stress are the byproducts generated by oxidative modification of proteins, such as carbonyl-groups 3-nitrotyrosine (formed by the reaction between the amino acid and peroxynitrite or NO), advanced oxidation protein products (AOPP), and those derived by modification of DNA, 8-oxo-7, 8-dihydro-2′-deoxyguanosine (8-OHdG), and RNA, 8-hydroxyguanosine (8-OHG), and carbohydrates (advanced glycation products, AGEs) (Dalle-Donne et al., 2006; Frijhoff et al., 2015).

As nicely reviewed by Frijhoff et al "additional value of oxidative stress biomarkers may come from being indicators of a disease mechanism common to several pathologies rather than diagnostic for a specific disease" (Frijhoff et al., 2015). The combination of biomarker of oxidative stress with those of inflammation may aid in risk stratification of several diseases, predicting and monitoring clinical progression in affected patients as well as in intercepting patient group that benefit from specific treatments.

CONCLUDING REMARKS

Here, we propose the term "OxInflammation" to describe a pre-pathological condition characterized by the well-documented chronic and systemic oxidative stress associated, within a vicious circle, to a mild-subclinical chronic inflammation (**Scheme 1**). The occurrence of a long-term sustained oxidative stress contributes to generate a permanent loss of the capacity to react by an adaptive homeostatic response, stabilizing and reinforcing a chronic induction of a pro-inflammatory status.

In other words, an initial stimulus, due to sporadic environmental events or originating from specific genetic features, triggers an inflammatory response. This response, if not properly quenched and terminated by appropriate negative feedback signals, results in a mild, low grade long term inflammation that is further propelled and stabilized by the sustained continuous production of pro-oxidant, electrophilic species. Electrophiles, such as reactive aldehydes (4-HNE), MDA, and F_2-isoprostanes, have a role as inducers of inflammatory cells infiltration and activation, but are also able to directly react with DNA to form exocyclic DNA adducts, which have been detected in a variety of inflammatory diseases (Uchida, 2003). Finally, MDA and 4-HNE can react with protein thanks to the close interaction between lipids and proteins resulting in the synthesis of neo-antigens that in turn can further initiate and exacerbate systemic immune reactions, activation of stellate cells in several organs including brain, liver, pancreas and bones and neutrophil chemotaxis.

Therefore, a "localized" original inflammatory response results in the systemic dysfunction of the adaptive control of redox status, generating in turn a dysfunction of antioxidant control involving several organs.

This condition, characterized by a derangement from the threshold of adaptive redox homeostasis predisposes the organism to a continuous damaging effect of oxidative stress. This oxidative damage primes in turn a continuous sub-clinical pro-inflammatory response that is a common feature for several diseases, at the pre-clinical and clinical levels, and that can have a role in the developing of the pathological conditions.

AUTHOR CONTRIBUTIONS

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

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Triplet Excited Carbonyls and Singlet Oxygen Formation During Oxidative Radical Reaction in Skin

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The skin is the largest organ in the body and is consistently exposed to aggressive environmental attacks (biological/physical/chemical, etc.). Reactive oxygen species (ROS) are formed during the normal oxidative metabolism which enhances to a lethal level under stress conditions referred to as oxidative stress. While, under normal conditions, cells are capable of dealing with ROS using non-enzymatic and enzymatic defense system, it can lead to a critical damage to cell system via the oxidation of cellular components under stress condition. Lipid peroxidation is a well-established mechanism of cellular injury in all kinds of organisms and it is often used as an indicator of oxidative stress in cells and tissues. In the presence of metal ions, ROS such as hydrogen peroxide (H_2O_2) produces highly reactive hydroxyl radical ($HO^{\bullet}$) via Fenton reaction. In the current study, we have used the porcine skin (intact pig ear/skin biopsies) as an *ex vivo*/*in vitro* model system to represent human skin. Experimental results have been presented on the participation of $HO^{\bullet}$ in the initiation of lipid peroxidation and thereby leading to the formation of reactive intermediates and the formation of electronically excited species eventually leading to ultra-weak photon emission (UPE). To understand the participation of different electronically excited species in the overall UPE, the effect of a scavenger of singlet oxygen ($^1O_2$) on photon emission in the visible and near-infrared region of the spectrum was measured which showed its contribution. In addition, measurement with interference filter with a transmission in the range of 340–540 nm reflected a substantial contribution of triplet carbonyls ($^3L{=}O^*$) in the photon emission. Thus, it is concluded that during the oxidative radical reactions, the UPE is contributed by the formation of both $^3L{=}O^*$ and $^1O_2$. The method used in the current study is claimed to be a potential tool for non-invasive determination of the physiological and pathological state of human skin in dermatological research.

Keywords: singlet oxygen, triplet excited carbonyl, ultra-weak photon emission, two-dimensional photon imaging, skin

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INTRODUCTION

The skin plays diverse essential functions such as protection against environment, metabolism, thermoregulation, sensation, and excretion (Zouboulis, 2009; Morrow and Lechler, 2015). The skin consists of the epidermis, which forms the outermost layer followed by dermis and subcutis/hypodermis which is the deepest layer (Meyer et al., 1994; Prost-Squarcioni, 2006;

Abbreviations: CCD, charge-coupled device; PMT, photomultiplier tube; ROS, reactive oxygen species.

Chartier et al., 2017). The whole epidermis constantly renews itself within few weeks and new cells are made in the lower layers of the epidermis (Rinnerthaler et al., 2015). The dermis contains extracellular molecules secreted by support cells that provide structural and biochemical support to the adjacent/surrounding cells and also consists of a dense network of tough elastic collagen fibres and bundles of proteins (elastin) found in the extracellular matrix. These make the skin strong and robust while at the same time elastic (Tepole et al., 2012). The subcutis/hypodermis is mostly made up of fat and connective tissue. In the subcutis, there are tiny cavities which are filled with storage tissue made out of fat and water (**Figure 1**). During the oxidative stress generated by abiotic stresses (toxic chemicals, exposure to ultraviolet irradiations, etc.), the epidermal and dermal cells are known to be most affected (Rinnerthaler et al., 2015; Ji and Li, 2016).

Apart from ethical reasons, there are also methodological difficulties to work with human skins and thus are generally replaced by an animal model for *in vivo* experimental research (Hikima et al., 2012; Abdullahi et al., 2014). The selection of an animal model may depend on factors such as its availability, ease of handling and, most importantly, functional and anatomical similarity to that of humans. For larger scale testing of new agents/cosmetics etc., small mammals are frequently used; however, these animals differ from humans in important anatomical and physiological ways (Kong and Bhargava, 2011). Based on the above consideration, porcine skin is considered to be the most appropriate model, from the perspective of dermatological investigation among all other experimental models. Several studies have demonstrated that porcine skin has important similarities in morphology, composition, and immunoreactivity to that of human skin (Avon and Wood, 2005). Porcine skin has thicker epidermis which is a striking similarity with human skin (Jacobi et al., 2007). The epidermis of the pig is reported to vary in thickness from 30 to 140 μm, thus being within a range similar to human skin which is in the range of 10 to 120 μm (Meyer et al., 1978; Morris and Hopewell, 1990; Avon and Wood, 2005).

During the last few decades, ultra-weak photon emission (UPE) detection techniques have been extensively used to study the oxidative metabolic processes in the different living system *in vivo*, *ex vivo,* and *in vitro* (Kobayashi, 2005; Cifra and Pospíšil, 2014; Ou-Yang, 2014; Poplova et al., 2017). Keratinocytes, fibroblast, skin homogenate, *ex vivo* skin tissues as well as malignant skin cells have been measured *in vitro* (Torinuki and Miura, 1981; Niggli et al., 2008; Madl et al., 2017). Spontaneous and induced UPE under exposure to stress factors such as ultraviolet irradiations, smoke and toxic chemicals have also been documented for human skin/animals cells model/organism and have been well-summarized in recent reviews (Sauermann et al., 1999; Ou-Yang, 2014).

Reactive oxygen species (ROS) has been reported to contribute to UPE via oxidation of biomolecules such as lipids, proteins

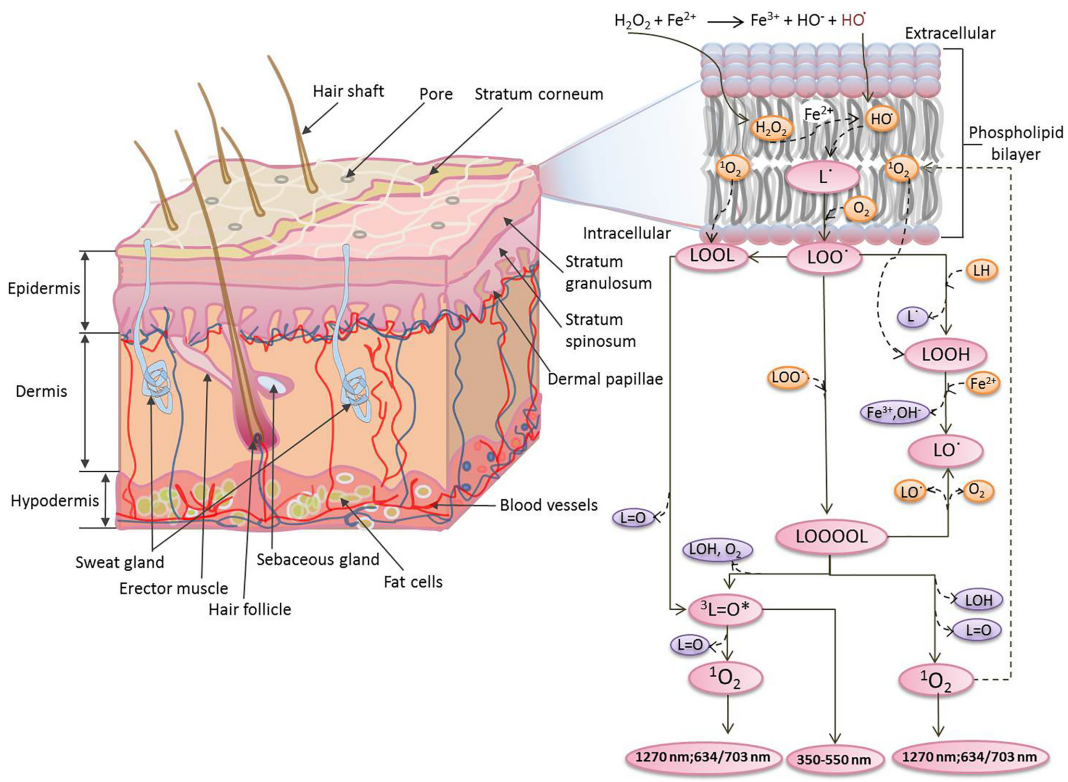

FIGURE 1 | Mechanism of the formation of electronically excited species by oxidative metabolic processes via oxidation of polyunsaturated fatty acid initiated by Fenton's reagent. The Fenton's reagent generates hydroxyl radical (HO•) at different locations within the vicinity of phospholipid bilayer.

and nucleic acids (Prasad and Pospíšil, 2011a,b; Rastogi and Pospíšil, 2011; Poplova et al., 2017). In the current study, we have used porcine ear and skin biopsies as a model system to represent human skin. We have measured the spontaneous and induced UPE from the porcine ear (as an *ex vivo* model system) and skin biopsies (as an *in vitro* model system). The induced UPE was measured under the exogenous application of Fenton's reagent generated chemically and applied topically on the skin before the start of photon emission measurement. Clinically, iron released by hemoglobin may initiate free radical chain reactions and may lead to ROS overproduction followed by lipid peroxidation (Sadrzadeh et al., 1984; Rifkind et al., 2015). As a result of iron-induced Fenton reaction, hydroxyl radical ($HO^{\bullet}$) is known to be produced, which is known to be among highly reactive and short-lived species. The iron in the free form favors the conversion of lipid hydroperoxides (LOOH) to lipid alkoxyl ($LO^{\bullet}$) radicals (**Figure 1**). The electronically excited species generated as a product of the oxidative radical reaction were investigated and their participation in the UPE has been presented.

MATERIALS AND METHODS

Porcine Skin

Intact pig ears were collected from a local slaughter house and transported at low temperature within first 30 min. Skin biopsies were prepared as per the procedure described with minor modifications (Chiu and Burd, 2005). For each set of measurements, fresh skin samples collected each day were used.

Chemicals

Fenton's reagent was prepared using hydrogen peroxide (H_2O_2) (Sigma-Aldrich Chemie GmbH, Germany) and ferrous sulfate ($FeSO_4.7H_2O$) (BDH Laboratory Supplies, United Kingdom). A fixed concentration of $FeSO_4$ (500 μM) and a variable concentration of H_2O_2 (100 μM/1 mM) was used. Spin trapping reagent, POBN [α-(4-Pyridyl 1-oxide)-*N*-tert-butylnitrone] was purchased (Sigma-Aldrich Chemie GmbH, Germany).

EPR Spin-Trapping Spectroscopy

To confirm the formation of $HO^{\bullet}$ during the Fenton reaction, electron paramagnetic resonance (EPR) spectra of POBN (4-pyridyl-1-oxide-*N*-tert-butylnitrone)-OH adduct was detected at 20 μM H_2O_2 in the presence of 100 μM $FeSO_4$ (**Figure 2**). Hydroxyl radical was detected using POBN [25 mM] utilizing spin-trapping in a glass capillary tube (Blaubrand intraMARK, Brand, Germany). EPR spectra were recorded using an EPR spectrometer MiniScope MS400 (Magnettech GmbH, Berlin, Germany) with following EPR conditions: microwave power, 10 mW; modulation amplitude, 1 G; modulation frequency, 100 kHz; sweep width, 100 G; scan rate, 1.62 G s^{-1}, gain, 100.

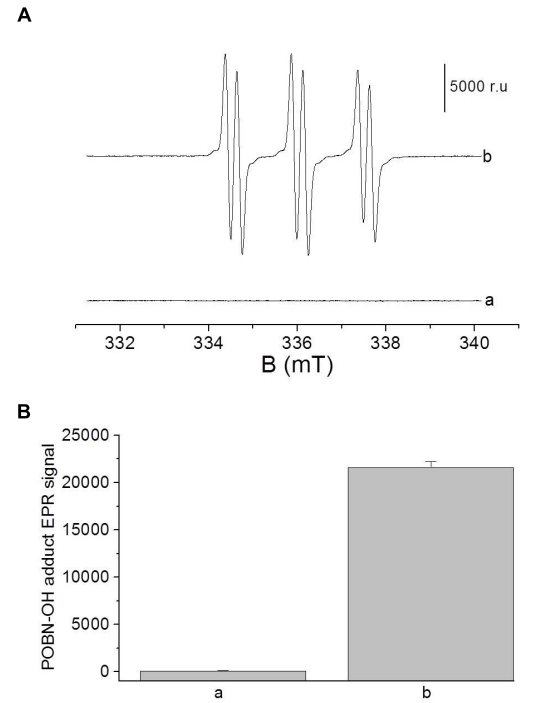

FIGURE 2 | (A) Electron paramagnetic resonance (EPR) spectra of POBN-OH adduct was detected in solutions containing 100 μM $FeSO_4$ in the absence (a) and presence of 20 μM (b) of H_2O_2. Bar represent 5000 relative units. **(B)** The data are presented as the mean and standard deviation of three measurements.

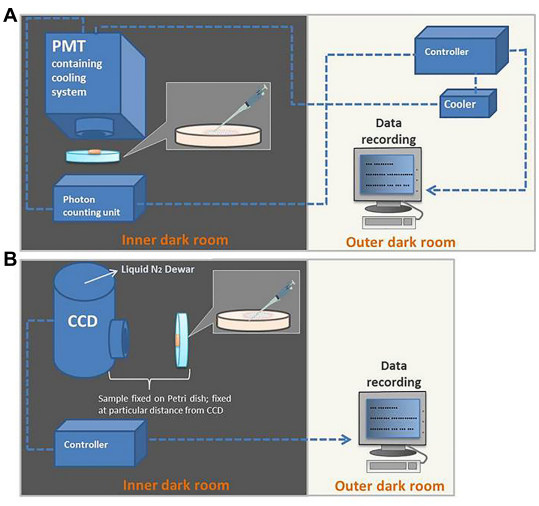

FIGURE 3 | Schematic illustration of the experimental setup for detection of kinetics of ultra-weak photon emission (UPE) using PMT **(A)** and two-dimensional imaging of UPE using CCD camera **(B)**.

Measurement Setup and Experimental Conditions

It is a pre-requisite to specifically design a dark room to avoid any kind of interference from stray photons. In

the current study, all UPE measurements were performed in an experimental dark room as described in Prasad and Pospíšil (2013). A schematic dark room and measurement setup are shown in **Figure 3**. All experiments were done in three replicates and the representative graph has been presented.

Fenton's Reagent-Induced Kinetic Measurement of Ultra-Weak Photon Emission From Skin

The skin biopsies were subjected to topical application of Fenton's reagent in the concentration of 500 μM $FeSO_4$ and 100 μM H_2O_2 (**Figure 4A**) or 1 mM H_2O_2 (**Figure 4B**). These concentrations of Fenton's reagent were chosen based on the pilot experiments in which the effect of different concentrations on photon emission was extensively explored (**Supplementary Figures S1**, **S2**). Fenton's reagent was always topically applied after the start of measurements (indicated by arrows). When required, 5 mM sodium ascorbate (Sigma-Aldrich Chemie GmbH, Germany) was added 20 s prior to the topical application of Fenton's reagent.

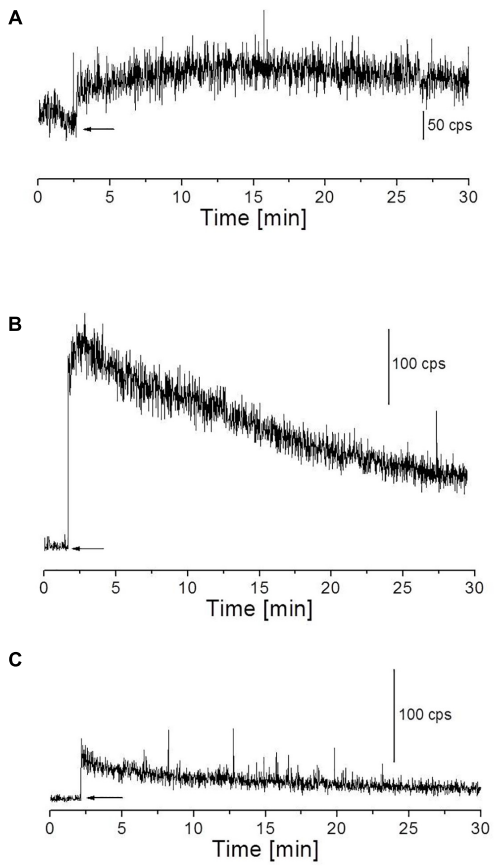

FIGURE 4 | Fenton's reagent-induced UPE measured using visible PMT from the porcine skin sample. **(A)** Kinetics of UPE was measured after the topical application of Fenton's reagent (100 μM H_2O_2 containing 500 μM $FeSO_4$). **(B)** Kinetics of UPE was measured after the topical application of Fenton's reagent (1 mM H_2O_2 containing 500 μM $FeSO_4$). **(C)** Kinetics of UPE was measured in the presence of sodium ascorbate (5 mM) applied to the skin prior to topical application of Fenton's reagent (1 mM H_2O_2 containing 500 μM $FeSO_4$). The decay curve was measured for a duration of 30 min. The arrow indicates the application of chemicals.

Fenton's Reagent-Induced Ultra-Weak Photon Emission in the Blue–Green Region of the Spectrum

To study the spectral distribution of ultra-weak photons emitted during the oxidative radical process mediated by Fenton's reagent, a blue–green interference filter type 644 (Schott & Gen., Jena, Germany) with a transmission in the range 340–540 nm was mounted in front of PMT window (**Figure 5A**). The kinetics of UPE was measured from the porcine skin biopsies after the topical application of Fenton's reagent (1 mM H_2O_2 containing 500 μM $FeSO_4$).

Ultra-Weak Photon Emission

Two-Dimensional Imaging of Ultra-Weak Photon Emission

Two-dimensional imaging of UPE was measured in porcine ear/skin biopsies using highly sensitive CCD camera. All samples were dark-adapted for 30 min to eliminate any interference by delayed luminescence. Other conditions are as per the procedure described in listed references (Prasad and Pospíšil, 2011b; Prasad et al., 2016). CCD camera VersArray 1300B (Princeton Instruments, Trenton, NJ, United States) with the spectral sensitivity of 350–1000 nm and almost 90% quantum efficiency

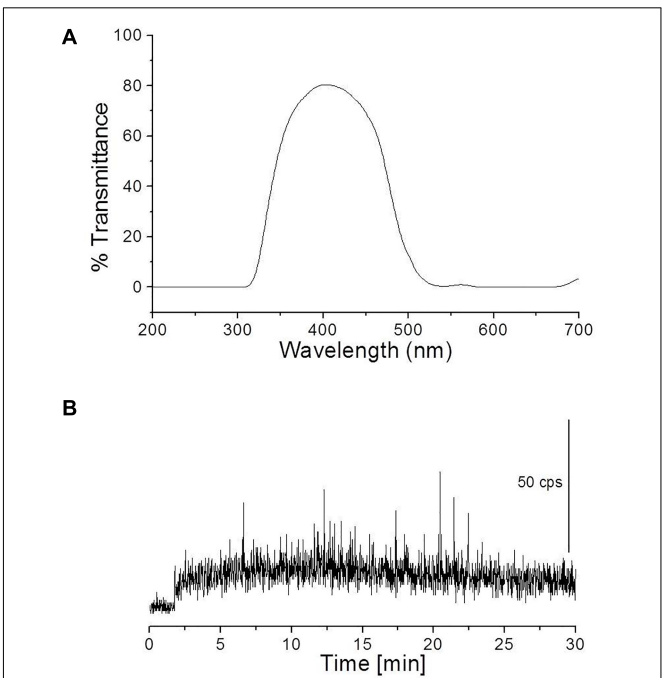

FIGURE 5 | Transmission spectrum of interference filter type 644 (Schott & Gen., Jena, Germany) **(A)** and kinetics of UPE measured after the topical application of Fenton's reagent (containing 1 mM H_2O_2 and 500 μM $FeSO_4$) in the presence of interference filter type 644 (340–540 nm) **(B)**. Other experimental conditions as described in **Figure 4**.

in the visible range of the spectrum was used under following parameters: scan rate, 100 kHz; gain, 2; an accumulation time, 30 min/45 min (porcine ear/skin biopsies). CCD camera was cooled down to −104°C using a liquid-nitrogen cooling system for reduction of dark current. Before each measurement, the data correction was made by subtracting the background noise.

Kinetics of Ultra-Weak Photon Emission in the Visible Region and Near-Infrared Region of the Spectrum

The kinetics of photon emission in the visible region was performed using PMT R7518P (spectral sensitivity: 185–730 nm; detection area: Ø 28 mm). PMT was cooled down to −30°C using thermoelectric cooler C9143 (Hamamatsu Photonics K.K., Iwata City, Japan) for reduction of thermal electrons. UPE in the near-infrared region was measured using a high-speed near-infrared PMT H10330C-45 (Hamamatsu Photonics K.K., Iwata City, Japan) (spectral sensitivity: 950–1400 nm; detection area: Ø 18 mm). The measurements were performed at room temperature. The photon counts were recorded using low-noise photon counting unit (C9744, Hamamatsu Photonics K.K., Iwata City, Japan).

RESULTS

Fenton's Reagent-Induced Kinetic Measurement of Ultra-Weak Photon Emission From Skin

The kinetics of UPE was measured from the porcine skin biopsies after the topical application of Fenton's reagent using visible PMT (**Figures 4A,B**). Prior to measurements, the dark count in the experimental dark room was measured and recorded to be ∼2 counts s^{-1} (**Supplementary Figure S3A**). As additional controls, the photon emission from H_2O_2, Fenton's reagent and scavenger were measured separately/in combinations to test any kind of interference/contribution in overall UPE. It was found that the contribution of chemicals (in the absence of skin sample) showed signal intensity corresponding to the photon count as observed in dark (**Supplementary Figures S3B–E**). When skin biopsies were subjected to topical application of Fenton's reagent in the concentration of 500 μM $FeSO_4$ and 100 μM H_2O_2 (**Figure 4A**) or 1 mM H_2O_2 (**Figure 4B**), it can be observed that the UPE was enhanced to ∼80 counts s^{-1} and ∼250 counts s^{-1} under exogenous application of lower and higher concentrations of

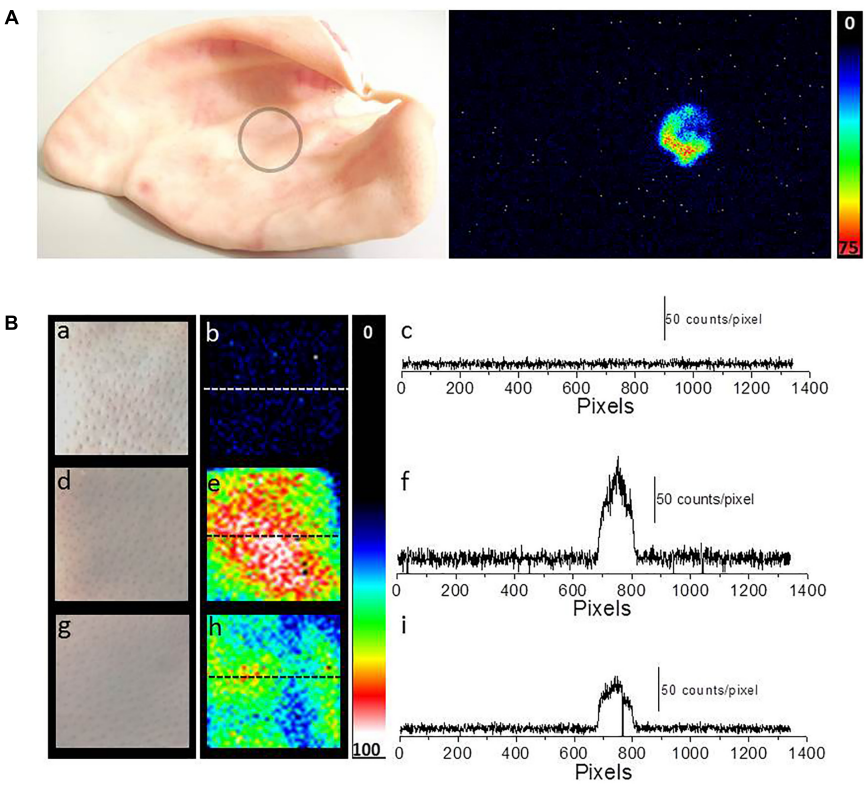

FIGURE 6 | Two-dimensional Fenton's reagent-induced UPE measured using CCD camera from the porcine ear/skin biopsies. **(A)** Photograph of pig ear (circle represents the area of the porcine ear where Fenton's reagent was topically applied) and two-dimensional UPE imaging measured after the topical application of Fenton's reagent (1 mM H_2O_2 containing 500 μM $FeSO_4$). **(B)** Photographs (a, d, and g) and corresponding images of UPE of spontaneous (b), induced with Fenton's reagent (1 mM H_2O_2 containing 500 μM $FeSO_4$) (e) and induced with Fenton's reagent (1 mM H_2O_2 containing 500 μM $FeSO_4$) in the presence of sodium ascorbate (5 mM) (h). Figure **(B)** (c, f, and i) shows the spatial profile of the photon emission in a single strip of the image (dashed line) in spontaneous (c), Fenton's reagent-induced (f) and Fenton's reagent-induced in the presence of sodium ascorbate (i). Y-axis reflects the number of photon counts accumulated after 30 min, whereas the X-axis denotes the pixel of the image.

Fenton's reagent, respectively which then decayed over time. Based on the current observation, it can be concluded that the concentration of Fenton's reagent which acts as an oxidant for the biomolecules (described in the later section) contributes as a key factor for ROS-mediated UPE. For all other results presented in the next section, we have chosen 1 mM H_2O_2 containing 500 μM $FeSO_4$ as the inducer of UPE (as otherwise indicated).

Fenton's Reagent-Induced Ultra-Weak Photon Emission in the Blue–Green Region of the Spectrum

To study the spectral distribution of ultra-weak photons emitted during the oxidative radical process mediated by Fenton's reagent, we mounted an interference filter (type 644 with a transmission in the range 340–540 nm) (**Figure 5A**) in the front of PMT window. Kinetics of UPE was measured from the porcine skin biopsies after the topical application of Fenton's reagent (1 mM H_2O_2 containing 500 μM $FeSO_4$). It can be observed that the application of Fenton's reagent resulted in UPE of ∼20 counts s^{-1} in contrary to 250 counts s^{-1} without interference filter (**Figures 4B**, **5B**). The current observation clearly indicates that not all UPE observed in Fenton's reagent-induced process is contributed by species emitting in the blue–green region of the spectrum but can be due to the involvement of other electronically excited species. A small decrease in photon emission; however, can be also contributed by % transmittance of the interference filter.

Fenton's Reagent-Induced Imaging of Ultra-Weak Photon Emission From Skin

Two-dimensional UPE imaging was measured from the porcine ear/skin biopsies after the topical application of Fenton's reagent using CCD camera (**Figure 6**). **Figure 6A** shows the photograph (left panel) and imaging of UPE (right panel) from an *ex vivo* porcine ear. In **Figure 6A**, UPE imaging was performed after the treatment with Fenton's reagent (1 mM H_2O_2) containing 500 μM $FeSO_4$. **Figure 6B** shows the photograph (left panel; a, d, and g), imaging of UPE (middle panel; b, e, and h) and intensity of UPE (right panel; c, f, and i) from skin biopsies. The imaging of UPE was measured in the absence (b) and presence of Fenton's reagent (e and h). In (e), Fenton's reagent was applied to skin biopsy and measured subsequently while in (h); sodium ascorbate (5 mM) which is a well-known scavenger of singlet oxygen ($^1O_2$) was added prior to the topical application of Fenton's reagent. It can be observed that the addition of sodium ascorbate prior to application of Fenton's reagent significantly suppressed the UPE from the skin biopsy. As evident from the intensity of UPE, the skin untreated with Fenton's reagent (c) does not show any increase while the skin treated with Fenton's reagent (1 mM H_2O_2) containing 500 μM $FeSO_4$ shows an intensity maximum of ∼150 counts/pixel which was found to be suppressed by ∼50% in the skin biopsy pre-treated with sodium ascorbate. Based on the current observation, it is evident that contribution of $^1O_2$ dimol photon emission in the overall UPE observed cannot be completely ruled out. The current observation was further validated by measuring the effect of sodium ascorbate on Fenton's reagent (1 mM H_2O_2) containing 500 μM $FeSO_4$ on skin biopsy. It was observed that in the presence of sodium ascorbate, the UPE was suppressed by ∼5 times (**Figure 4C**).

Fenton's Reagent-Induced Ultra-Weak Photon Emission in the Near-Infrared Region of the Spectrum

We measured the kinetics of UPE in the near-infrared region using a high-speed near-infrared PMT with a spectral sensitivity in the range of 950–1400 nm. The skin biopsy was subjected to topical application of Fenton's reagent in the concentration of 100 μM H_2O_2 (A) and 1 mM H_2O_2 (B) containing 500 μM $FeSO_4$. It can be observed that application of lower concentration (100 μM) of Fenton's reagent did not enhance detectable range of UPE while application of higher concentration (1 mM) of Fenton's reagent enhanced the UPE to about 500 counts s^{-1} which then decayed in the time range of 0–2 min (**Figures 7A,B**). Using near-infrared PMT, Fenton's reagent-induced kinetics of UPE was measured subsequently in the presence of sodium ascorbate (**Figure 7C**). It can be observed that the addition of sodium ascorbate prior to application of Fenton's reagent significantly suppressed the UPE as in agreement with results obtained in **Figure 6B** (h and i) and **Figure 4C**.

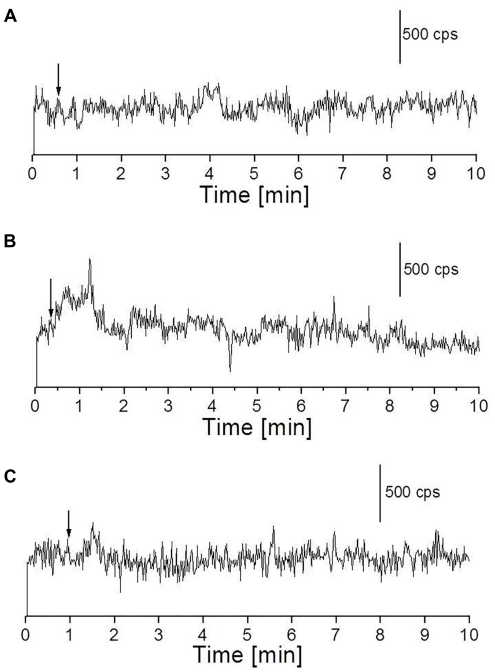

FIGURE 7 | Fenton's reagent-induced UPE measured using near-infrared PMT from the porcine skin sample. **(A)** Kinetics of UPE was measured after the topical application of Fenton's reagent (100 μM containing 500 μM $FeSO_4$). **(B,C)** Kinetics of UPE was measured after the topical application of Fenton's reagent (1 mM H_2O_2 containing 500 μM $FeSO_4$) added between 30 s and 1 min of the start of measurement (indicated by arrow) in the absence **(B)** and presence **(C)** of sodium ascorbate (5 mM), respectively added prior to treatment with Fenton's reagent. The decay curve was measured for a duration of 10 min.

DISCUSSION

Oxidative Radical Reaction and Triplet Excited Carbonyls in Overall Ultra-Weak Photon Emission

The oxidation of polyunsaturated fatty acid mediated by $HO^{\bullet}$ initiates with the hydrogen abstraction from the hydrophobic tail of the lipid molecule (L) resulting in the formation of an alkyl radical ($L^{\bullet}$), which in the presence of molecular oxygen (O_2) forms lipid peroxyl radical ($LOO^{\bullet}$) (Halliwell and Gutteridge, 2007). The epidermal and the dermal layer of the porcine or human skin consists of a high distribution of this target and thus, access to the lipid molecules and further oxidation is very probable. With the formation of $LOO^{\bullet}$ and further accumulation, the interaction with another $LOO^{\bullet}$ becomes feasible. Self-reaction of $LOO^{\bullet}$ yields triplet carbonyls ($^3L{=}O^*$) and O_2 or the ground state of carbonyls (L=O) and $^1O_2$ via the formation of tetroxide (LOOOOL) (**Figure 1**) (Russell, 1957; Cadenas and Sies, 2000; Miyamoto et al., 2014). In addition, $LOO^{\bullet}$ can react with neighboring lipid molecule and can lead to the formation of LOOH. Alternatively, cyclic high-energy intermediates dioxetanes (LOOL) can be formed by the cyclisation of $LOO^{\bullet}$ (Corey and Wang, 1994). As a result of oxidative metabolic processes, electronically excited species such as $^3L{=}O^*$ are formed by the decomposition of high-energy intermediates (LOOL and LOOOOL) (**Figure 1**) (Adam and Cilento, 1982; Cilento and Adam, 1995). The suppression of Fenton's reagent-induced UPE from the porcine skin in the presence of blue–green filter was significant. It clearly indicates that $^3L{=}O^*$ is one of the major contributors in the overall UPE (**Figure 5**). The involvement of $^3L{=}O^*$ in UPE have been recently reported in several studies (Havaux, 2003; Footitt et al., 2016); however, the participation of other molecules cannot be completely ruled out. The decomposition of high-energy intermediates (LOOL and LOOOOL) leads to the formation of $^3L{=}O^*$ which can undergo an electronic transition from the triplet excited state to the ground state emitting ultra-weak photons in the near UVA and blue–green regions of the spectrum (350–550 nm).

Oxidative Radical Reaction and Singlet Oxygen in Overall Ultra-Weak Photon Emission

In the presence of O_2, the excitation energy can be transferred from $^3L{=}O^*$ to O_2 via triplet-singlet energy transfer and can lead to the formation of $^1O_2$ (Kellogg, 1969). The spontaneous collision of two $^1O_2$ results in the dimol photon emission in the red region of the spectrum at the wavelengths of 634 and 703 nm or $^1O_2$ can undergo from singlet excited state to ground triplet state accompanied by the monomol photon emission in the near IR region of the spectrum at the wavelengths of 1270 nm (Cadenas et al., 1980; Mathew and Roy, 1992; Miyamoto et al., 2007; Suzuki et al., 2008; Massari et al., 2011; Pospíšil et al., 2014). Our observation that UPE was significantly suppressed with the topical application of sodium ascorbate in Fenton's reagent-induced UPE from porcine skin indicates that $^1O_2$ can contribute either directly through dimol emission or indirectly can be involved in the formation of LOOL (**Figure 1**) to contribute to overall UPE. In agreement to this, two-dimensional imaging of Fenton's reagent-induced UPE shows significant suppression in the presence of sodium ascorbate (**Figure 6B**). Our observation that UPE under the effect of Fenton's reagent was enhanced in the near-infrared region of the spectrum and subsequently suppressed by the exogenous application of sodium ascorbate confirms the generation of $^1O_2$ during the oxidative radical reaction.

CONCLUSION

The current study presents the mechanism on the oxidation of polyunsaturated fatty acid which is one of the primary targets of ROS in the skin. It is aimed to clarify the participation of different electronically excited species ($^3L{=}O^*$ and $^1O_2$) in UPE during the oxidative radical reactions. The results presented by means of UPE kinetic measurement and two-dimensional imaging provides a series of evidence showing the contribution of these species in the overall UPE. The methodology used to obtain the information/results clearly indicates the potential of UPE as a non-invasive tool without the involvement of any probes, etc. The changes in UPE were observed to reflect the oxidative stress which can serve as a potential tool for monitoring the physiological and pathological state of a biological system. However, technical advancement with respect to sensitivity of PMT and CCD camera is essential for its wide application in different areas such as dermatological research and/or clinical applications.

AUTHOR CONTRIBUTIONS

AP and PP contributed to the conception and design of the work. AP analyzed, interpreted the data, and drafted the manuscript. AB participated in the drafting of the manuscript. PP revised it critically for important content. All authors approved the final version of the manuscript.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01109/full#supplementary-material

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BRIEF RESEARCH REPORT
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Salivary Oxidant/Antioxidant Status in Chronic Temporomandibular Disorders Is Dependent on Source and Intensity of Pain – A Pilot Study

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Temporomandibular disorders (TMD) have been associated with altered salivary oxidative status, but the relation with pain source and pain severity isn't clarified. With the aim to assess their interaction with TMD, we compared levels of selected salivary oxidative stress (OS) markers (glutathione peroxidase, superoxide dismutase, total antioxidant capacity (TAC), uric acid, 8-hydroxydeoxyguanosine, malondialdehyde) and salivary cortisol (SC) as a stress indicator, between 20 TMD patients and 15 healthy control subjects. In order to record differences relating to pain source and severity, patients were respectively classified according to specific diagnoses (myofascial pain or disc displacement (DD)), and pain intensity (high or low). TAC was significantly higher in TMD patients than in controls (morning $p = 0.015$; afternoon $p = 0.005$). Significant differences were also observed when TAC levels between high-intensity pain patients and controls were compared, as well as between DD patients and controls. In logistic regression analysis, higher levels of TAC were related to DD (morning OR: 1.66, 95%CI: 1.05–2.64, $p = 0.029$; afternoon OR: 2.10, 95%CI: 1.11–3.98, $p = 0.021$) and to high-intensity pain (morning OR: 1.81, 95%CI: 1.04–3.15, $p = 0.037$; afternoon OR: 1.79, 95%CI: 1.02–3.14, $p = 0.043$). We also found that morning SC was positively correlated with antioxidant parameters in TMD patients. Our data suggest compensatory mechanism as response to higher level of stress. This stress could be extrinsic and lead toward TMD, or intrinsic, emerging from established TMD, or could be both. The intensity and the source of pain should be considered important factors in future investigations evaluating salivary OS markers in TMD patients.

Keywords: orofacial pain, temporomandibular disorders, salivary diagnostics, oxidative stress, antioxidants, cortisol

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INTRODUCTION

Oxidative stress (OS) has been implicated in the pathophysiology of many diseases, including oral diseases (Agha-Hosseini et al., 2012; Lopez-Jornet et al., 2014; Almerich-Silla et al., 2015; Babaee et al., 2016; Ahmadi-Motamayel et al., 2017; Kumar et al., 2017).

Different biospecimen, including saliva (Nagler et al., 2002; Chiappin et al., 2007), are used to assess the (dis)balance in oxidative status for its potential role in the onset and/or the progression of a disease. The role of OS has also been studied in temporomandibular disorders (TMD), the most common orofacial pain disorders of non-dental origin. Their etiopathophysiology is multifactorial, involving a combination of factors such as parafunctions, micro- and macro traumas, genetic influences, physiological and psychological stressors (Sharma et al., 2011). TMD mostly affect women between 20 and 40 years (Warren and Fried, 2001).

Because pain-related TMD impact individual's daily activities, psychosocial functioning, and quality of life, it is important to understand their pathophysiology and the mechanisms involved in provoking pain and determining its intensity in order to determine factors predictive for disease severity and enhance therapeutic strategies for these patients. Advances in our understanding of the disorder and the mechanisms of pain allow the possibility of providing personalized care for TMD patients (Harper et al., 2016).

Several studies suggest that OS plays a role in the TMD and the experience of pain related to TMD. Rodríguez de Sotillo et al. (2011) reported increased OS products in TMD patients and a significant association between TMD pain and salivary OS markers. De Almeida and Amenábar (2016) determined lower total antioxidant capacity (TAC) in patients with pain-related TMD, but they found no correlation between TAC and pain intensity. Basi et al. (2012) as well as Etöz et al. (2012) support the role of OS in the intensity of pain in TMD but specimen other than saliva were used in these studies.

The aim of our study was to compare the levels of selected salivary OS markers and salivary cortisol (SC) between patients with chronic pain-related TMD and healthy controls and to assess differences relating to the source (muscle or joint) and the intensity (low or high) of pain. We also evaluated the relationship between OS markers and SC, a biological marker for stress response, since it is suggested that TMD patients have biological predisposition for enhanced stress reactivity (Rollman and Gillespie, 2000). On the other hand, TMD might impact cortisol levels (Jones et al., 1997), and cortisol changes might lead to altered redox changes (Aschbacher et al., 2013).

We hypothesized that we would encounter higher salivary oxidant levels and lower salivary antioxidant levels in TMD patients than in control group, depending on the source and intensity of pain, and that salivary OS markers would correlate with SC concentrations.

MATERIALS AND METHODS

Subjects

This case-control study was performed at the School of Dental Medicine, University of Zagreb. The study was approved by the Ethics Committee (01-PA-26-6/15, item 3.2) and conducted in accordance with the Helsinki Declaration. All subjects were informed of the procedures involved in the study and provided written consent. Recruitment of participants was performed between May 2017 and April 2018.

Power analysis, performed to estimate sample size, was based on the data from the pilot study (Rodríguez de Sotillo et al., 2011). Minimal number of 30 participants (10 per group) was calculated with power set at 80% and a significance level of 5%. The effect size was hypothesized to be 0.58 based on previous studies.

Participants were recruited from patients seeking treatment for TMD and orofacial pain. Inclusion criteria were painful disc displacement (DD) or myofascial pain (MP) according to the diagnostic criteria for TMD (DC/TMD) (Schiffman et al., 2014) and reports of ongoing pain lasting more than 6 months.

Exclusion criteria were other orofacial pain conditions including dental pain, smoking, poor oral hygiene, gum swelling, periodontitis, oral lesions, chronic medical conditions (cardiovascular disease, cancer, diabetes, autoimmune diseases), pregnancy, use of supplements and medications known to affect the results of our tests. Patients displaying combined MP and DD and patients with degenerative joint disease were also excluded. Twenty TMD patients and 15 healthy age matched control subjects were included. All participants were women. Different groups relating to pain source (10 DD and 10 MP) were formed in order to explore its interaction with OS.

Assessment of Pain Intensity

Characteristic pain intensity (CPI) was assessed using the graded chronic pain scale by computing the means of three items (current pain, worst pain, average pain) and multiplying them by 10. CPI < 50 was considered to be low-intensity pain (LIP), and CPI ≥ 50 was considered to be high-intensity pain (HIP). Subsequently, we formed another division of TMD patients according to pain severity (10 HIP and 10 LIP).

Sample Collection

The methods of sample collection and analysis were described in detail and validated by our group in a prior study (Alajbeg et al., 2017). The subjects were instructed to fast before saliva collection in the morning and to not eat or drink anything but water at least 2 h before sampling in the afternoon. Brushing teeth before saliva sampling was forbidden to avoid blood contamination. Five mL of whole, unstimulated saliva sample was collected in a graduated tube (50 mL, self-standing centrifuge tubes, Ratiolab, Germany). Saliva aliquots (1 mL) were stored at −80°C until analysis. Since some of the markers showed significant diurnal variations, saliva was collected both in the morning (7 AM) and in the afternoon (5 PM).

Salivary Analysis

Saliva samples were used to analyze the following OS markers: 8-hydroxydeoxyguanosine (8-OHdG), malondialdehyde (MDA), TAC, glutathione peroxidase (GPX), superoxide dismutase (SOD), and uric acid (UA). For these assays, analytical performance, including intra- and inter-assay variability, was assessed. The data and a detailed description of the methodology is available in our previous study (Alajbeg et al., 2017).

TABLE 1 | Differences in salivary oxidative stress markers and salivary cortisol between the control group and TMD subgroups.

	Marker	Control (*N* = 15)		TMD (*N* = 20)							
				Diagnosis (source)				Pain intensity			
				MP (*N* = 10*)		DD (*N* = 10*)		HIP (*N* = 10**)		LIP (*N* = 10**)	
		Mean	(95%CI)	Mean	(95%CI)	Mean	(95%CI)	Mean	(95%CI)	Mean	(95%CI)
Morning	GPX (U/g)	91.74^{AC}	(56.05–126.88)	71.57	(17.77–125.38)	41.61^{A}	(12.08–71.15)	66.23	(18.83–113.63)	46.96^{C}	(6.08–87.82)
	SOD (U/g)	2873.48	(2167.5–3579.4)	3196.38	(1227.7–5165.1)	5773.14	(2327.21–9219.1)	6255.86	(2761.7–9749.9)	2713.66	(1349.1–4078.2)
	TAC (mmol/g)	2.65^{AD}	(1.743–3.56)	4.94	(1.9–7.98)	6.01^{A}	(3.2–8.82)	6.93^{D}	(3.55–10.3)	3.82	(242–5.23)
	UA (μmol/g)	375.12	(223.39–526.84)	670.54	(137.16–1203.92)	619.43	(337.67–901.20)	825.94	(278.38–1373.49)	464.04	(297.96–630.12)
	8-OHdG (μg/g)	1.94	(0.98–2.91)	1.24	(0.3–2.21)	2.29	(0.52–4.06)	2.31	(0.45–4.17)	1.22	(0.46–1.99)
	MDA (nmol/g)	329.97	(115.40–544.54)	341.65	(58.79–624.51)	483.30	(23.41–990.04)	635.37	(123.03–1147.71)	189.59	(36.31–342.87)
	SC (μg/g)	24.16	(15.11–33.21)	43.74	(22.19–65.28)	38.40	(18.91–57.89)	49.59	(24.92–74.25)	32.55	(19.85–45.26)
Afternoon	GPX (U/g)	141.35	(35.59–247.12)	52.16	(29.21–75.10)	61.50	(32.72–90.28)	50.95	(27.42–74.46)	62.72	(34.67–90.77)
	SOD (U/g)	2646.70	(1603.6–3689.7)	2424.7	(631.73–4217.7)	3797.8	(2189.8–5405.7)	3574.17	(1832.9–5315.4)	2648.35	(898.9–4397.8)
	TAC (mmol/g)	2.93^{ACD}	(1.83–4.03)	4.65^{B}	(2.49–6.81)	6.39^{AB}	(3.79–8.98)	5.49^{D}	(3.35–7.64)	5.45^{C}	(2.65–8.25)
	UA (μmol/g)	402.32	(284.12–520.53)	638.20	(197.33–1079.08)	795.18	(302.81–1287.56)	722.55	(304.16–1140.93)	710.84	(192.45–1229.23)
	8-OHdG (μg/g)	2.06	(1.03–3.08)	1.37	(0.2–2.56)	2.50	(0.82–4.18)	2.18	(0.77–3.59)	1.69	(0.2–3.29)
	MDA (nmol/g)	727.55	(359.38–1095.71)	738.29	(136.57–1613.16)	1209.99	(704.39–1715.61)	1090.55	(201.79–1979.31)	857.74	(329.54–1385.93)
	SC (μg/g)	7.16	(3.34–10.98)	6.55	(2.27–10.83)	14.11	(2.91–25.32)	9.15	(3.32–14.98)	11.51	(0.34–22.68)

**Pooled regardless of pain intensity; **pooled regardless of pathology source (diagnostic subgroup).*

TMD, temporomandibular disorders; MP, myofascial pain; DD, disc displacement; HIP, high-intensity pain; LIP, low-intensity pain; GPX, glutathione peroxidase; SOD, superoxide dismutase; TAC, total antioxidant capacity; UA, uric acid; 8-OHdG, 8-hydroxydeoxyguanosine; MDA, malondialdehyde; SC, salivary cortisol; CI, Confidence Interval; Mann-Whitney U test was used for comparisons.

[A] *P-values between control vs. DD: $P_{morning\ GPX} = 0.012$, $P_{morning\ TAC} = 0.027$, $P_{afternoon\ TAC} = 0.005$.*

[B] *P-values between MP vs. DD: $P_{afternoon\ TAC} = 0.041$.*

[C] *P-values between control vs. low-intensity pain: $P_{morning\ GPX} = 0.011$, $P_{afternoon\ TAC} = 0.027$.*

[D] *P-values between control vs. high-intensity pain: $P_{morning\ TAC} = 0.012$, $P_{afternoon\ TAC} = 0.016$.*

TABLE 2 | Correlations* between salivary cortisol and salivary oxidative stress markers.

			Marker					
			GPX (U/g)	**SOD (U/g)**	**TAC (mmol/g)**	**UA (μmol/g)**	**8-OHdG (μg/g)**	**MDA (nmol/g)**
Control (*N* = 15)	Morning	SC (μg/g)	−0.22	0.23	0.01	−0.02	0.24	−0.33
	Afternoon		−0.22	0.08	0.40	0.30	0.11	0.05
TMD (*N* = 20)	Morning		0.59[A]	0.45[B]	0.64[C]	0.45[D]	0.19	0.39
	Afternoon		−0.07	0.35	0.39	0.32	0.20	0.08

**Spearman correlation coefficient; GPX, glutathione peroxidase; SOD, superoxide dismutase; TAC, total antioxidant capacity; UA, uric acid; 8-OHdG, 8-hydroxydeoxyguanosine; MDA, malondialdehyde; SC, salivary cortisol.*
[A]P-value = 0.005.
[B]P-value = 0.044.
[C]P-value = 0.002.
[D]P-value = 0.044.

Free SC was analyzed using a competitive ELISA kit (Demeditec Diagnostics GmbH, Germany). The intra- and inter-assay variabilities of this assay kit are 5.8 and 6.4%, respectively, according to the manufacturer. All results were normalized to the total protein concentration. Sample analysis was performed at the Department of Laboratory Diagnostics, University Hospital Center Zagreb.

Statistical Analysis

Analyses were performed using SPSS 17.0 (Chicago, IL, United States) with the alpha set at $p < 0.05$. Data distribution was tested using the Shapiro-Wilk test. Student's *t*-test was used for comparison of two groups (TMD vs. controls) and analysis of variance (ANOVA) for comparison of three groups (controls vs. TMD subgroups), for the normally distributed data (age and CPI). Mann-Whitney U test and Kruskal-Wallis test were used for comparison of two and three groups, respectively, if the data were not normally distributed (OS markers and SC).

Spearman correlation evaluated the association between salivary OS products and antioxidative enzymes with SC. Logistic regression analysis determined the association between each marker, which were the independent variables, and the groups, for which the dependent variables were study groups (TMD and controls), source of pain (MP or DD), and pain intensity (LIP or HIP).

RESULTS

No statistically significant age differences were noted between 20 TMD subjects (39.30 ± 12.07) and 15 controls (34.33 ± 7.86) ($t = -1.38$; $p = 0.175$) nor between the control subjects and the TMD subgroups of 10 MP (42.6 ± 12.65) and 10 DD (36.00 ± 11.10) subjects ($F = 2.01$, $p = 0.15$). The mean CPI of MP subjects (46.03 ± 15.92) and DD subjects (45.4 ± 19.77) were not statistically different ($t = 0.078$, $p = 0.938$). Five MP and 5 DD subjects had LIP (34.2 and 28.2, respectively). The difference between groups was non-significant ($t = 0.963$, $p = 0.36$). Similarly, no significant difference ($t = -0.819$, $p = 0.43$) was shown between 5 MP and 5 DD subjects with HIP (57.8 and 62.6, respectively). Therefore, patients were pooled in pain intensity subgroups regardless of the source. Conversely, patients were pooled in pain source subgroups regardless of pain intensity.

The levels of salivary GPX among TMD subjects were significantly lower than among the controls (morning: $Z = 2.43$; $p = 0.014$; afternoon: $Z = 1.98$; $p = 0.047$). The levels of salivary TAC were significantly higher in TMD patients compared to the controls (morning: $Z = -2.41$; $p = 0.015$; afternoon: $Z = -2.76$; $p = 0.005$). Morning SC was significantly higher ($Z = -2.01$, $p = 0.043$) in the TMD group than in the control group. Differences in salivary OS markers and SC between the control group and TMD subgroups are presented in **Table 1**.

In TMD patients, antioxidant parameters were positively correlated with morning SC (**Table 2**). Among control subjects, no significant correlation was found between SC and OS markers.

TAC was positively associated with TMD (morning OR: 1.66, 95%CI: 1.03–2.66, $p = 0.034$; afternoon OR: 1.86, 95%CI: 1.07–3.21, $p = 0.025$).

When evaluating the association between each salivary OS marker and the source of pain, higher levels of TAC in the morning (OR: 1.66, 95%CI: 1.05–2.64, $p = 0.029$) and in the afternoon (OR: 2.10, 95%CI: 1.11–3.98, $p = 0.021$) were positively associated with DD. No such association was found for MP. Higher TAC was also related with HIP (morning OR: 1.81, 95%CI: 1.04–3.15, $p = 0.037$; afternoon OR: 1.79, 95%CI: 1.02–3.14, $p = 0.043$).

DISCUSSION

The results of our study did not confirm lower levels of salivary TAC in TMD patients as we expected, based on the results of previous studies (Rodríguez de Sotillo et al., 2011; De Almeida and Amenábar, 2016). On the contrary, TAC was significantly higher in our TMD patients in comparison with controls. Considering that they experienced pain lasting more than 6 months, higher TAC might imply a compensatory increase of the antioxidant enzymes in response to changing levels of OS as a prerequisite for efficient defense (Sies, 1993). De Almeida and Amenábar (2016) did not report how long their patients experienced TMD pain, and it is unclear from the report by Rodríguez de Sotillo et al. (2011) whether all

their patients had chronic pain. With longer duration of the disorder, antioxidant systems might regenerate (if their level was decreased in comparison with healthy subjects) or increase, probably as a compensatory mechanism (as found in our study). Duration of the TMD might thus, at least partly, explain why this result differs from previous studies. TAC increase could be greater in patients experiencing more severe symptoms, as suggested by our finding of higher TAC levels in subjects with HIP. However, individual antioxidants might show less ability to adapt, as suggested by the finding of a significantly lower GPX in TMD patients. Regarding the source of pain, higher TAC was positively related only to pain of joint origin.

Rodríguez de Sotillo et al. (2011) reported significantly higher levels of 8-OHdG and MDA in TMD patients, and an association between higher levels of these OS markers with higher scores of pain intensity. In our study, the concentrations of MDA and 8-OHdG were higher in specific subgroups of TMD patients (HIP and DD) compared to controls, but the difference was not statistically significant. Previous investigations did not report salivary biomarker levels normalized for the total protein concentration in saliva, which partly explains the inconsistencies between our results and those of previous studies.

The mechanisms by which OS may modulate pain in TMD are not known but are probably diverse. Ray et al. (2015) showed that the production of oxidatively modified lipoproteins induces nociception and Medow et al. (2013) suggested direct alteration of local sensory nerve activity by certain reactive oxygen species as a mechanism of their influence on the generation and maintenance of pain-associated symptoms and myalgias in subjects with chronic fatigue syndrome. The latter finding is particularly interesting in the light of a strong clinical association between muscular TMD and fibromyalgia and chronic fatigue syndrome as reported by Korszun et al. (1998). This association relates to the perturbation of the hypothalamic-pituitary-adrenal axis and subsequent hyperreactivity to stress which then manifests as one or more stress-related conditions. Higher SC levels and a positive correlation of antioxidants with SC in TMD patients, observed in our study, might indicate that stress either has a role in the development of TMD, or that pain-related TMD might produce additional stress to the body and further enhance cortisol secretion.

Number of subjects and high variability of OS markers clearly represent study's limitations, and demand caution in the interpretation of results. Nevertheless, results encourage research on the relationship between OS and TMD, aiming to better understand the disturbed antioxidative/oxidative balance and TMD, either as the consequence or the cause of one another. In conclusion, both the intensity and source of pain as well as time of saliva sampling should be considered in future investigations to determine the diagnostic utility of OS markers in the saliva of TMD patients.

DATA AVAILABILITY

The datasets generated and analyzed during the current study are not publicly available, because this is a pilot study and further study will include more TMD patients who are still being recruited. However, all data from this study is available from the corresponding author on reasonable request.

AUTHOR CONTRIBUTIONS

EV and LV collected the samples and wrote the manuscript. IZA designed the study, analyzed the data, and wrote the manuscript. IL measured the samples and co-wrote the manuscript. DR contributed substantially to the concept and design, and measured the samples. AAR collected the samples and coordinated specimen storage. DI analyzed and interpreted the data. DKZ collected the samples and analyzed the data. TB planned the analyses and collected the samples. IA designed the study and critically reviewed the manuscript.

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REVIEW
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Mitochondria, Oxidative Stress and Innate Immunity

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Canonical functions of mitochondria include the regulation of cellular survival, orchestration of anabolic and metabolic pathways, as well as reactive oxygen species (ROS) signaling. Recent discoveries, nevertheless, have demonstrated that mitochondria are also critical elements to stimulate innate immune signaling cascade that is able to intensify the inflammation upon cytotoxic stimuli beyond microbial infection. Here we review the expanding research field of mitochondria and oxidative stress in innate immune system to highlight the new mechanistic insights and discuss the pathological relevance of mitochondrial dysregulation induced aberrant innate immune responses in a growing list of sterile inflammatory diseases.

Keywords: mitochondria, innate and adaptive immune response, inflammasome, ROS, thioredoxin

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INTRODUCTION

Mitochondria are multifunctional organelles that collaborate with their host cells in biosynthesis, metabolism, and cell death or survival functions. Recent growing evidence has highlighted expanded roles of mitochondria in modulating oxidative stress as well as innate immune responses. Since mitochondria is the major resource of reactive oxygen species (ROS) that emerges as a convergent signaling hub that regulates diverse developmental, environmental, and pathological stimuli. Along with mitochondrial dysfunction and excessive ROS observed in various disease conditions including cardiovascular diseases, autoimmune diseases, metabolic syndromes as well as tumor, aberrant innate immune responses were also identified, which is considered to be a common factor which drives the inflammatory pathology of these conditions. In this review, we present a comprehensive landscape regarding the role of mitochondria in innate immunity. We also detail recent advances in understanding novel function of mitochondria in inflammasome machinery, highlighting their emerging roles in driving the sterile inflammatory responses upon injured or damaged tissues and cells.

MITOCHONDRIAL FUNCTION AND ROS

Mitochondria are ubiquitous multifunctional organelles with a double-membrane structure, which are present in majority of mammalian cell types. They are dynamic, branched networks with continuous cycles of fission and fusion (Mishra and Chan, 2014). Mitochondria bear the residual genome (mitochondrial DNA, mtDNA) that is essential for their activity of oxidative phosphorylation (OXPHOS) and a protein library containing with 1,200 proteins (Calvo and Mootha, 2010) that are varied substantially between cell and tissue types (Scarpulla, 2008).

It is believed that mitochondrial biogenesis and homeostasis, including mtDNAs, are under tight regulation of nucleus. This needs bi-directional signaling pathways that mediate crosstalk between the nucleus and mitochondria (Shadel and Horvath, 2015). Such mitochondrial retrograde signaling pathways are highly conserved from prokaryotic to eukaryotic cells which potentially can trigger both favorable and maladaptive responses. Therefore, the number, morphology, distribution, and activity of mitochondria are constantly altered in respond to physiological, developmental, and environmental stimuli.

As the sites of the tricarboxylic acid (TCA) cycle and OXPHOS, mitochondria produce substantial ATP using the electrochemical gradient by the electron transport chain (ETC), whereas mitochondria could also produce ROS predominately at the ETC complex I and complex III (Murphy, 2009). ROS were initially considered to exert damage-promoting, detrimental effects; however, recent studies have depicted it as an emerging central signaling molecule. Therefore, beyond the traditional roles of mitochondria in metabolism such as glucose oxidation as well as biosynthesis of fatty acid, amino acid and hormones, mitochondria are also actively involved in ROS signaling, apoptosis and innate immunity (Shadel and Horvath, 2015).

Given its intrinsic complicated nature of mitochondria, mitochondrial dysfunction can induce distinct stress signals. For example, reduced OXPHOS and ETC activity can result in disturbed mitochondrial ROS (mtROS) production, eliminated mitochondrial membrane potential, or reduced cellular adenosine 5′-triphosphate (ATP) or energy (Butow and Avadhani, 2004; Sena and Chandel, 2012). As the major generator of ROS, mitochondria are also prone to become the target of ROS followed by pathological consequences. For example, elevated free radicals associated with mtDNA oxidative damage trigger cell apoptosis by inducing mitochondrial stress and downstream signaling (Scheibye-Knudsen et al., 2015; West et al., 2015). Finally, dynamic morphology and distribution of mitochondria within cells can also elicit distinct forms of stress that are associated with mitochondrial elimination by mitophagy or autophagy (Labbe et al., 2014). However, mitochondria contain potent anti-oxidant systems that protect mitochondria from ROS-mediated damages, including mitochondrial superoxide dismutase (SOD2) and the thioredoxin system made of thioredoxin-2 (Trx2), thioredoxin reductase-2 and peroxidase 3.

One of the most studied mitochondrial protein is Trx2, a small redox protein with double redox-active sites (C90 and C93). Trx2 is ubiquitously presented in tissues with high metabolic activity, including liver, brain and heart. Trx2 is able to maintain cell in a reduced state by reversible oxidation to Cys disulfide (Trx-S2) via the transfer of reducing equivalents from the catalytic site Cys residue to a disulfide protein substrate (protein-S2). By such way, Trx2 regulates a large number of apoptosis related molecules and critical transcription factors, such as apoptosis signal-regulating kinase 1 (ASK1) (Zhang et al., 2004) and nuclear factor kappa B (NF-κB) (Hansen et al., 2006). Trx2 depletion leads to cytosolic release of cytochrome c from mitochondria and subsequently activation of caspase-3 and -9. Moreover, Trx2 is able to attenuate tumor necrosis factor (TNF)-dependent elevated mtROS and apoptosis (Zhang et al., 2004; Hansen et al., 2006), suggesting that Trx2 modulates TNF-dependent redox signaling in mitochondria (Benhar et al., 2008). Trx2 not only protects against oxidative stress in mitochondria, but also induces the cells insensitive in response to ROS-induced apoptosis. In femoral artery ligation model, mitochondrial Trx2 in the endothelium cells is able to inhibit ASK1-induced apoptosis by elimination of ROS to increase nitric oxide (NO) bioavailability and inhibition of ASK1 activity (Zhang et al., 2004, 2007; Dai et al., 2009). Moreover, a similar role of Trx2 in cardiomyocytes was also identified. The absence of Trx2 in cardiomyocytes exhibits the disorganized mitochondrial arrays and swelling as well as impaired ATP generation, whereas ASK1 is required for Trx2 deletion triggered apoptosis, dysfunctional mitochondria, excessive ROS production observed in cardiomyocytes. Our findings in small animal model were further also validated in the clinical samples (Huang et al., 2015). Reduced Trx2 expression, elevated levels of phosphorylated ASK1 and activated caspase-3 were found in cardiomyocytes of patients with dilated cardiomyopathy (DCM) compared to that of healthy organ donors, indicating human Trx2 is also required to inhibit ASK1-dependent apoptosis signaling. Taken together, as exemplified by Trx2, mitochondrial proteins are capable to modulate cellular activities by maintaining cellular redox state and limiting ROS production.

Recent evidence has highlighted the essential role of mitochondria in activities of immune cells. Mitochondrial physiology, morphology, and metabolism tightly regulate immune cell fate during immune responses. For example, T cell development toward memory or effector phenotypes is tightly modulated by fission and fusion activities of mitochondria (Buck et al., 2016). OXPHOS and ROS production is required for T cell activation, while activated T cells can use either OXPHOS or glycolysis for proliferation. T cell mitochondrial dysfunction has been considered as a signature for infectious diseases and some autoimmune disease. During hepatitis B virus (HBV) infection, various cellular processes centered on mitochondrial activities and ROS were substantially downregulated in CD8 T cells, which might contribute to functional exhaustion of HBV CD8 T cells. Mitochondrion targeted antioxidants can elicit a notable improvement and restore anti-HBV CD8 function (Fisicaro et al., 2017). Mitochondrial hyperpolarization, ATP depletion and elevated ROS production has been observed from T cells from systemic lupus erythematosus (SLE) patients (Gergely et al., 2002), which might result from abnormal NO generation derived from monocytes (Nagy et al., 2003). CD4+ T cells from rheumatoid arthritis patients also exhibited elevated autophagy, ATP depletion, and impaired redox status (Yang et al., 2013, 2016). CD4+ T cells from multiple sclerosis patients displayed remarkable abundance in mitochondrial inner membrane lipid cardiolipin (Vergara et al., 2015). Potential therapeutic strategies include the inhibition of the mitochondrial oxidation and glycolytic rate to restore CD4+ T cell function in autoimmune diseases (Yin et al., 2015).

Beyond immune cells, mitochondrial dysfunction also extensively influences the function of non-immune cells. Taking endothelial cells for example, a representative mechanism that links to sterile inflammation is the so-called senescence

associated secretory phenotype (SASP) (Tchkonia et al., 2013). It has been shown that upon mitochondrial dysfunction, endothelial cells secrete multiple pro-inflammatory cytokines includes interleukin (IL)-1, IL-6, and TNF-α, and upregulate intercellular adhesion molecule-1 (ICAM-1) expression which attracts monocyte activation and adhesion (Choi et al., 2018). Indeed, our recent unpublished data have also revealed that Trx2-deletion induced mitochondrial dysfunction also lead to endothelial cell senescence and SASP *in vivo* and *in vitro*, which might further recruit innate immune cells to augment inflammatory responses.

THE ACTIVATION OF INNATE IMMUNE RESPONSES BY MITOCHONDRIA-DERIVED ROS

Innate immunity provides a front line of host defense through direct engagement of pathogen or environmental insult, which further initiates the development of an adaptive immune response. However, the ability of innate immune system to resist a subsequent challenge with either the same or a different insult remains unaltered. Although the innate immune system lacks the fine specificity of adaptive immunity that is necessary to produce immunological memory, it can distinguish self from non-self by pattern recognition receptors (PRRs), germline-encoded receptors located at the cell surface and within endosomes (Kawai and Akira, 2006), serving as a sensor to monitor signs of infection or tissue injury. Such evolutionarily ancient PRRs sense a wide range of exogenous pathogen-associated molecular patterns (PAMPs) and endogenous danger-associated molecular patterns (DAMPs). DAMPs include ROS, heat shock proteins, oxidized lipoproteins, and cholesterol crystals. DAMPs could trigger sterile inflammation by binding with PPRs such as Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), retinoic acid–inducible gene (RIG)-I-like receptors (RLRs), and absent in Melanoma (AIM) 2-like receptors (ALRs) (Sellge and Kufer, 2015). Engagement of these receptors further recruit immune cells to the site of infection or injury in an attempt to clear pathogens or amply inflammatory responses within the host.

The innate immune receptors might also modulate OXPHOS, mitochondrial function (**Figure 1**), ROS and apoptosis present in tissue injury. For example, NLR family member X1 (NLRX1) is a designated innate immune receptor localized in mitochondria. During ischemia-reperfusion injury, NLRX1 is capable to protect against mortality by attenuating mitochondrial impairment and oxidative stress-induced apoptosis of epithelial cells (Stokman et al., 2017). By this way, the innate immune receptor could also control the metabolism of epithelial cells under cytotoxic stress.

An essential member of downstream cytosolic surveillance is the inflammasome, a large multimolecular complex that intensifies the inflammation when sensing microbial components and endogenous danger signals (Kanneganti et al., 2007; Takeuchi and Akira, 2010). The activated inflammasome modulates the proteolytic enzyme caspase-1, result in the maturation of proinflammatory cytokines, IL-1ß and IL-18 (Martinon et al., 2002; Kayagaki et al., 2011). To date, at least two major types of inflammasomes were identified, NLR families and the ALR families (Lamkanfi et al., 2007). NLRs, including the NLRP1, NLRP3 and NLR family caspase recruitment domain (CARD) domain containing 4 (NLRC4) inflammasomes, are characterized by a central located NOD domain that is flanked at N-terminal CARD, pyrin domain (PYD), acidic transactivating domain, or baculovirus inhibitor repeat (BIR) (Franchi et al., 2009; Sutterwala et al., 2014; Tschope et al., 2017). These motifs are capable to bound NLRs with adapter proteins and effectors such as apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1. C-terminal of NLRs contain leucine-rich repeat (LRR) motifs that regulate NLR activity (Lamkanfi et al., 2007). AIM2 inflammasome, including AIM2 and interferon-gamma inducible factor 16 (IFI16), contains a prototypical DNA binding HIN200 domain and an amino-terminal pyrin motif, through which AIM2 recruits ASC and caspase-1.

The assembly and activation of these inflammasomes are dependent on specific pathogens and endogenous insults (Kanneganti, 2010). For example, the NLRC4 inflammasome is activated in response to bacterial infection such as *Salmonella typhimurium* (Zhao et al., 2011). The NLRP1b and NLRP12 inflammasomes are triggered by anthrax lethal toxin and *Y. pestis* infection, respectively (Levinsohn et al., 2012; Vladimer et al., 2012). NLRP6 is widely present in the intestine that is critical to maintain the intestinal homeostasis (Elinav et al., 2011), whereas NLPR7 inflammasome has been shown to recognize diacylated lipopeptides in human macrophages (Khare et al., 2012). Compared to the NLR family members mentioned above, NLRP3 inflammasome is the most well characterized inflammasome. NLRP3 inflammasome is activated by diverse stimuli including both RNA and DNA viruses, presence of cytosolic bacterial RNA during infection, injury-induced stress molecules such as ROS, ATP and the release of mitochondrial DNA, and harmful environmental substances such as silica and asbestos, K^+ efflux, and lysosomal destabilization (Piccini et al., 2008; Martinon et al., 2009; Broz et al., 2010; Franchi et al., 2012). Activation of NLRP3 requires two-step signals: firstly priming with either TLR or NLR ligands to enhance NF-κB-driven transcriptional level of NLRP3, and subsequently exposing to microbial toxins and ionophores or endogenous alarmins to trigger inflammasome assembly (Tschopp and Schroder, 2010). Meanwhile, during microbial infection of macrophages, two-step signaling required by NLRP3 inflammasome activation may occur simultaneously (Tschopp and Schroder, 2010). Interestingly, although adenoviral DNA triggers assembly of the NLRP3 inflammasome activation, upon exposure with transfected non-viral cytosolic DNA, caspase-1 activation and IL-1β secretion, however, was dependent on ASC rather than NLRP3 (Muruve et al., 2008). Given such diverse stimuli NLRP3 recognizes, it is believed that the NLRP3 inflammasome was able to recognize cytosolic nucleic acids and other endogenous danger signals indirectly. Indeed, it has been suggested that NLRP3 activation might be triggered by perturbed cell membrane (Barlan et al., 2011).

Given diverse stimuli converge on the activation of NLRP3 including ROS, here we hypothesize that ROS might be the

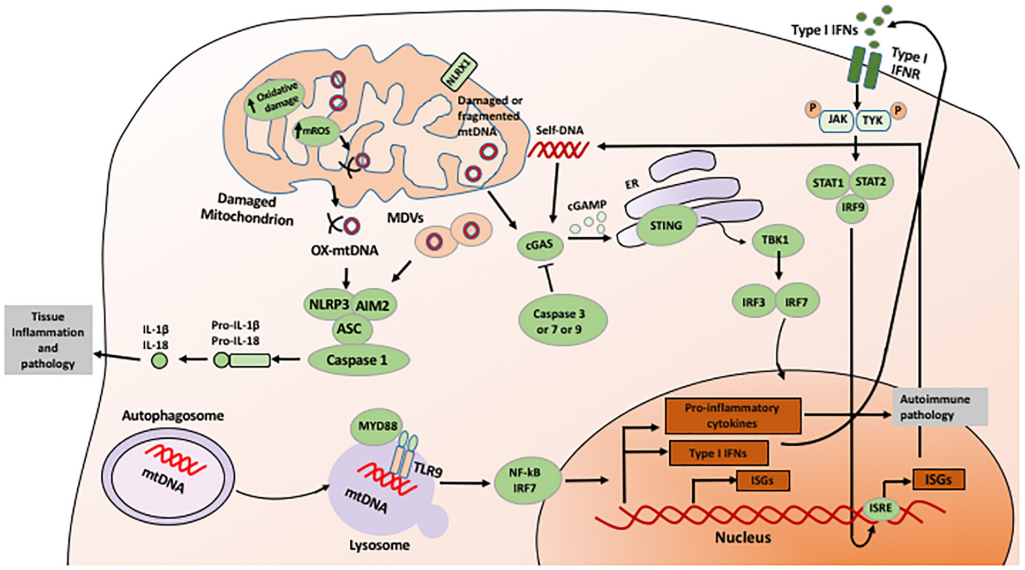

FIGURE 1 | Mechanism of mitochondria-mediated signaling in innate immunity. Mitochondria-derived ROS and released mtDNA directly induces activation of innate immune responses, including activation of inflammasome, sGAS-STING, and NF-kB signaling pathways. See text for details.

direct mediator to trigger NLRP3. Crystal structural analysis of NLRP3 has identified an unexpected, however, highly conserved disulfide bond between the PYD motif and the nucleotide-binding site motif, that is super sensitive to disturbed redox status (Bae and Park, 2011), indicating a crucial redox role for NLRP3. Indeed, ROS is identified as a potent endogenous ligand for NLRP3, since the early reports has demonstrated that the inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-induced ROS inhibited NLRP3 activation in macrophages treated with ATP (Cruz et al., 2007); meanwhile, the absence of the p22*phox* subunit within NADPH oxidase substantially attenuated IL-1β production when macrophages exposed with asbestos (Dostert et al., 2008). In addition to NADPH oxidase-derived ROS, mtROS has also demonstrated to elevate NLRP3 activation (Bulua et al., 2011; Zhou et al., 2011; Wu et al., 2013). Interestingly, NLRP3 is present in the cytosol and endoplasmic reticulum (ER) during rest state but is migrated to mitochondria upon activation, which is mediated by the association of mitochondrial anti-viral signaling protein (MAVS) (Subramanian et al., 2013). Specific inhibition of mtROS, instead of the inhibition of NADPH oxidase, was able to prevent inflammasome-dependent IL-1β expression triggered by cyclic stretch in alveolar macrophage (Wu et al., 2013).

THE ACTIVATION OF INNATE IMMUNE RESPONSES BY MITOCHONDRIA-DERIVED DNA

The innate immune system is ultrasensitive to dsDNA. Cellular sources of immune stimulatory self-DNA include nucleus DNA, mtDNA, and DNA from phagolysosomal compartments. Mitochondria in most cells have approximately 2–10 copies of mtDNA, characterized as circular, closed, dsDNA with size of 15,000 base pairs (Kukat and Larsson, 2013). Upon mitochondrial stress induced apoptosis, Bcl2 antagonist/killer (BAK) and Bcl2-associated X protein (BAX) induce permeabilization of mitochondrial outer-membrane (MOMP), leading to cytochrome c release and activation of apoptotic caspases. During this process, mitochondrial network is compromised and newly appeared BAK/BAX macropores allows mitochondrial matrix component containing mtDNA leakage into the cytosol. Similar to NLR inflammasomes, ALR inflammasomes also induce caspase-1 activation and IL-1β cytokine maturation. However, AIM2 inflammasome has been identified as a cytosolic receptor for DNA, including intracellular bacteria *F. tularensis* and mouse cytomegalovirus (Fernandes-Alnemri et al., 2010); while AIM-2 related inflammasome, IFI16 is in the nucleus, which has also implicated in forming an inflammasome complex during herpes simplex virus infection of endothelial cells (Kerur et al., 2011). Contrast with NLR inflammasomes, ALR inflammasomes is able to bind their ligand directly, dsDNA. Further, AIM2 and IFI16 lack CARD domains and recruitment of ASC is required for their activation. Of note, AIM2 is the first inflammasome where a direct receptor:ligand interaction has been formally demonstrated (Fernandes-Alnemri et al., 2009; Hornung et al., 2009). Intriguingly, AIM2 recognizes cytosolic DNA with a minimum length of 80 base pairs in a sequence-dependent fashion (Rathinam et al., 2012). When DNA binding with its C-terminal HIN200 domain, AIM2 undergoes oligomerization and further engages caspase-1 via ASC and secretes IL-1β. In Aim2-deficient mice, it has been shown that the AIM2 inflammasome plays a non-redundant role to induce defense responses against DNA viruses and intracellular bacterial infections (Fernandes-Alnemri et al., 2010). Moreover,

AIM2 contributes to the adjuvanticity of DNA vaccines (Suschak et al., 2015) and enhance autoimmune disorders such as SLE via host DNA recognition (Panchanathan et al., 2011). During Nelfinavir treatment, an HIV aspartyl protease, AIM2 could also be activated upon DNA release from the nucleus with a compromised nuclear envelope integrity (Di Micco et al., 2016). Until recently, there is a few reports suggesting a role of AIM2 in caspase-1 activation trigger by mtDNA. The depletion of AIM2 in bone marrow-derived macrophages (BMDMs) leads to reduced IL-1β with mtDNA transfection compared to control BMDMs (Nakahira et al., 2011). A recent study elegantly demonstrated that cholesterol overload causes impaired mitochondrial metabolism and mtDNA release, which further triggers AIM2 inflammasome in activated BMDMs (Dang et al., 2017).

NLRP3 inflammasome could also been directly activated by mtDNA. This was first identified by the observation that the autophagic proteins were able to regulate NLRP3-dependent inflammation by maintaining mitochondrial integrity (Nakahira et al., 2011). Upon stimulation of lipopolysaccharide (LPS) and ATP, the deficiency of autophagy proteins beclin 1 and LC3B in BMDMs leads to dysfunctional mitochondrial and cytosolic translocation of mtDNA. The later event was strictly relied on NALP3 inflammasome activation and mtROS, ultimately enhancing IL-1β and IL-18 secretion. A subsequent study by Shimada et al. (2012) suggested that the essential role of mROS during the process of NLRP3-dependent cytosolic release of mtDNA might be contributed by the intrinsic characteristics of NLRP3, which preferentially binds oxidized mtDNA and further stabilizes it in the cytoplasm after release.

Cyclic GMP-AMP synthase (cGAS) is a prominent cytosolic DNA sensor (Gao et al., 2013). The nucleotidyl transferase enzyme cGAS detects cytoplasmic DNA and produces the second-messenger, cyclic AMP-GMP (cGAMP), which is associated with and subsequently activates stimulator of interferon gene (STING) (Chen et al., 2016). Activated STING is able to recruit TANK-binding kinase 1 (TBK1), which phosphorylates interferon regulatory factor 3 (IRF3) to enhance its homodimerization and migrate into the nucleus, followed by induction of interferon β (IFNβ) and interferon-stimulated genes (ISGs) (Schoggins et al., 2014; Storek et al., 2015; Watson et al., 2015). Despite cGAS-STING signaling pathway has considered as a major defense mechanism against microbial infection, it is also essential to orchestrate type I interferon (IFN) and proinflammatory responses to self-DNA, driving type I IFN induction and ultimately resulting in autoimmune responses as well as antitumor immunity (Barber, 2015; Crow and Manel, 2015; Gray et al., 2015; Roers et al., 2016). Genetic studies in humans have demonstrated that mutations in DNA nucleases such as three prime repair exonuclease 1 (Trex1) lead to cytosolic DNA accumulation which activates the cGAS–STING pathway (Rice et al., 2015). With exposure of genotoxic stress, the collapsed micronuclear envelope help cGAS get access to the damaged nuclear DNA followed by activation of cGAS–STING pathway (Harding et al., 2017; Mackenzie et al., 2017). Interesting enough, it has been demonstrated that mtDNA leaked into the cytosol can activate the cGAS–STING pathway and type I IFN production in the absence of active caspases (West et al., 2015; McArthur et al., 2018).

Here comes an interesting question: since there are billions of cells that undergo apoptosis per day in our body, during which DNA is released into cytosol, how the immune system effectively keep immunologically silent from apoptosis triggered stimuli? A recent unexpected finding is that mitochondria and downstream caspases can determine the immunological status of cell death (Rongvaux et al., 2014). It has long been considered that the highly regulated caspase-dependent apoptosis is immunologically silent, in which the effector caspases including caspase-3, -7, and -9, were necessary to inhibit mtDNA triggered activation of STING (White et al., 2014; West et al., 2015). In contrast, cell death independent of caspase provokes an inflammatory response through releasing DAMPs into the local microenvironment. The activation of such DAMPs further recruits inflammatory cells such as granulocytes, monocytes, and macrophages. Specifically, without the presence of active caspases, Bax and Bak induced MOMP leads the activation of mitochondrial DNA-dependent cGAS–STING pathway followed by the potent induction of type I IFNs and a state of viral resistance. These unexpected mechanistic findings indicated an essential role of mitochondria and caspases, not only on the decision of the cell fate but also on the choice decease in an inflammatory or immunologically silent manner.

It worth mentioning that mtDNA uniquely affects the innate immunity distinct from NADPH oxidase-induced ROS and mtROS. NADPH oxidase-induced ROS serves as an alarm signal in the cytosol that induces efficient defense signal transduction pathways that use hydrogen peroxide as secondary messenger. Although NADPH oxidase-induced ROS could be partially scavenged by a versatile antioxidant system, it also results extensive cellular damage and necrosis. NADPH oxidase-induced ROS can exert oxidative damage on cellular proteins, lipids and nucleic acids, nevertheless, they are also crucial secondary messengers in innate immune responses. ROS is required for heightened sensing by innate immune receptors including TLRs, RLRs, and NLRP3 in an indirect manner via interaction with and modification of other molecules such as mtDNA (Banoth and Cassel, 2018). As reviewed above, mtROS directly contribute to inflammatory cytokine production and innate immune responses. mtROS can directly activate adenosine monophosphate-activated protein kinase (AMPK) and mitogen-activated protein kinase (MAPKs) (Bulua et al., 2011). Phosphorylation of their substrate can directly affect diverse metabolic pathways, regulation of gene expression by transcription factors, and direct activation or inhibition of specific target proteins including innate immune molecules such as RLRs (Tal et al., 2009) and NLRP3 (Zhou et al., 2011). In the absence of any mtROS, mtDNA upon releasing into cytoplasm, extracellular space or circulation during cell death or mitochondrial damage could be sensed by multiple PRRs including cGAS-STING, TLR9, NLRP3, NLRC4, and AIM2 in cell-type and context-dependent manners to trigger pro-inflammatory and type I IFN responses.

MITOCHONDRIA-MEDIATED INNATE IMMUNE RESPONSES IN THE CONTEXT OF STERILE INFLAMMATION RELATED DISEASES

Recent studies have illustrated a deleterious role of innate immunity in sterile inflammation, which we have summarized in **Table 1**. Tissue injury would result in cell death accompanied with nucleus and mitochondrial DNA damages. Recently, AIM2 has been shown to be involved in ionizing radiation induced hematopoietic syndrome and severe injury to gastrointestinal (GI) tract. During this process, AIM2 is capable to recognize nucleus DNA damage triggered by radiation and caspase-1 dependent death of bone marrow cells and intestinal epithelial cells (Hu et al., 2016). Blockage of AIM2 inflammasome activity might be an effective therapeutic regimen especially for cancer patients suffering from hematopoietic or GI toxicity. Similarly, using permanent coronary ligation after myocardial infarction (MI) in mice, King et al. (2017) reported a harmful role of IRF3-dependent innate immune response on ventricular remodeling, which supports the key molecules in innate immunity as a new target for protection against MI. Mechanistically, during MI a great number of dying ischemic cell from the heart catastrophically releases large quantities of DAMPs, such as self-DNA, that trigger the innate immune response. Phagocytic macrophages in the heart trigger a fatal response against MI by sensing DNA via cGAS, followed by IRF3-IFN signaling axis activation. Secreted type I IFNs is able to further amplify the response through ISGs by diffusing to the local microenvironment and signaling to cells with interferon-α/β receptor (IFNAR). Indeed, in contrast to wild type (WT) mice after MI, mice with genetic deficiency of cGAS, IRF3, or IFNAR suffer less ventricular dilation and rupture, greater contractile ability and improved survival time. Further, in cardiomyocytes, mitochondrial DNA that escapes degradation specifically activate TLR9-mediated inflammatory response, resulting in exacerbated heart failure (Oka et al., 2012).

TABLE 1 | Summary of sterile inflammation diseases triggered by mitochondrial dysregulation-induced aberrant innate immune responses and potential therapeutic strategies.

Human disease	Target cells	Mechanism of action	Potential therapeutic strategies	Reference
Radiation-induced hematopoietic GI toxicity	Bone marrow cells, intestinal epithelial cells	AIM2 recognizes nucleus DNA damage triggered by radiation and caspase-1 dependent death	AIM2 inhibitor	Hu et al., 2016
Myocardial infarction	Macrophage	DAMPs from dying ischemic cells trigger cGAS-IRF3-interferon signaling axis	Inhibition of cGAS-IRF3 signaling pathway or inhibition of DAMPs release	King et al., 2017
Heart failure	Cardiomyocyte	mtDNA activates TLR9 mediated inflammatory responses	Blockage of TLR9 signaling	Oka et al., 2012
Atherosclerosis	Foam cells, macrophages	NLRP3 activation; NLRP3 facilitates mtDNA oxidative damage	Blockage of NLRP3 signaling pathway	Yu et al., 2013; Zheng et al., 2014; Shi et al., 2015; Afrasyab et al., 2016; Lin et al., 2018
	Macrophage	CD36 coordinates the intracellular conversion of endogenous ligands (such as oxLDL) into crystals or fibrils, which result in lysosomal disruption and NLRP3 inflammasome activation	CD36 inhibitor	Py et al., 2013; Sheedy et al., 2013
	Macrophage	8-oxoguanine glycosylase (OGG1) deficiency leads to oxidized mDNA, cyto c release, apoptosis, and IL-1 secretion	Removal of oxidative DNA	Tumurkhuu et al., 2016
STING-associated vasculopathy with onset in infancy (SAVI)	Endothelial cells	Gain of function mutation of STING leading to JAK activation	JAK inhibitor	Liu et al., 2014
Obesity and insulin resistance	Endothelial cells and adipose tissue	Obese caused mitochondrial damage and mtDNA leakage activate STING-IRF3 pathway	STING inhibitor	Mao et al., 2017

These studies elegantly demonstrate the molecular inflammation events during pathogenesis of myocardial disease.

During early atherosclerosis, endothelial cells are activated by the elevation of ATP synthesis-uncoupled but proton leak-coupled mtROS without causing mitochondrial damage and EC death (Li et al., 2016, 2017), highlighting a novel regulatory network triggered by mtROS among mitochondrial metabolism, physiological EC activation, patrolling cell migration, and pathological inflammation. This suggests that mitochondrial antioxidants are promising therapies for vascular inflammation and cardiovascular diseases. Indeed, during the progression of atherosclerosis, excessive oxidative stress, dysfunctional mitochondria (Victor et al., 2009), ER stress (Chistiakov et al., 2014), and lysosome rupture (Yuan et al., 2000) are considered as one of the critical driver responsible for inflammasome activation. Clinical evidence has shown that the level of NLRP3 from peripheral blood monocyte is highly associated with the severity of coronary atherosclerosis in patients (Afrasyab et al., 2016), especially NLRP3 inflammasome has been observed to localize in the cytoplasm of foam cells and macrophages (Shi et al., 2015). Blockage of NLRP3 signaling inhibits the progression of atherosclerosis in apolipoprotein E-deficient ($ApoE^{-/-}$) mice treated with a high-fat diet. NLRP3 knockdown also reduces macrophages and lipids while increases smooth muscle cells and collagen deposition of the plaque, contributing to plaque stabilization (Zheng et al., 2014). Interestingly, hydrogen sulfide which has shown to have anti-oxidative properties is able to also attenuate oxidative stress induced NLRP3 inflammasome activation via S-sulfhydrating c-Jun in macrophages (Lin et al., 2018). The priming event of NLRP3 is initiated by the cooperation of CD36 and a heterodimer of TLR4–TLR6 to convert intracellular ligands to crystals or fibrils followed by lysosomal disruption and NLRP3 activation (Sheedy et al., 2013). CD36 is an archetypal PRR which has been involved in the pathogenesis of atherosclerosis via modified endogenous danger signals such as oxidized low density lipoprotein (LDL) (oxLDL). $CD36^{-/-}ApoE^{-/-}$ mice had a reduced serological IL-1β and plaque cholesterol crystal accumulation. TLR and ROS signaling can increase the levels of NLRP3 via BRCA1/BRCA2-containing complex subunit 3 (BRCC3) which mediates deubiquitination of the LRR domain of NLRP3 and such modification is essential for its activation (Py et al., 2013). NLRP3 might also facilitate atherosclerosis via sensing oxidative mtDNA (Tumurkhuu et al., 2016). Increased mtDNA damage has been indicated in human atherosclerotic plaques compared to normal vessels. Further, leukocyte mtDNA damage was associated with higher-risk plaques in humans (Yu et al., 2013). 8-oxoguanine glycosylase (OGG1) is one of the major DNA glycosylase that is responsible for removing oxidative DNA. Ogg1 deficiency in atherosclerotic mice induces larger plaque and greater amount of lipid content, while both knockout of Ogg1 and NLRP3 rescue the enhanced atherosclerosis observed in $Ogg1^{-/-}$ mice. Specifically, $Ogg1^{-/-}$ macrophages showed increased oxidized mtDNA, cytochrome c, apoptosis, and IL-1 secretion. All these studies highlight NLRP3 as a potential therapeutic target for atherosclerosis (Tumurkhuu et al., 2016).

Chronic inflammation of endothelial cells initiates cardiovascular disease (Liao, 2013). Mitochondrial damage induced by palmitic acid and mitochondrial DNA release activates the STING–IRF3 pathway, which further trigger ICAM-1 expression and endothelial inflammation. Clinical evidence also identified STING-associated vasculopathy with onset in infancy (SAVI), an autoinflammatory disease result from gain-of-function mutations of STING (Liu et al., 2014). With the identified STING expression, endothelial cells exposed with cGAMP, the STING ligand, result in cellular activation indicated by elevated inducible nitric oxide synthase (iNOS) and E-selectin followed by apoptosis. Since mutant STING increased level of phosphorylated signal transducer and activator of transcription 1 (STAT1), the elucidation of such mechanism also suggests Janus kinase (JAK) inhibitor as a potential therapeutic strategy for SAVI, which is currently under clinical assessment. STING pathway is also involved in adipose tissue inflammation and insulin resistance. In obese mice triggered by high-fat diet, STING was found in adipose tissue and was actively involved in tissue inflammation and insulin resistance (Mao et al., 2017).

CONCLUSION

Mitochondria are not only house machineries that support cellular essential activities, but also important sources of endogenous DAMPs including ROS as well as necessary triggers for inflammasome signaling. A large number of evidence has emerged linking dysfunctional mitochondria to aberrant innate immune responses. Nevertheless, our understanding of precise roles the inflammasomes in response to mitochondrial malfunction and ROS are still lacking. Many significant questions regarding the molecular machineries which initiate inflammasome activation upon mitochondria disorder and ROS remain to be addressed. Further elucidation of the interplay of ROS, mitochondrial function and inflammasome pathways might open up a new horizon for the development of immunotherapeutic strategy for chronic inflammation diseases such as cardiovascular diseases.

AUTHOR CONTRIBUTIONS

YC, ZZ, and WM wrote the paper.

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frontiers in Physiology

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α_1-Microglobulin Protects Against Bleeding-Induced Oxidative Damage in Knee Arthropathies

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Knee injury increases the risk of developing knee osteoarthritis (OA). Recent evidence suggests involvement of oxidative stress induced by inflammation and bleeding in the joint. This study investigates the role in this process of α_1-microglobulin (A1M), a plasma and tissue antioxidant protein with reducing function, and heme- and radical-binding properties. We studied matched knee synovial fluid (sf) and serum (s) samples from 122 subjects (mean age 40 years, 31% females): 10 were knee healthy references, 13 had acute inflammatory arthritis (AIA), 79 knee injury 0–10 years prior to sampling, and 20 knee OA. Using immunoassays, we measured sf-A1M and s-A1M, sf-hemoglobin (sf-Hb), sf-total free heme (sf-Heme), and sf-carbonyl groups (sf-Carbonyl). We explored associations by partial correlation, or linear regression models with adjustments for age, sex and diagnosis, and evaluated diagnostic capacity by area under the receiver operator characteristics curve (AUC). The AIA group had 1.2- to 1.7-fold higher sf-A1M and s-A1M concentrations compared to the other diagnostic groups; other biomarkers showed no between-group differences. sf-A1M and s-A1M were with AUC of 0.76 and 0.78, respectively, diagnostic for AIA. In the injury group, the amount of bleeding in the joint was inversely correlated to time after injury when measured as sf-Heme ($r = -0.41$, $p < 0.001$), but not when measured as sf-Hb ($r = -0.19$, $p = 0.098$). A similar inverse association with time after injury was noted for sf-A1M ($r = -0.30$, $p = 0.007$), but not for s-A1M and sf-Carbonyl. Linear regression models showed that sf-Heme was more strongly associated with sf-A1M and sf-Carbonyl than sf-Hb. Independent of diagnosis, sf-Heme explained 5.7% of the variability in sf-A1M and 3.0% in the variability in sf-Carbonyl, but appeared unrelated to s-A1M. High sf-A1M and low sf-Heme or sf-Hb were independently associated with low sf-Carbonyl. In conclusion, our results demonstrate that independent of disease, Hb and heme within a knee joint correlates with an increased sf-A1M concentration that appears to be protective of oxidative damage, i.e., a reduction in carbonyl groups. High concentrations of A1M in synovial fluid and serum was further diagnostic for AIA.

Keywords: α_1-microglobulin, heme, hemoglobin, hemolysis, knee injury, knee osteoarthritis, oxidative stress, synovial fluid

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INTRODUCTION

Knee osteoarthritis (OA) is the most common joint disease and is a major cause of pain and disability in the older population worldwide (Liu et al., 2015). It is a complex and multifactorial disorder that represents a pathological imbalance of degenerative and regenerative processes involving the whole joint (Loeser et al., 2012). Obesity, genetic factors, and aging are some of the most prominent risk factors (Sharma et al., 2006), and in the younger population, previous knee injury is an important contributor to increased prevalence of knee OA – as much as half of those sustaining a knee injury in their 20s had developed post-traumatic knee OA in the following 12 to 14 years (Lohmander et al., 2004, 2007; von Porat et al., 2004).

Lately, much evidence has emerged to support a central role for participation of local inflammatory pathways in the disease processes (Scanzello, 2017), particularly so after knee injury (Goldring and Otero, 2011). As a part of the inflammatory response after injury, oxidative stress due to formation of reactive oxygen species (ROS) is involved both in direct damage of cartilage components and as integral factors in cell signaling leading to cartilage degradation (Henrotin et al., 2003). In addition to inflammation, cell-free hemoglobin (Hb) released via hemolysis is a potent inducer of oxidative stress. Spontaneous decomposition of Hb via auto-oxidation leads to the formation of ROS, free heme groups and free iron, which are highly reactive and have the ability to damage lipids, proteins, and DNA (Olsson et al., 2012).

Protection from Hb and heme toxicity exists on several levels, both by mechanisms removing cell-free Hb and heme, and by a complex network of antioxidant mechanisms that inhibit and eliminate the oxidative compounds and repair the oxidative damage caused. On the extracellular level, within the circulation, haptoglobin (Hp) and hemopexin (Hpx) are two of the most prominent scavenger proteins, with antioxidative properties through their capacity to remove cell-free Hb (by Hp) and heme (by Hpx). A major intracellular antioxidant is heme oxygenase-1 (HO-1) which, through its heme-degrading activity, plays a critical role in the protection of cells. However, during disease and damage these antioxidant defense systems are not sufficient to protect against harmful reactive compounds, damage to DNA, lipids and proteins arises. Oxidation of proteins result in stable carbonyl groups on amino acid side chains, and protein carbonyl content is one of the most widely used marker of protein oxidation and a general indicator of oxidative stress (Dalle-Donne et al., 2003).

Another protein that in the last decade has been shown to play a central role as a protective protein against oxidative stress is α_1-microglobulin (A1M) (Åkerström and Gram, 2014). It is a 26 kDa plasma and tissue protein with the capacity to bind free radicals and heme groups, and the capacity to repair oxidative damage by chemical reduction. A1M is mainly produced in the liver, and a rapid flow of A1M through the blood and tissues binds harmful molecules and delivers them to the kidneys where they are degraded or excreted in the urine. In addition to the liver, smaller quantities are also expressed in most other cells in the body (Åkerström and Gram, 2014). Of particular interest for this investigation is that the expression of A1M has been shown to be up-regulated in several cells and tissues during oxidative stress conditions in response to elevated levels of ROS, Hb, and free heme (Olsson et al., 2007, 2011).

The main objective of this work was to study the role of A1M and bleeding-induced oxidative stress in the development of osteoarthritis after knee injury. Using a cross-sectional knee arthropathy cohort with matched serum and synovial fluid samples, we measured A1M and protein carbonyl groups as biomarkers of oxidative stress and Hb and free heme as biomarkers of bleeding, hypothesizing that increased A1M buffers against oxidative damage in synovial fluid.

MATERIALS AND METHODS

Subjects and Samples

From a convenience cohort of previously studied individuals (Lohmander et al., 1989, 1999; Larsson et al., 2009; Kumahashi et al., 2015), we selected 122 subjects with matched knee synovial fluid and serum samples that were diagnosed as knee healthy references, having acute inflammatory arthritis (AIA) in the knee, knee injury (anterior cruciate ligament tear and/or meniscus injury), or knee osteoarthritis (**Table 1**). Diagnosis was made by arthroscopy, radiography, assessment of synovial fluid and clinical examination (Lohmander et al., 1989). Serum was prepared from whole blood that had been allowed to clot at room temperature (RT), with the clot removed by centrifuging at 1800 *g* for 10 min. Synovial fluid samples were aspirated without lavage, centrifuged at 3000 *g* for 10 min. Aliquots of serum and synovial fluid supernatants were stored at −80°C. All patient-related procedures were approved by the ethics review committee of the Medical Faculty, Lund University.

α_1-Microglobulin (A1M)-Concentrations

We used a radioimmunoassay (RIA) developed in-house for the detection of A1M in serum (s-A1M) and synovial fluid (sf-A1M) (Gram et al., 2015). Radiolabeling of A1M with ^{125}I (Perkin Elmer Life Sciences) was done using the chloramine T method

TABLE 1 | Characteristics of the study subjects.

Study group	*n* (% women)	Age, mean years (*SD*, range)	Time after injury, range in weeks (years)
All subjects	122 (31)	40 (18, 14–86)	–
Reference	10 (10)	60 (11, 45–77)	–
Acute inflammatory arthritis[a]	13 (46)	63 (15, 29–81)	–
Injury[b]	79 (28)	31 (12, 14–69)	0–549 (0–10.5 years)
Osteoarthritis	20 (45)	51 (16, 24–86)	–

[a] 12 had pyrophosphate arthritis, and 1 Yersinia-triggered reactive arthritis.[b] 12 had isolated anterior cruciate ligament (ACL) injury, 46 ACL injury with concomitant meniscus injury, and 21 had isolated meniscus injury.

(Greenwood et al., 1963). Protein-bound iodine was separated from free iodide by gel-chromatography on a Sephadex G-25 column (PD10, GE Healthcare, Stockholm, Sweden). A specific activity of around 0.1–0.2 MBq/μg protein was obtained. The RIA was performed as described (Plesner et al., 1975). Briefly, goat antiserum against human A1M (diluted 1:6000) was mixed with ^{125}I-labeled A1M (approximately 0.05 pg/ml) and unknown patient samples (serum, diluted 400x; synovial fluid, diluted 100x) or calibrator A1M-concentrations. After incubating overnight at RT, antibody-bound antigen was precipitated by adding bovine serum and 15% polyethylene glycol, centrifuged at 1600 *g* for 40 min, after which the ^{125}I activity of the pellets was measured in a Wallac Wizard 1470 gamma counter (Perkin Elmer Life Sciences).

Total Hemoglobin (Hb-Total) Concentrations

To determine the concentration of total Hb in synovial fluid (sf-Hb) we used a Human Hb ELISA Quantification Kit from Genway Biotech, Inc. (San Diego, CA, United States) according to the manufacturer's instructions. Samples were analyzed diluted 1:2000, at which the lowest detection limit for samples was 12.5 μg/ml. Absorbance was read at 450 nm using a Wallac 1420 Multilabel Counter.

Heme Concentrations

To determine the concentration of heme in synovial fluid (sf-Heme) we used QuantiChrom™ Heme Assay kit (Gentaur BVBA, Brussels, Belgium; cat. no. DIHM-250) following instructions in the kit and diluting samples 10x with PBS.

Protein Carbonyl Groups

Determination of the concentration of protein carbonyl groups in synovial fluid (sf-Carbonyl) was done by an ELISA, modified from the original description by Buss et al. (1997). Briefly, samples were diluted 4x in 6 M guanidine-HCl, 0.5 M K_3PO_4, pH 2.5, containing 10 mM DNP-hydrazine, incubated at RT for 45 min, mixed 1:200 with PBS and coated overnight in 96-well microtiter plates at 4°C. After washing and blocking for 1 h at RT with 0.1% reduced bovine serum albumin (BSA) in PBS, the plates were incubated with rabbit anti-DNP (Invitrogen, diluted 1000x) in PBS, 0.1% reduced BSA, 0.25% Tween-20 for 1 h at RT, washed and incubated 1 h at RT with horseradish peroxidase-conjugated porcine anti-rabbit IgG (Dako, diluted 2000x) in the same buffer. The wells were finally washed and developed by addition of a ready-to-use 3,3′,5,5′-tetramethylbenzidine substrate solution (TMB, Life Technologies, Stockholm, Sweden), stopping the reaction after 5 min by addition of 1 M HCl. The absorbance was read at 450 nm using a Wallac 1420 Multilabel Counter (Perkin Elmer Life Sciences, Waltham, MA, United States). Reduced BSA for blocking and incubation buffers, and oxidized BSA for standard carbonyl groups, were prepared as described (Buss et al., 1997). Measured concentrations are described as absorbance at 450 nm divided by total protein content of the sample (abs./tot. prot.). Total protein content in synovial fluid (diluted 10x) was determined with Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Inc.).

Statistical Analysis

Synovial fluid samples with Hb concentrations below the lower limit of detection (12.5 μg/ml) were given the concentration 12.5 μg/ml. We created the ratio of A1M concentrations in synovial fluid to serum (A1M ratio sf/s) for evaluation of the relative influence of local versus systemic concentrations of A1M. Normal distribution was evaluated by visual inspection of histograms, normal q–q plots and box plots. Student's *t*-test was used to compare A1M concentrations between sexes. For comparisons of biomarker concentrations between diagnostic groups we used univariate ANCOVA adjusted for age and sex with evaluation of residuals in histograms and normal p–p plots. Adjustments were included since groups were heterogeneous for these variables, and s-A1M was shown to increase with increasing age (Takagi et al., 1980), and to be higher in males compared to females (Takagi et al., 1980; Itoh and Kawai, 1990). For the same reason, we adjusted for age and sex in partial correlation analysis between biomarkers and time after injury (where longer time after injury was associated with higher age, $r = 0.357$, $p = 0.001$). Age, sex, and diagnosis was adjusted for in (a) linear regression models exploring joint bleeding (sf-Hb or sf-Heme) as explanatory factors for other biomarkers, and (b) linear regression models exploring the interaction of joint bleeding (sf-Hb or sf-Heme) and sf-A1M as explanatory factors for oxidative damage in the joint (sf-Carbonyl). In the linear regression models, sf-Heme, sf-Hb, sf-Carbonyl and the interaction variables ratio sf-A1M/sf-Heme and ratio sf-A1M/sf-Hb were log10 transformed to get linear relationships between variables, with evaluation of residuals in histograms and normal p–p plots.

In receiver operator characteristics (ROCs) analyses we evaluated A1M concentrations as a diagnostic tool for correctly diagnosing subjects with AIA. Evaluations were based on the area under the ROC curve (AUC) with cut-offs chosen to maximize the sum of the sensitivity and specificity.

We used SPSS version 24 for all statistical calculations, and made no adjustment for multiple comparisons due to the exploratory nature of the study.

RESULTS

α1-Microglobulin by Diagnosis

α1-Microglobulin concentrations were normally distributed within the diagnostic groups, and were overall 2.6 times higher in serum compared to synovial fluid (**Figure 1A**), without difference between males and females ($p = 0.80$ and 0.62 for sf-A1M and s-A1M, respectively). The highest concentrations of A1M, both in synovial fluid and serum, were found in the AIA (**Figure 1A**). The between-group-differences were largest in synovial fluid, where the mean A1M concentration in the AIA group was 1.5-fold higher compared to the OA and knee injury groups ($p = 0.005$ and 0.045, respectively), while the 1.7-fold difference in means between AIA and the reference group failed to reach statistical

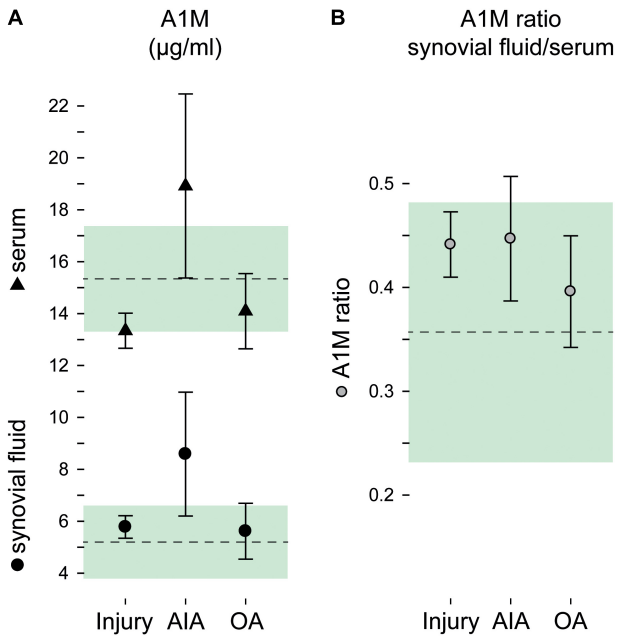

FIGURE 1 | A1M by diagnostic groups. Concentrations of A1M in **(A)** synovial fluid and serum and **(B)** as a ratio in concentrations synovial fluid to serum. Group means and 95% confidence intervals are plotted as circles and triangles with error bars for the injury, acute inflammatory arthritis (AIA) and osteoarthritis (OA) groups, and as a dashed line and green area for the reference group. (Plotted data and statistics are reported in full in **Supplementary Table S1**).

significance (p = 0.068). In serum, the A1M concentration in the AIA group was between 1.3- and 1.4-fold higher compared to the injury and osteoarthritis groups (p = 0.035 and 0.030, respectively), whereas the 1.2-fold higher mean concentration compared to the reference group was not statistically significant (p = 0.15; **Figure 1A**).

The A1M ratio in synovial fluid to serum ranged between 0.36 and 0.45 in the four diagnostic groups, without statistically significant differences between groups (p = 0.36; **Figure 1B**).

Since the AIA group was standing out as the only group with increased A1M levels in synovial fluid as well as in serum, we performed a ROC curve analysis for having AIA using A1M in both fluids as the diagnostic tool. We found that with similar AUC close to 0.8, sf-A1M had higher specificity but lower sensitivity than s-A1M (**Table 2**).

TABLE 2 | Receiver operator characteristic (ROC) curve analysis of sf-A1M and s-A1M as diagnostic for having acute inflammatory arthritis.

	sf-A1M	s-A1M
Cut off, µg/ml	7.625	15.4
AUC (95% CI)	0.763 (0.619, 0.908)	0.780 (0.648, 0.912)
sensitivity	0.615	0.692
specificity	0.844	0.734

ROCs, receiver operator characteristics; AUC, area under the ROC curve.

Markers of Bleeding and Oxidative Damage

Fifty-three out of 122 synovial fluid samples (43%) had sf-Hb concentrations below the lower limit of detection. Synovial fluid concentrations of Hb, total heme, and protein carbonyl groups were skewed, but were approximately normally distributed after log10 transformation. Intra-articular bleeding was more evident in the injury group, especially when measured as total heme (**Figures 2A,B**), but no statistically significant between-group differences were noted (p = 0.58 and 0.90 for sf-Heme and sf-Hb, respectively).

Oxidative damage in the joints, measured as protein carbonyl groups in synovial fluid, was highly similar and without statistically significant differences between diagnostic groups (p = 0.49) (**Figure 2C**).

Temporal Variation in Biomarkers After Injury

In the injury group, the levels of the biomarkers of bleeding, Hb and heme, in the joint was highest early after injury, and longer times after injury were associated with less bleeding (**Figure 3A**). This inverse relationship was strongest when measured as sf-Heme, for which we (after adjusting for age and sex) observed a statistically significant inverse correlation with time after injury (r = −0.46), and less accentuated, and not statistically significant, when measured as sf-Hb (r = −0.19) (**Figure 3A**). A similar temporal pattern and inverse association with time after injury was noted for sf-A1M (r = −0.30) and for the ratio of sf-A1M/s-A1M (r = −0.47) but not for s-A1M and sf-Carbonyl (**Figures 3B,C**).

Bleeding in the Joint as Explanatory Factor for Concentrations of A1M and Protein Carbonyl Groups Independent of Diagnosis

In hierarchical linear regression models including all 122 subjects, we examined how bleeding in the joint, independent of diagnosis, affected biomarker response after adjusting for age, sex and diagnostic group. This analysis showed that sf-Heme had a greater influence than sf-Hb on biomarkers of oxidative stress, since sf-Heme concentrations were associated with statistically significant effects on sf-A1M, the A1M ratio sf/s and sf-Carbonyl, whereas sf-Hb was only associated with an effect on the A1M ratio sf/s (**Table 3**). All associations had positive regression coefficients, i.e., higher concentrations of sf-Heme were associated with higher concentrations of sf-A1M, A1M ratios sf/s and sf-Carbonyl, and higher concentrations of sf-Hb were associated with higher A1M ratios sf/s (**Table 3**).

The relative influence of sf-Heme and sf-Hb on sf-A1M, A1M ratio sf/s and sf-Carbonyl can be evaluated by comparing the standardized effect sizes, partial correlations and R^2 changes in the models. These comparisons show that sf-Heme had the largest influence on sf-A1M and A1M ratio sf/s (with standardized effects and partial correlation coefficients of approximately 0.26), explaining 5.7 and 5.6% of the variability

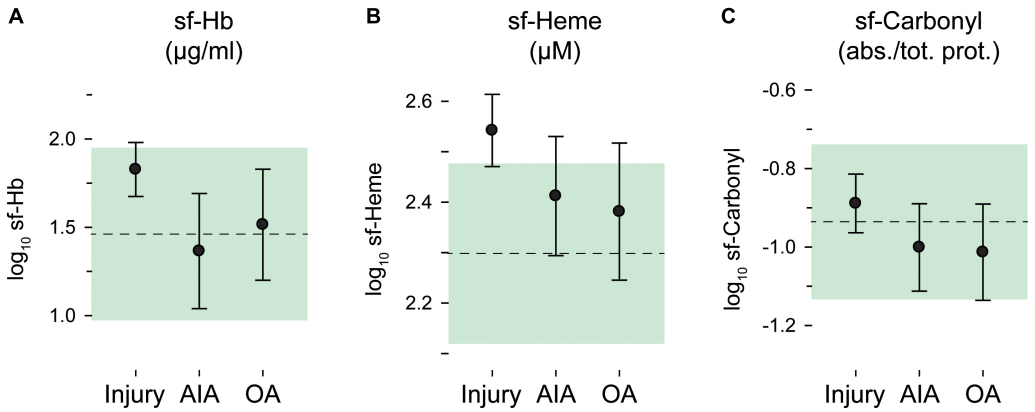

FIGURE 2 | Oxidative stress in synovial fluid by diagnostic groups. Log_{10} transformed concentrations in synovial fluid (sf) of **(A)** hemoglobin (Hb), **(B)** total heme, and **(C)** carbonyl groups. Group means and 95% confidence intervals are plotted as circles with error bars for the injury, AIA and osteoarthritis (OA) groups, and as a dashed line and green area for the reference group. sf-Carbonyl values are given relative to total protein concentration. (Plotted data and statistics are reported in full in **Supplementary Table S1**).

in sf-A1M and A1M ratio sf/s, respectively (R^2 change = 0.057 and 0.056) (**Table 3**). The relative influence of sf-Heme on sf-Carbonyl was slightly lower (standardized effect size and partial correlation both close to 0.20), where the R^2 change indicated that sf-Heme explained 3.5% of the variability of sf-Carbonyl in the model (**Table 3**). The lowest relative influence was noted for sf-Hb, which explained 2.5% of the variability of the A1M ratio sf/s (R^2 change = 0.025, with standardized effect size and partial correlation below 0.2), without statistically significant influence on the individual concentrations of sf-A1M or s-A1M (**Table 3**).

Interaction Between A1M and Hb or Heme as Explanatory Factors for Oxidative Damage Measured as Protein Carbonyl Groups Independent of Diagnosis

To investigate the combined effect of intra-articular bleeding and A1M on oxidative damage in the joint, we created two interaction variables, ratio sf-A1M/sf-Heme and ratio sf-A1M/sf-Hb. In two linear regression models, using heme as proxy for bleeding in model 1 and Hb in model 2, we then (in two steps, or blocks) explored the relationship between sf-Carbonyl and bleeding and sf-A1M alone (block 1) or in combination with the two interaction variables (block 2). Results in both models show that, when entered without an interaction variable, higher sf-Heme or sf-Hb were independently associated with higher oxidative damage measured as sf-Carbonyl (statistically significant positive effects), and higher sf-A1M was independently associated with lower sf-Carbonyl (statistically significant negative effects) (blocks 1, **Table 4**). Although different in direction, the relative influence of bleeding in the joint and sf-A1M on sf-Carbonyl was comparable in size (as judged by similarities in the magnitude of the standardized effects sizes and partial correlation coefficients in block 1 in both models) (**Table 4**). The model including sf-Heme and sf-A1M without an interaction variable explained 9.4% in the variability in sf-Carbonyl (adjusted R^2 = 0.094) and the model including sf-Hb and sf-A1M explained 6.5% (**Table 4**).

Adding the interaction variables (in block 2 of both models) increased the power of the models to explain variability in sf-Carbonyl from 9.4 to 14.1% when adding the ratio sf-A1M/sf-Heme, and from 6.5 to 10.4% when adding the ratio sf-A1M/s-Hb (**Table 4**). Based on the statically significant negative regression coefficients (unstandardized and standardized effects) (**Table 4**), higher ratios sf-A1M/sf-Heme or sf-A1M/sf-Hb were independently associated with lower concentrations of sf-Carbonyl.

When adding the interaction variables in block 2, multicollinearity between variables and their ratios was substantial in both models, as indicated by variance inflation factors (VIF) far beyond 10 (**Table 4**). This influenced the magnitudes of the effect sizes, making comparisons of effect sizes with and without the interaction variable present difficult. However, partial correlation coefficients for the interaction variables (ratios) and the individual concentrations were of the same magnitude (ranging between 0.20 and 0.26) (**Table 4**). This indicates that the relative influence on sf-Carbonyl of the ratio between sf-A1M and sf-Heme or sf-Hb is similar to that of the individual concentrations of sf-A1M, sf-Heme and sf-Hb.

DISCUSSION

This exploratory cohort study of oxidative stress in joint disease reveals a complex interplay between pro- and antioxidative molecular markers in knee injury, inflammation, or osteoarthritis. The study focuses on antioxidative reactions and protection from bleeding in the knee joint, and its main finding is that—without disease dependency—bleeding in the knee joint was associated with increased oxidative damage (in the form of higher concentrations of protein carbonyl groups in synovial fluid), as well as with higher concentrations of the

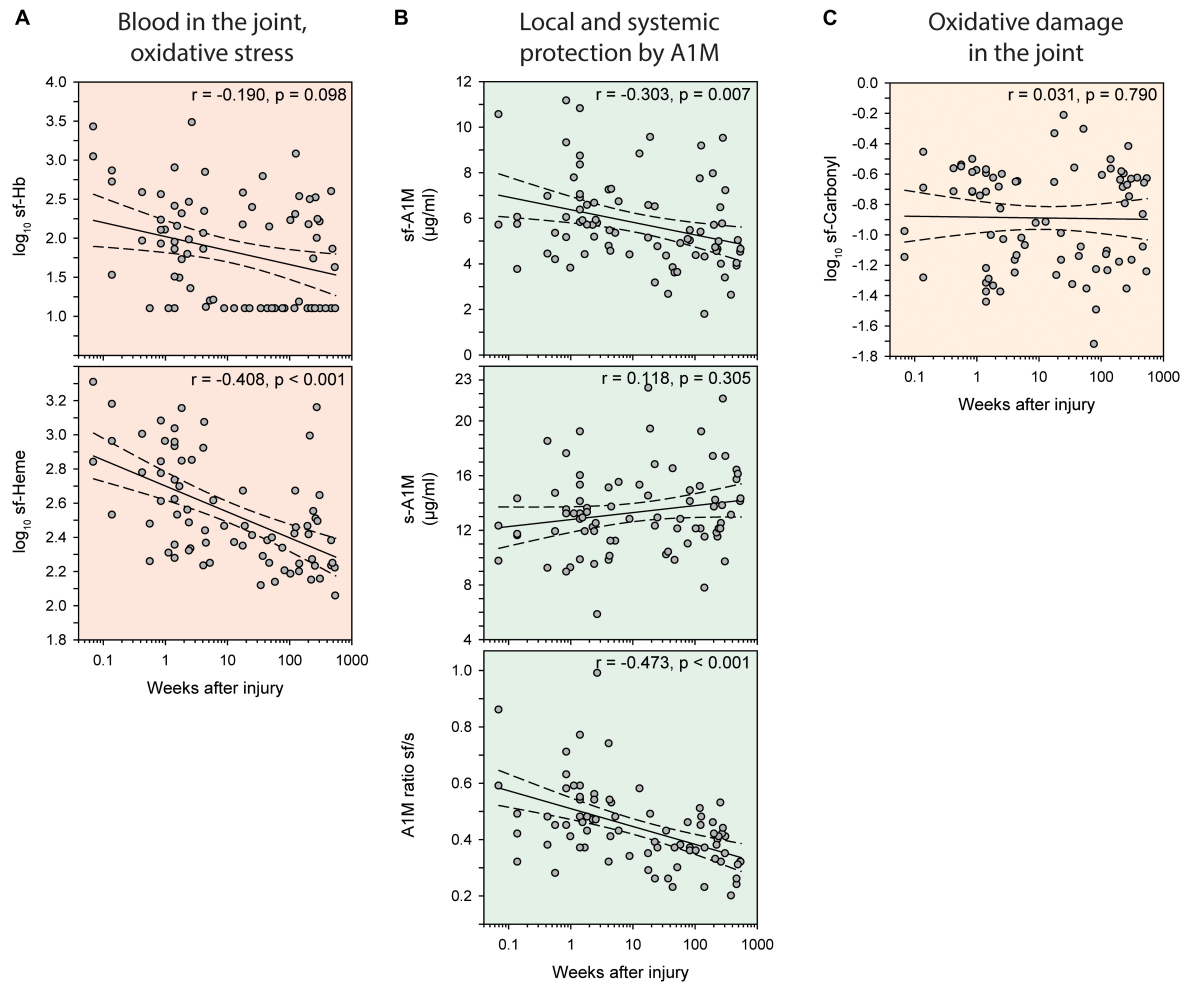

FIGURE 3 | Temporal variations in biomarkers of oxidative stress after knee injury. Bivariate scatterplots of biomarker concentrations against weeks after injury with regression lines (solid) and 95% confidence intervals of the regression lines (dashed). (**A, red**) Blood in the joint measured as hemoglobin (Hb) or total heme in synovial fluid (sf). (**B, green**) Concentrations of A1M in synovial fluid and serum, and the ratio of A1M in synovial fluid to serum. (**C, yellow**) Oxidative damage measured as protein carbonyl groups in synovial fluid. Partial correlation coefficients (r) indicate correlations adjusted for age and sex.

antioxidative protein A1M. In addition, we saw that the net amount of oxidative damage was dependent not only on the absolute concentrations of Hb, heme or A1M, but that the ratios between pro- and antioxidative molecules were as important as their absolute concentrations. When adjusted for their respective absolute concentrations, the higher the proportion sf-A1M to sf-heme or sf-Hb, the less oxidative damage measured as protein carbonyl groups in synovial fluid. These novel data support a role for A1M in the protection against oxidative damage due to extracellular Hb and heme in the joint in various knee arthropathies.

The diagnostic group for which these findings may be of greatest value is the injury group, which had the highest synovial fluid concentrations of extracellular Hb and heme. For this group, we noted that the time-related pattern of cell-free Hb and heme in synovial fluid after injury was mimicked by a similar pattern of A1M in synovial fluid, whereas serum A1M and synovial fluid carbonyl groups appeared as unaffected by time after injury. Altogether, our best interpretation of these data is that increased levels of cell-free Hb and heme in synovial fluid early after injury triggered an increase in the synovial fluid A1M concentration that appeared to be protective of oxidative damage.

The ratio of A1M in synovial fluid to serum followed a similar temporal pattern after injury and was more strongly associated with time after injury than the synovial fluid concentration by itself. This provides support that the reaction to knee injury and/or cell-free Hb and heme is localized to the injured knee, rather than being a systemic response. Such a local response may be the result of an upregulated local production of A1M, similar to previous reports of an upregulation of the protein in liver, skin, placenta, retina, and blood cells upon oxidative stimulation (Olsson et al., 2007, 2011; Cederlund et al., 2013). However, it cannot be excluded that an increased influx of systemic A1M also contributes to the synovial accumulation of A1M, since (i) the overall concentration of A1M in serum is three times higher compared to synovial fluid and (ii) reports have shown that the

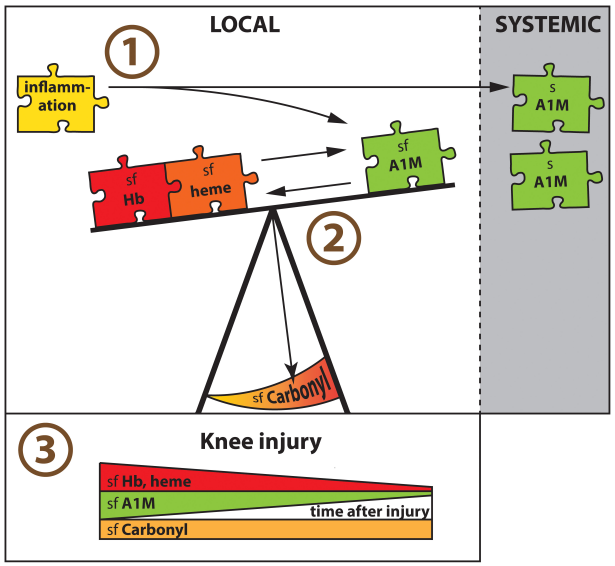

FIGURE 4 | Major findings in summary. **(1)** Having AIA in a knee joint was associated with increased A1M concentrations locally in synovial fluid (sf) and systemically in serum (s). With area under the ROC curve of close to 0.8, A1M was a biomarker for AIA in both synovial fluid and serum. **(2)** Independent of disease, cell-free Hb and heme within a knee joint triggered an increased synovial concentration of A1M that appeared to be protective of oxidative damage measured as sf-Carbonyl. The net amount of local oxidative damage (sf-Carbonyl) was dependent not only on the absolute concentrations of oxidative Hb and heme, or antioxidative A1M, but the ratio between pro- and antioxidative molecules appeared as important. **(3)** In knee injured there was a similar temporal pattern in the synovial concentrations of both oxidants (Hb and heme) and antioxidants (A1M), which were highest early after injury, without a corresponding temporal change in oxidative damage (sf-Carbonyl).

permeability of the synovial membrane and its blood vessels of small molecules like A1M, is increased in the early phases after joint injury and by hemorrhage (Soren et al., 1978; Hettinga, 1979).

There are many examples of studies that have shown increased A1M in serum or urine to be a biomarker of diseases such as preeclampsia (Anderson et al., 2011, 2016; Gram et al., 2015), and kidney failure (Terzi et al., 2014; Kang et al., 2015). Our study extends those findings to include elevated A1M in both serum and synovial fluid as a biomarker of AIA, as suggested by the ROC-curves with AUCs close to 0.8. It has previously been speculated that the plasma concentration of A1M does not change during inflammation unless it is secondary to disorders affecting the liver and kidney functions in humans (Itoh and Kawai, 1990), or rats (Falkenberg et al., 1997). Although we have no record of illnesses secondary to the AIA that the subjects of our study were seeking health care for, and therefore cannot rule out involvement of kidney or liver disease in some of the subjects, we find it unlikely that illnesses secondary to the AIA would explain the elevations of A1M seen in these subjects at group level in both serum and synovial fluid.

Studies on A1M in synovial fluid are rare. The only two reports we find are proteomic studies of subjects with knee OA

TABLE 3 | Hierarchical linear regression models exploring joint bleeding as explanatory factors for other oxidative stress biomarkers in all 122 subjects with Heme or Hb entered last after age, sex, and diagnostic group.

	log10 sf-Heme as explanatory					log10 sf-Hb as explanatory				
Dependent factors	[a]Effect, unstandardized (95%CI)	[b]Standardized effect	*p*-Value	Adjusted R^2 for model	Adjusted R^2 change when adding sf-Heme	Partial correlation	[a]Effect, unstandardized (95%CI)	[b]Standardized effect	*p*-Value	Adjusted R^2 for model	Adjusted R^2 change when adding sf-Hb	Partial correlation
sf-A1M	**2.169 (0.634, 3.705)**	**0.267**	**0.006**	**0.063**	**0.057**	**0.257**	0.420 (−0.250, 1.091)	0.118	0.217	0.009	0.005	0.114
Serum-A1M	1.934 (−0.272, 4.141)	0.150	0.085	0.227	0.013	0.163	0.263 (−0.674, 1.201)	0.047	0.579	0.206	−0.005	0.051
A1M ratio sf/serum	**0.121 (0.036, 0.207)**	**0.264**	**0.006**	**0.083**	**0.056**	**0.257**	**0.038 (0.001, 0.075)**	**0.190**	**0.045**	**0.046**	**0.025**	**0.185**
Log10 sf-Carbonyl	**0.214 (0.015, 0.414)**	**0.206**	**0.036**	**0.035**	**0.030**	**0.198**	0.082 (−0.002, 0.166)	0.183	0.056	0.026	0.023	0.176

[a]*Effect (regression coefficient): the estimate in average change in a dependent factor that corresponds to a 1-unit change in the explanatory factor log10-transformed sf-Heme or sf-Hb.*
[b]*Standardized effect: the estimate in average change in a dependent factor expressed in standard deviations that corresponds to a 1 standard deviation change in the explanatory factor log10-transformed sf-Heme or sf-Hb.*
Numbers in bold face indicate statistically significance at the 0.05 level.

TABLE 4 | Linear regression models exploring the interaction of joint bleeding and A1M as explanatory factors for oxidative damage in the joint in all 122 subjects.

		Explanatory factors	Dependent factor: log10 sf-Carbonyl					
			[a]Effect, unstandardized (95%CI)	[b]Standardized effect	*p*-Value	Adjusted R^2 for model	Partial correlation	Collinearity statistics, [c]VIF
Model 1 Heme and A1M	Block 1	log10 sf-Heme	0.288 (0.088, 0.488)	0.277	0.005	0.094	0.263	1.2
		sf-A1M	−0.034 (−0.058, −0.010)	−0.267	0.005		−0.263	1.1
	Block 2	log10 ratio sf-A1M/sf-Heme	−1.549 (−0.392, −2.706)	−1.522	0.009	0.141	−0.246	44.1
Model 2 Hb and A1M	Block 1	log10 sf-Hb	0.094 (0.011, 0.178)	0.210	0.027	0.065	0.205	1.1
		sf-A1M	−0.028 (−0.050, −0.005)	−0.220	0.016		−0.222	1.0
	Block 2	log10 ratio sf-A1M/sf-Hb	−1.386 (−0.272, −2.500)	−3.106	0.015	0.104	−0.225	212.7

Age, sex, and diagnosis were included as confounders in all models with Heme as proxy for bleeding in model 1 and Hb in model 2. In both models age, gender, diagnosis, sf-A1M and log10 sf-Heme or log10 sf-Hb was entered in the first block, with the interaction variable log10 ratio sf-A1M/sf-Heme or log10 ratio sf-A1M/sf-Hb entered together with the other variables in block 2.

[a]Effect (regression coefficient): the estimate in average change in the dependent factor log10 sf-Carbonyl that corresponds to a 1-unit change in the explanatory factors in the models.

[b]Standardized effect: the estimate in average change in the dependent factor log10 sf-Carbonyl expressed in standard deviations that corresponds to a 1 standard deviation change in the explanatory factors in the models.

[c] VIF (variance inflation factor): VIF higher than 10 indicates multicollinearity between variables.

(Gobezie et al., 2007; Sohn et al., 2012). One of those studies reported presence of A1M without comparison to other diseases or references (Sohn et al., 2012), the other found A1M to be upregulated in synovial fluid in subjects with OA compared to normal synovial fluid (Gobezie et al., 2007). In similarity with both studies, we found detectable concentrations of A1M in synovial fluid in subjects with knee OA. However, we did not see different concentrations between healthy and osteoarthritic subjects as was reported by Gobezie et al. (2007), which may be due to differences in methodology (mass spectrometry versus RIA), in the selection of patients, or in the power between the studies.

Expression and synthesis of A1M has been shown to be upregulated in cells after exposure to heme and ROS (Olsson et al., 2007, 2011). This is consistent with our findings here that both heme and Hb were associated with increased levels of synovial fluid A1M. Thus, we may speculate that local production of A1M in the knee joint is induced by synovial fluid free Hb or heme. However, with A1M increased in both synovial fluid and serum in AIA, the present study further suggests that, in addition to heme and ROS, A1M may be regulated by inflammatory cytokines. In further corroboration for an inflammatory link, we note that the temporal pattern of synovial fluid A1M seen after injury in this cross-section study, is highly similar to findings in a longitudinal study of ACL injured subjects, in which synovial fluid levels of inflammatory cytokines were initially elevated with subsequent decreasing levels over 5 years (Struglics et al., 2015; Larsson et al., 2017). This type of regulation by cytokines has been proposed for another major scavenger of ROS, extracellular superoxide dismutase (EC-SOD or SOD3) (Strålin and Marklund, 1994, 2000).

α_1-Microglobulin has been proposed as a therapeutic or diagnostic tool for several different conditions and diseases. The focus has so far mainly been on two areas of application: diagnosis and treatment of preeclampsia (Gunnarsson et al., 2017), and kidney protection in radiation therapy (Ahlstedt et al., 2015). The present study suggests that treatment with A1M following knee injury may prevent development of OA. In addition to the obvious protective benefit of exogenous A1M binding and neutralizing cell-free Hb and heme, it may also be beneficial as a repair mechanism in already damaged cartilage, since *in vitro* studies showed that collagen fibrils were restored to normal by addition of recombinant A1M after destruction already had begun (Olsson et al., 2011).

One of the novelties and strengths of this study—that we studied biomarkers of oxidative stress in matched samples of serum and synovial fluid—also proved to be a limitation, since it drastically reduced the number of available subjects to include from our convenience cohort of subjects with various knee-related illnesses. This may have increased the risk for spurious results, and reduced the likelihood of finding statistically significant differences between diagnostic groups. The study was further limited by its cross-sectional design, which reduced the possibility to draw firm conclusions on temporal change after injury since no repeated sampling was made within individual subjects. Lack of information on secondary or concomitant illnesses that may have influenced our results represents a further limitation. Finally, the presence of imputed values for sf-Hb may

have influenced and/or concealed associations with time after injury, or with other biomarkers.

CONCLUSION

Our results suggest (as summarized in **Figure 4**) that independent of disease, cell-free Hb and heme within a knee joint triggers an increased synovial concentration of A1M that appears to be protective of oxidative damage. We further conclude that the net amount of oxidative damage depends not only on the absolute concentrations of oxidative Hb and heme, or antioxidative A1M, but that the ratios between pro- and antioxidative molecules appears as important. Finally, we note that in AIA A1M is increased both locally in the joint fluid and systemically in serum, whereas in knee injury A1M is increased only in the joint fluid.

AUTHOR CONTRIBUTIONS

SL drafted the manuscript and full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. SL, BÅ, LL, and AS were involved in study conception and design. BÅ and SL in the acquisition of data. All authors were involved in revising it critically for important intellectual content, approved the final version to be published, and the analysis and interpretation of data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01596/full#supplementary-material

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Heme Induces Endoplasmic Reticulum Stress (HIER Stress) in Human Aortic Smooth Muscle Cells

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Accumulation of damaged or misfolded proteins resulted from oxidative protein modification induces endoplasmic reticulum (ER) stress by activating the pathways of unfolded protein response. In pathologic hemolytic conditions, extracellular free hemoglobin is submitted to rapid oxidation causing heme release. Resident cells of atherosclerotic lesions, after intraplaque hemorrhage, are exposed to heme leading to oxidative injury. Therefore, we raised the question whether heme can also provoke ER stress. Smooth muscle cells are one of the key players of atherogenesis; thus, human aortic smooth muscle cells (HAoSMCs) were selected as a model cell to reveal the possible link between heme and ER stress. Using immunoblotting, quantitative polymerase chain reaction and immunocytochemistry, we quantitated the markers of ER stress. These were: phosphorylated eIF2α, Activating transcription factor-4 (ATF4), DNA-damage-inducible transcript 3 (also known as C/EBP homology protein, termed CHOP), X-box binding protein-1 (XBP1), Activating transcription factor-6 (ATF6), GRP78 (glucose-regulated protein, 78kDa) and heme responsive genes heme oxygenase-1 and ferritin. In addition, immunohistochemistry was performed on human carotid artery specimens from patients who had undergone carotid endarterectomy. We demonstrate that heme increases the phosphorylation of eiF2α in HAoSMCs and the expression of ATF4. Heme also enhances the splicing of XBP1 and the proteolytic cleavage of ATF6. Consequently, there is up-regulation of target genes increasing both mRNA and protein levels of CHOP and GRP78. However, TGFβ and collagen type I decreased. When the heme binding proteins, alpha-1-microglobulin (A1M) and hemopexin (Hpx) are present in cell media, the ER stress provoked by heme is inhibited. ER stress pathways are also retarded by the antioxidant N-acetyl cysteine (NAC) indicating that reactive oxygen species are involved in heme-induced ER stress. Consistent with these findings, elevated expression of the ER stress marker GRP78 and CHOP were observed in smooth muscle cells of complicated lesions with hemorrhage compared to either

atheromas or healthy arteries. In conclusion, heme triggers ER stress in a time- and dose-dependent manner in HAoSMCs. A1M and Hpx as well as NAC effectively hamper heme-induced ER stress, supporting their use as a potential therapeutic approach to reverse such a deleterious effects of heme toxicity.

Keywords: heme, vascular smooth muscle cell, heme induced endoplasmic reticulum stress, atherosclerosis, hemopexin, alpha-1-microglobulin

INTRODUCTION

Vascular diseases including atherosclerosis remain one of the major causes of death worldwide (Roth et al., 2017). Atherosclerosis is associated with various pathological manifestations like ischemic heart disease, ischemic stroke, and peripheral arterial disease remaining the leading public health issue (Herrington et al., 2016). A more complete understanding of the pathophysiology would lead to new interventions that are urgently needed especially to treat the advanced, irreversible stage of atherosclerosis. Type IV atherosclerotic lesions, which are characterized by pronounced extracellular lipid accumulation, easily progress into more complicated lesions by the rupture of the fibrous cap and subsequent hematoma formation or intraplaque hemorrhage (Barger et al., 1984; Stary et al., 1995; Kolodgie et al., 2003; Moreno et al., 2006, 2012). The highly oxidative environment of these complicated lesions contain lipid peroxidation products such as lipid hydroperoxides, oxysterols, aldehydes and carbonyls, which are extremely toxic to cells of the vascular wall. Our group has been interested in this area, especially the role of heme in the pathogenesis of plaque progression (Balla et al., 2007).

In cell free models, heme was able to cause lipid peroxidation characterized by extensive cross-linking with subsequent amino acid oxidation (Gutteridge and Smith, 1988; Vincent, 1989). Moreover, heme catalyzes the degradation of proteins to small peptide fragments (Aft and Mueller, 1984). Our group was the first in the literature providing evidence that free heme, having amphipathic nature, can enter to lipid domains of living cells sensitizing them toward any types of reactive oxygen species (ROS) derived from activated neutrophils or inorganic-, organic hydroperoxides leading to cell death (Balla G. et al., 1991). We also demonstrated that oxidation of low density lipoprotein can be highly catalyzed by free heme facilitating modification of plasma apolipoprotein B-100 and the end products of this process lead to vascular endothelial cell injury (Balla G. et al., 1991; Miller and Shaklai, 1994; Li et al., 2006). The origin of the extracellular free heme might be red blood cells (RBCs) often captured in death zones of intraplaque hemorrhage. Plaque lipids of complicated lesions interacts with RBCs causing hemolysis resulting in more free heme inside of the vessel wall (Nagy et al., 2010). During the catalytic process, the porphyrin ring opens, degrades, and iron is liberated leading to the accumulation of footprints of heme catalyzed oxidative reactions including oxidized lipids and proteins, as well as cellular responses and injuries (Balla et al., 2007; Nagy et al., 2010; Jeney et al., 2014).

The importance of protection against heme-catalyzed cell injury is underlined by the existence of natural heme binding proteins, which protect molecules, cells, and the whole organism from the deleterious effects of free heme. Hemopexin (Hpx) binds heme with a stoichiometry of one to one molar ratio with one of the highest known affinities (Kd 10^{-13} M) (Hrkal et al., 1974). Internalization of Hpx-heme complex takes place in part via the scavenger receptor, LDL receptor-related protein1/CD91, although not exclusively (Hrkal et al., 1974; Herz and Strickland, 2001; Hvidberg et al., 2005; Smith and McCulloh, 2015). After intravenous injection of heme-Hpx complexes in rats, uptake was predominantly by hepatocytes (Smith and Morgan, 1978, 1979). It is well established that in plasma and other biological fluids binding of heme by Hpx prevents heme catalyzed oxidative reactions such as LDL oxidation and endothelial cell death (Gutteridge and Smith, 1988; Vincent et al., 1988; Balla G. et al., 1991). Alpha-1-microglobulin (A1M), a radical scavenger and reductase in the plasma and extravascular tissues, is also an effective binder of heme (Kd 10^{-6} M) (Larsson et al., 2004; Åkerström et al., 2007; Åkerström and Gram, 2014). A1M binds heme in plasma, extravascular fluids and cells. A1M binds heme with two binding sites, with a relatively weak one and a stronger, covalent interaction (Larsson et al., 2004; Allhorn et al., 2005). Several reports described the *in vitro* protective effects of A1M in cell cultures against hemoglobin-, heme-, and ROS-induced cell- and tissue damage (Olsson et al., 2008, 2011). Because these two heme binding proteins, A1M and Hpx, protect cells and biological molecules from heme toxicity, they have been proposed as therapeutic agents in pathophysiological conditions where free heme is present; and this has been established in several studies with cell and animal models of human diseases (Schaer et al., 2013, 2014; Vinchi et al., 2016).

The nature of the lethal cellular injury provoked by uptake of "free" heme, *i.e.,* non-protein bound heme is not fully understood. At the present time, the main form of cell death is generally considered to be necrosis caused by heme sensitization to ROS. Heme has been reported to induce ROS in a variety of cell types such as small intestine epithelial cells, neutrophils, macrophages and human umbilical vein endothelial cells provoking various pathophysiological conditions such as intestinal mucosa dysfunction, necrosis, and inflammation (Porto et al., 2007; Fortes et al., 2012; Barcellos-de-Souza et al., 2013; Erdei et al., 2018). One of the major targets of ROS are proteins leading to excessive protein modifications with often detrimental consequences on protein structure and function (Berlett and Stadtman, 1997; Davies, 2016). Because raising intracellular heme can generate ROS resulting in loss off protein functions and fragmentations (Aft and Mueller, 1984; Vincent et al., 1988; Alvarado et al., 2015), we consider that heme-induced ROS triggers endoplasmic reticulum stress (ER stress) together

with unfolded protein response (UPR) through ROS-mediated protein damage. We hypothesize that these protein modifications might trigger ER stress and UPR. If protein folding is inhibited or the demand for folding exceeds the folding capacity of the ER, the pathways of UPR are activated leading to ER stress (Schröder and Kaufman, 2005; Walter and Ron, 2011). Evidence is accumulating for an association between vascular diseases and ER stress as well as between atherosclerosis and oxidative stress (Ivanova and Orekhov, 2016; Hong et al., 2017; Kattoor et al., 2017; Yang et al., 2017). However, to date there is no evidences for the role of ER stress in plaque progression. Furthermore, heme has not previously been studied as a trigger of ER stress/UPR in vascular research.

During ER stress, at least three distinct arms of the UPR are initiated, pancreatic ER kinase-like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring enzyme 1 (IRE1), all of which are activated by GRP78 (glucose-regulated protein 78 kDa) (Walter and Ron, 2011). Early activation of PERK/phosphorylated eIF2α/ATF4 pathway reduces the protein load of the ER and enables cells to recover from this form of stress (Hamanaka et al., 2005; Raven and Koromilas, 2008). ATF4 initiates the transcription of genes involved in the functional UPR, including those linked to amino acid metabolism, redox homeostasis, and even ER stress-induced apoptosis (Harding et al., 2003; Ameri and Harris, 2008). Prolonged or severe ER stress leads to cell death mediated by CHOP, which is predominantly activated by the PERK/phosphorylated eIF2/ATF4 pathway (Zinszner et al., 1998). The second major player of ER stress is IRE1a, a proximal sensor of ER stress, splices X-box binding protein (XBP1) mRNA by an unconventional mechanism using its endoribonuclease activity (Tirasophon et al., 1998; Lee et al., 2002). Canonical targets of spliced XBP1 include ER chaperones and components of endoplasmic reticulum-associated degradation (ERAD), which eliminate unfolded or misfolded proteins (Acosta-Alvear et al., 2007; He et al., 2010). Prolonged ER stress can also activate apoptosis *via* IRE1-ASK1-JNK pathway (Nishitoh et al., 2002). ATF6 is a transmembrane glycoprotein of ER. Upon ER stress, ATF6 is cleaved and a 50 kDa fragment translocates to the nucleus (Ye et al., 2000; Liu and Kaufman, 2003). ATF6 activates the expression of a number of genes like the ER chaperones including Grp78, Grp94, protein disulfide isomerase, and the components of ERAD and XBP1 (Dorner et al., 1990; Haze et al., 1999; Yoshida et al., 2001; Hirota et al., 2006; Thuerauf et al., 2007; Todd et al., 2008). Overall, these three arms either regulate the expression of numerous genes that restore homeostasis in the ER or may even induce apoptosis (Walter and Ron, 2011).

Endoplasmic reticulum stress was shown to suppress the expression of TGFβ and downstream product collagen type I. TGFβ enhances plaque stability, reduces atherosclerotic plaque size (Bobik, 2006; Chen et al., 2006, 2016; Bot et al., 2009; Reifenberg et al., 2012; Hassan et al., 2018), and is limitedly present in advanced atherosclerotic plaques (Grainger et al., 1995; Bobik et al., 1999; McCaffrey et al., 1999).

The purpose of this study was to investigate whether free heme, in addition to causing intracellular "heme stress" (by raising redox active heme and iron), might also induce ER stress. If so, this would add a new insight into the heme-mediated vessel wall injury in the pathogenesis of atherosclerosis. One of our goals was to demonstrate the close proximity of heme to smooth muscle cells, and the signs of ER stress in these cells in the depth of atherosclerotic plaques in human samples. Using *in vitro* cell culture experiments we mimicked this *in vivo* phenomenon in human aortic smooth muscle cells (HAoSMCs) evaluating heme as a trigger for ER stress using changes in key target proteins of the three arms of the UPR. The final goal was to confirm our second hypothesis, that A1M and Hpx are efficient protectants against this type of complex, toxic heme stress.

MATERIALS AND METHODS

Reagents

Reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States) unless otherwise specified. Hemin chloride stock solution (2 mM) was prepared in sterile 20 mM NaOH on the day of use for each experiment. Human recombinant wild-type A1M was expressed in *E. coli*, purified and refolded as described with an additional ion-exchange chromatography step as outlined (Kwasek et al., 2007; Ahlstedt et al., 2015). Rabbit hemopexin was isolated and purified as previously described (Morgan et al., 1993; Eskew et al., 1999).

Tissue Samples

Carotid arteries from patients who underwent carotid endarterectomy were obtained from the Department of Surgery at the University of Debrecen. The sample collection was approved by the Scientific and Research Ethics Committee of the Scientific Council of Health of the Hungarian Government under the registration number of DE OEC RKEB/IKEB 3712-2012. Written informed consents were received from the participants. Specimens were examined by trained pathologist and classified according to AHA guidelines. Type I (healthy), IV (atheromatous) and VI (complicated) lesions were selected for further investigation.

Cell Culture

Human aortic smooth muscle cells were obtained from Cell Applications (San Diego, CA, United States) and Lonza (Allendale, NJ, United States). Cells were grown in low glucose (1g/L) DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and amphotericin B. They were grown to 90% confluence and used from passages 5 to 7. The medium was changed every 2 days. Heme treatments were carried out in serum- and antibiotic-free DMEM. Briefly, hemin chloride stock solution in sterile 20 mM NaOH was diluted in serum- and antibiotic-free DMEM. Cells were washed twice with Hank's Balanced Salt Solution pH 7.4 supplemented with Ca^{2+} and Mg^{2+} (HBSS +) and treated with different hemin concentrations for 60 min. Cells were washed with HBSS + and fresh DMEM with 10% fetal bovine serum (FBS) and antibiotics were added and cells were further incubated for 3, 6, and 16 h in a CO2 (5%) incubator. For the A1M and Hpx studies, the proteins were added to hemin diluted in serum- and antibiotic-free

DMEM to a concentration of 12.5 μM of A1M (two heme binding sites) and 25 μM of Hpx (one heme binding site), gently mixed then incubated at room-temperature in the dark with gentle agitation for 30 min. Cells treated with 1 μM of thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) as positive controls of ER stress for 3, 6, and 16 h.

For N-acetyl cysteine (NAC), cells were pretreated with NAC (10 mM) in complete growth medium 1 h prior to the heme exposure. Cells were then washed with HBSS+ and treated with NAC (10 mM), heme (25 μM) or with NAC (10 mM) and heme (25 μM) together for 60 min in serum-free DMEM. HAoSMCs were then rinsed with HBSS+ and incubated in complete growth medium alone or supplemented with NAC (10 mM) for 3 h. ER stress markers were then analyzed with q-RT-PCR or immunoblot as described above.

Cell Lysis and Western Blot

Cells were washed with cold phosphate buffered saline pH 7.4 then lysed with RIPA buffer containing protease and phosphatase inhibitors (50 mM Tris pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 1% Sodium-deoxycholate, 0.1% SDS, 1× Complete Mini Protease Inhibitor Cocktail, 2 × PHOSSTOP phosphatase inhibitor cocktail, and incubated for 15 min on ice. Lysates were clarified by spinning at 16000 × *g*, 4°C for 15 min. Protein content was determined using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, United States).

Cell extracts (30 μg protein) were electrophoresed on 10% Tris-glycine SDS-PAGE gels, then the proteins were transferred to 0,22 μm PVDF membrane (Bio-Rad, Hercules, CA, United States membrane) and blocked with 5% w/v BSA for 60 min. Primary antibodies against phospho-eIF2α, eIF2α, ATF4, ATF6, XBP1s, and ferritin heavy chain (FTH) from Cell Signaling Technology (Danvers, MA, United States) were diluted 1:1000, while Grp78 and HO1 from Proteintech Group (Manchester M3 3WF, United Kingdom) and diluted 1:5000 in blocking solution. In order to ascertain equivalent protein loading in the samples, the membranes were stripped and reprobed with a mouse anti-human GAPDH antibody (Novus Biologicals, LLC, Littleton, CO, United States) at a dilution of 1:5000. Antigen-antibody complex was detected by WesternBright ECL HRP substrate (Advansta, Menlo Park, CA, United States). Protein bands were quantified with ImageJ software and normalized to GAPDH (Rasband, 1997–2005).

RNA Isolation and XBP1 Splicing PCR

Cells were grown on six-well plates and total RNA was isolated with Tri Reagent (Zymo Research) and reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA, United States). XBP1 and GAPDH were amplified with Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, United States). The splice variant of XBP1 was evaluated using the primers and conditions as described by Kosakowska-Cholody et al. (2014). Amplimers were quantified with ImageJ software and normalized to GAPDH.

Quantitative Reverse Transcription-Polymerase Chain Reaction

Cells were grown on six-well plates and total RNA was isolated and reverse transcribed as described above. ATF4, CHOP, Grp78, and HO1 mRNA expressions were determined by TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, United States) and were normalized to GAPDH (ATF4: Hs00909569_g1; CHOP: Hs00358796_g1; Grp78: Hs00607129_gH; HO1: Hs01110250; GAPDH: Hs02758991_g1; TGFβ: Hs00998133_m1; collagen type I: Hs00164004_m1). Reverse transcriptions and qPCRs were carried out using the C1000 Thermal Cycler with CFX 96 Real Time PCR System (Bio-Rad, Hercules, CA, United States). Relative mRNA expressions were calculated with the ΔΔCt method using GAPDH as internal control.

Confocal Microscopy

Cells on coverslips were exposed to heme in serum- and antibiotic-free DMEM for 60 min, rinsed twice and then cells were incubated for 3 h in DMEM containing 10% FBS and antibiotics. Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4 for 15 min. Coverslips were washed with PBS and samples were blocked with 5% goat serum in PBS supplemented with 0.3% Triton X-100 for 60 min. Samples were then incubated with primary antibody against CHOP (Proteintech Group Manchester M3 3WF, United Kingdom) at a 1:500 dilution or against ATF4 (Cell Signaling Technologies, Danvers, MA, United States) at a 1:500 dilution overnight at 4°C in antibody dilution buffer (1% BSA in PBS supplemented with 0.3% Triton X-100). The secondary antibody was a goat anti-rabbit IgG conjugated to Alexa Fluor® 532 (Thermo Fisher Scientific, Waltham, MA, United States) used at a 1:1000 dilution in antibody dilution buffer and incubated for 60 min at room temperature. Nuclei were visualized with Hoechst. Cells treated with 1 μM of thapsigargin for 3 h was used as positive control. Nuclear translocation of CHOP and ATF4 was investigated with TCS SP8 STED microscope using Leica Application Software X (Leica, Mannheim, Germany).

Detection of Crosslinked Hemoglobin by Western Blot

Detection of crosslinked hemoglobin in three healthy carotid arteries and three complicated carotid lesions by Western blot was performed as described in our previous study using HRP-conjugated goat anti-human Hb polyclonal antibody (ab19362-1 Abcam, Cambridge, United Kingdom) at a dilution of 1:15000 (Nagy et al., 2010).

Determination of Oxidized Hemoglobin and Heme Content in Healthy Carotid Arteries and Complicated Carotid Lesions

Determination of oxidized hemoglobin concentration in healthy carotid arteries and complicated carotid lesions was performed

according to the method of Winterbourne CC (Winterbourn, 1990). Heme measurements in tissue samples were performed as described by Huy et al. (2005).

Immunohistochemistry

The common carotid artery specimens were fixated with PBS buffered formaldehyde (4%) solution (4%) at pH 7.4 for 1 to 3 day – based on the size of the sample. In case of calciphicated samples 1.0 M/l EDTA/Tris buffer was used for the decalcification after fixation. The vascular segments were embedded in paraffin wax, than 3–5 um thick slides were prepared through deparaffination used by xylene and ethanol. After inhibition of endogenous peroxidase (0.5% for 20 min) activity slides were subjected to antigen retrieval in a buffer solution (pH 9.0, RE7119, Leica, Wetzlar, Germany). For immunohistochemistry, samples were incubated with Dako EnVision FLEX Peroxidase-Blocking Reagent (Dako, Glostrup, Denmark) for 5 min in a wet chamber. Slides were then washed with EnVision^{TM} FLEX Wash Buffer, Tris-buffered saline solution containing Tween 20, pH 7.6 ($\pm$ 0.1). Antigen retrieval was performed in the epitope retrieval solution (RE-7119, Tris/EDTA–based buffer containing surfactant, Leica, Wetzlar, Germany) at pH 9 using a pressure cooker. Slides were then washed with distilled water and EnVision^{TM} FLEX Wash Buffer, Tris-buffered saline solution containing Tween 20, pH 7.6 ($\pm$0.1). Next slides were incubated with CHOP (clone: rabbit polyclonal 15204-1-AP Proteintech Group, Rosemont, IL 60018, United States) primary polyclonal antibody at a dilution of 1:1000 used OPTIVIEW DAB DETECTION KIT based on protocol No. 6396500001. Serial sections next slides were incubated with GRP78/BIP antibody (clone: rabbit polyclonal 11587-1-AP Proteintech Group, Rosemont, IL 60018, United States) primary polyclonal antibody at a dilution of 1:2000 used ULTRAVIEW UNIVERSAL DAB DETECTION KIT based on protocol No. 5269806001. The intensity and distribution of CHOP and GRP78/BIP specific immunostaining was assessed by light microscopy (Leica DM2500 microscope, DFC 420 camera and Leica Application Suite V3 software, Leica). Counterstain with Gill Hematoxylin solution (105175 Merck Millipore, Billerica, MA, United States). Rinse in running tap water for 2–5 min. Dehydrate through 95% ethanol for 1 min, 100% ethanol for 2 $\times$ 3 min. Clear in xylene for 2 $\times$ 5 min. The slices were coverslip with mounting medium.

Determination of ROS

Reactive oxygen species generated by heme was measured using the chloromethyl derivative of 2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Life Technologies, Carlsbad, CA, United States). Cells were seeded in a clear- bottom black 96-well plate overnight in DMEM medium containing 10% FBS and antibiotics. Cells were washed three times and incubated in HBSS + supplemented with CM-H2DCFDA (10 μM) for 30 min in a CO2 incubator. Cells were washed three times and incubated with corresponding to vehicle control (VC), thapsigargin (1 μM) ER stress positive control (PC) or with various doses of heme (1, 10, and 25 μM) for 60 min. Cells were washed thoroughly, and fluorescence intensity was measured applying 485 nm excitation and 530 nm emission wavelengths. In some experiments, heme (25 μM) was pre-incubated either with hemopexin (25 μM) or recombinant A1M (12.5 μM) in HBSS + for 30 min before the treatments. To mitigate oxidative stress, ROS was scavenged by the addition of NAC (10 mM) to the experimental medium before and during the treatments. Briefly, cells were pre-incubated with ROS scavenger NAC (10 mM) for 60 min in HBSS+ then exposed to heme (25 μM) together with NAC (10 mM).

Cell Viability Assay

Cell viability was determined by the MTT assay. Briefly, cells were cultured and treated in 96-well plates for the indicated time. Then cells were washed with PBS, and 100 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (0.5 mg/mL) solution in HBSS was added. After a 90-min incubation, the MTT solution was removed, formazan crystals were dissolved in 100 μL of DMSO and optical density was measured at 570 nm.

Statistical Analysis

Data are shown as mean $\pm$ SD. Statistical analysis was performed by one-way ANOVA test followed by Bonferroni correction or unpaired *t*-test. A value of $p < 0.05$ was considered significant.

RESULTS

The ER Chaperone Grp78 and Cell Death Marker CHOP Are Both Upregulated in Hemorrhaged Complicated Lesions Compared to Atheromas and Healthy Carotid Arteries

First, we analyzed whether hemorrhaged complicated lesions show increased ER stress marker expression compared to healthy carotid arteries or atheromas (**Figure 1**). Macroscopic features and hematoxylin-eosin-stained slides covered the full spectrum of atherosclerotic lesions within the collected carotid endarterectomy specimens. Healthy vessels, atheromas and complicated, freshly hemorrhagic lesions are shown. The selected samples were stained for smooth muscle actin, Grp78 and CHOP using immunohistochemistry. Normal appearing carotid wall tissue showed weak level of Grp78 and CHOP expression, mostly located to the nucleolar and perinucleolar regions. Simple (stabile) atheromas displayed separated layers of smooth muscle cell bunches surrounding the lipid deposition. CHOP and Grp78 protein expression in these cells was nearly the same compared with the healthy control carotid samples. Ruptured, hemorrhagic complicated lesions are presented with highly increased CHOP and Grp78 immunopositivity appearing in both the cytoplasm and the nucleolus of the polygonal activated smooth muscle cells in the interstitium of the artery.

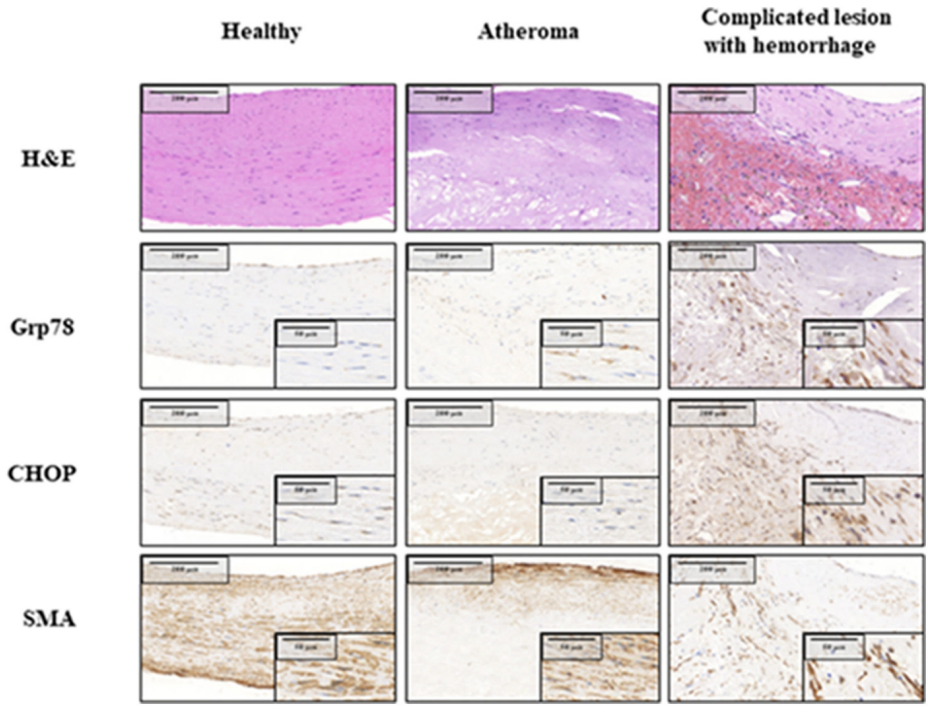

FIGURE 1 | Expression of ER chaperone Grp78 and death signal protein CHOP in healthy carotid wall, atheromatous plaque and complicated lesion with hemorrhage. Immunomorphological features associated with different stages of atherosclerotic process: serial sections of healthy carotis wall **(Left)**, atheromatous plaque **(Middle)** and complicated (hemorrhagic) lesion **(Right column)** are shown. H&E staining, SMA, CHOP and Grp78 immunohistochemistry are presented from the same tissue areas, all pictures and all inserts with the same magnification.

Oxidation of Hemoglobin Occurs in Complicated Lesions Resulting in Accumulation of Free Heme

Our previous studies revealed that complicated lesions with hemorrhage contained oxidized forms of hemoglobin (Nagy et al., 2010). Here, we also demonstrate that in specimens of such atherosclerotic plaques, the hemoglobin was oxidized (methemoglobin containing ferric heme) as reflected by the accumulation of cross-linked dimers, tetramers and multimers (**Figure 2A**). As expected, methemoglobin readily released its heme moieties (**Figures 2B,C**). Since cells are exposed to heme in the complicated hemorrhaged atherosclerotic plaques, we next addressed whether ER stress in vascular smooth muscle cells was provoked by heme.

Heme Activates the PERK/eIF2/ATF4/CHOP Arm of ER Stress Sensors

CHOP is the terminal effector protein of the PERK pathway and our first aim was to investigate its expression in HAoSMCs in response to heme. CHOP mRNA was measured in extracts of cells made 3, 6, and 16 h after exposure to heme (1, 10, and 25 μM). Thapsigargin-treated (1 μM) cells were used as positive controls. As shown in **Figure 3A**, heme induced CHOP mRNA expression in a time- and dose-dependent manner. Significant enhancement was observed after HAoSMCs were exposed to heme (10 and 25 μM) 3 h after the treatment and declined there after back to the control level 16 h after heme treatment. Thapsigargin treated cells showed more robust CHOP activation compared to heme even after 16 h of incubation where heme induced CHOP levels declined to the control level (**Supplementary Figure 1**). The expression of CHOP at the protein level together with its nuclear translocation were also assessed with immunofluorescence 3, 6, and 16 h after heme exposure. Untreated cells as well as vehicle controls had very low fluorescence as did the cells exposed to low levels of heme (1 μM). Cells treated either with 10 or 25 μM of heme showed increased fluorescence signal consistent with higher expression, and, furthermore, nuclear translocation of CHOP 3 h after the heme exposure demonstrating ER stress (**Supplementary Figure 2**). Fluorescence signals in cells treated with 10 μM of heme markedly declined thereafter within the next 3 h similar to the controls, while levels remained elevated for up to 16 h in the cells exposed to 25 μM (**Supplementary Figures 3**, **4**). CHOP is predominantly up-regulated *via* the PERK-eIF2α-ATF4 signaling branch of the UPR; therefore, to gain a deeper insight into the mechanism of CHOP activation in response to heme, we analyzed eIF2α phosphorylation as well as ATF4 expression. These two proteins are the upstream regulatory element of CHOP. Heme at 10 and 25 μM, but not 1 μM, transiently induced the phosphorylation of eIF2α (p-eIF2α) and subsequently ATF4 expression too, both at the mRNA and protein levels **(Figures 3B–E**). As with the induction of CHOP mRNA, eIF2α phosphorylation

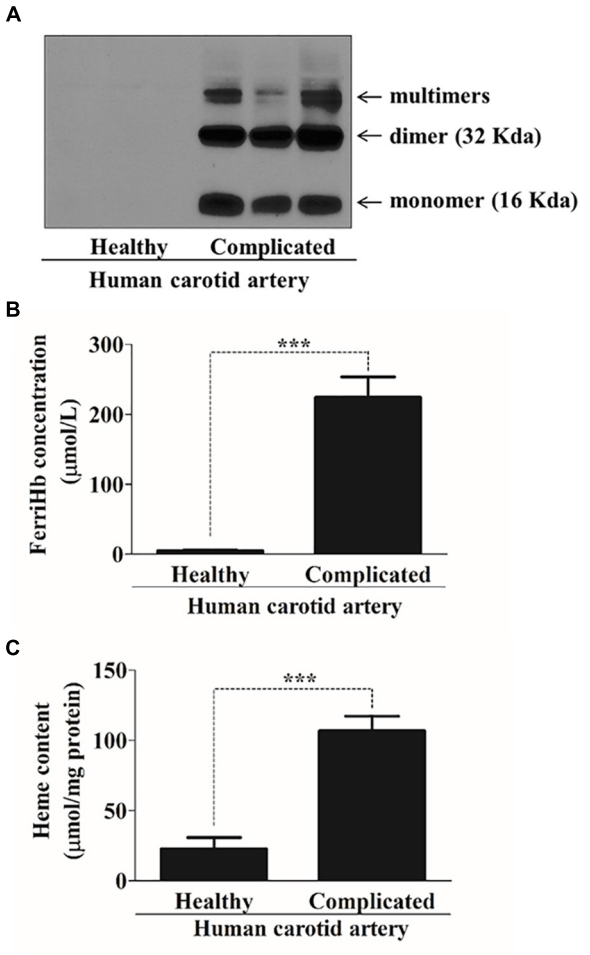

FIGURE 2 | Complicated lesions contain oxidized forms of hemoglobin and are characterized by high heme content. **(A)** Representative Western blot (one out of five performed) showing Hb expression in three human healthy and complicated carotid artery (20 μg/lane). **(B)** A spectral scan of human healthy ($N = 17$) and complicated ($N = 11$) carotid artery tissue lysates were taken and concentrations of ferriHb (μmol/L) were calculated based on the visible spectra. Values were normalized to protein content of the tissue lysates. ***$p < 0.0001$ using unpaired t-test. **(C)** Total heme concentrations (μmol/mg protein) of human healthy ($n = 14$) and complicated ($n = 11$) carotid artery tissue lysates were determined spectrophotometrically and calculated according to the heme standard curve. ***$p < 0.0001$ using unpaired t-test.

as well as ATF4 mRNA expression showed a dose- and time dependency. Phosphorylation of eIF2α was significantly increased 3 h after heme treatment and was only detectable 6 h after the treatment when the highest heme doses were applied (25 μM; **Figures 3C,D**). eIF2α phosphorylation as well as ATF4 expression declined to basal levels 16 h after the heme challenge **(Figure 3E**). ATF4 mRNA levels showed slightly different kinetics from CHOP mRNA expression with a peak at 3 h after 10 μM heme treatment and remained constant up to 6 h. ATF4 levels increased gradually up to 6 h after the treatment when HAoSMCs were exposed to 25 μM heme (**Figure 3B**). Accordingly, the ATF4 was translocated to the nucleus in cells incubated with heme (10 and 25 μM) further supporting activation of the PERK pathway within 3 h after the heme exposure (**Supplementary Figure 5**).

Heme Also Activates the IRE1/XBP1s Arm of ER Stress Sensors in a Time- and Dose-Dependent Manner

Upon activation of the UPR, IRE1 IRE1α splices XBP1 mRNA resulting XBP1s protein, which is a potent transcriptional activator that induces expression of many UPR responsive genes. To examine whether heme activates this arm of the classical ER stress in addition to the PERK arm, we measured XBP1 splicing catalyzed by activated IRE1 after challenging the HAoSMCs with different concentrations of heme. As previously, control cells were treated with 1 μM of thapsigargin. The PCR results showed that heme (10 and 25 μM) induced the activation of the canonical IRE1/XBP1s pathway in a time- and concentration-dependent manner, which was demonstrated by a transient increase in the ratio of XBP1 spliced (XBP1s) and XBP1 unspliced (XBP1u) levels (**Figures 4A–F**). IRE1/XBP1 activation was the most pronounced 3 h after heme exposure (not shown). The level of XBP1s mRNA markedly decreased 6 h after the treatment when HAoSMCs were exposed to 10 μM of heme, but remained induced when cells were treated with 25 μM of heme (**Figures 4C,D**). XBP1s levels decreased to the control level 16 h after the treatment (**Figures 4E,F**). Neither vehicle nor low dose heme (1 μM) activated IRE1/XBP1 pathway (**Figures 4A–F**) consistent with our data on the PERK pathway. The protein expression of the spliced form of XBP1 correlated well with the mRNA expression and showed time- and dose-dependence (**Figures 4G–I**). These findings suggest that the IRE1 pathway was activated by a certain level of heme but transiently, in contrast to the sustained activation of the PERK pathway.

Heme Also Activates the ATF6 Arm of ER Stress in Time- and Dose-Dependent Manner

ATF6 is a membrane bound transcription factor that activates genes in response to ER stress because as unfolded proteins accumulate. ATF6 is cleaved and its cytoplasmic domain enters the nucleus, where it induces a set of chaperones (Grp78, Grp94, calreticulin) that are involved in stress adaptation and survival. To investigate whether heme induces ATF6-mediated stress adaptive response, we assessed the extent of proteolytic activation of ATF6 by immunoblotting. Heme at concentrations of 10 and 25 μM, but not at 1 μM, induced a dose-dependent proteolytic activation of ATF6 (**Figures 5A–F**) causing a time- and dose-dependent decrease in ATF6 protein levels. Noticeably, the ATF6 pathway was still activated 16 h after heme challenge indicating a robust and long-lived response (**Figures 5E,F**). Thus, the adaptive ATF6 pathway is also activated in response to heme.

Heme Increases the Expression of the Grp78, an ER Chaperone

The trafficking of ATF6 from the ER to the Golgi for processing by site 1 and site 2 proteases is controlled by the ER chaperone BiP/Grp78. Thus, this protein is considered the master regulator

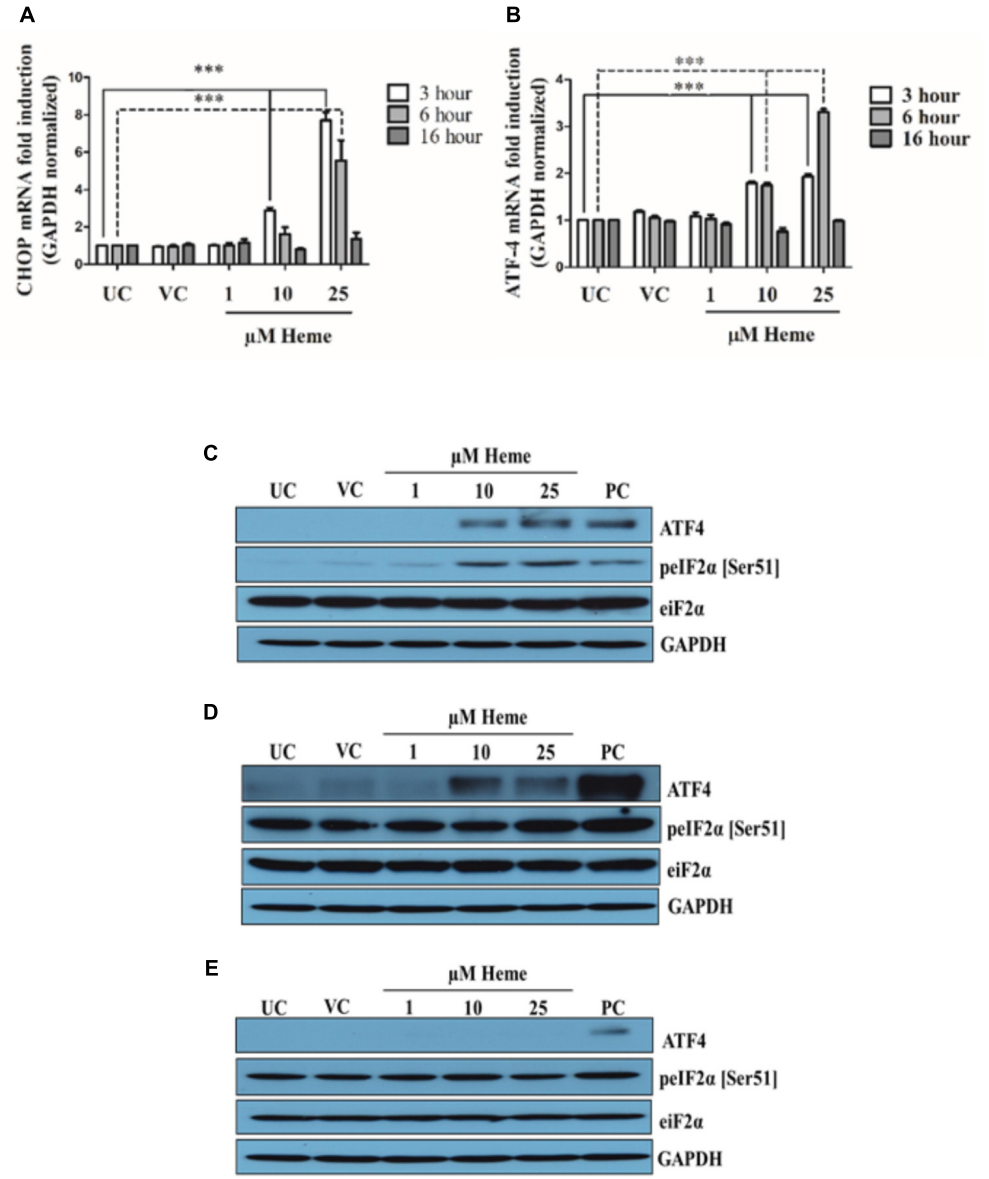

FIGURE 3 | Heme activates the PERK/ATF4/CHOP arm of ER stress in a time- and dose-dependent manner. HAoSMCs were treated with various doses of heme (1,10, and 25 μM) or corresponding vehicle solution to highest heme dose (25 μM) in serum-free DMEM for 60 min, then medium was changed to DMEM with 10% FCS and antibiotics. ER stress markers were measured after 3, 6, or 16 h. Thapsigargin (1 μM) treated cells were used as positive control **(A–E)**. **(A,B)** Relative expressions of CHOP **(A)** and ATF4 **(B)** mRNA levels were determined by qRT-PCR, normalized to GAPDH and compared to the untreated controls at each time points. UC, untreated control; VC, vehicle control; PC, positive control, thapsigargin treated. Results are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.001$. **(C–E)** Representative Western blots of whole cell lysates from five independent experiments are shown representing eIF-2 phosphorylation and ATF4 protein levels **(C)** 3, **(D)** 6, and **(E)** 16 h after the heme treatment. UC, untreated cells; VC, vehicle control cells; PC, positive control cells.

of ER stress as well as a remarkable chaperone that is itself up-regulated during ER stress. Therefore, we next examined whether or not Grp78 responded to heme. Heme treatment increased the expression of Grp78 mRNA 3 h after heme exposure (**Figure 6A**). Importantly, a trend in Grp78 mRNA induction was apparent even in response to low dose heme (1 μM) within 3 h (**Figure 6A**). Grp78 mRNA levels gradually rose up to 6 h after treatment when HAoSMCs were exposed to either 10 or 25 μM of heme (**Figure 6A**). Grp78 mRNA levels returned to control levels after 16 h unless cells had been treated with 25 μM heme in which case Grp78 protein remained significantly increased ($p \leq 0.05$). Accordingly, Grp78 protein levels were considerably up-regulated when cells were treated with heme (10 and 25 μM) compared to control cells (**Figures 6B–D**). Notably, Grp78 protein levels for heme treatments and vehicle controls did not differ 3 h after the heme exposure showing an induction phase of stress adaptation (**Figure 6B**). Parallel with Grp78 mRNA induction, a robust increase in Grp78 protein levels

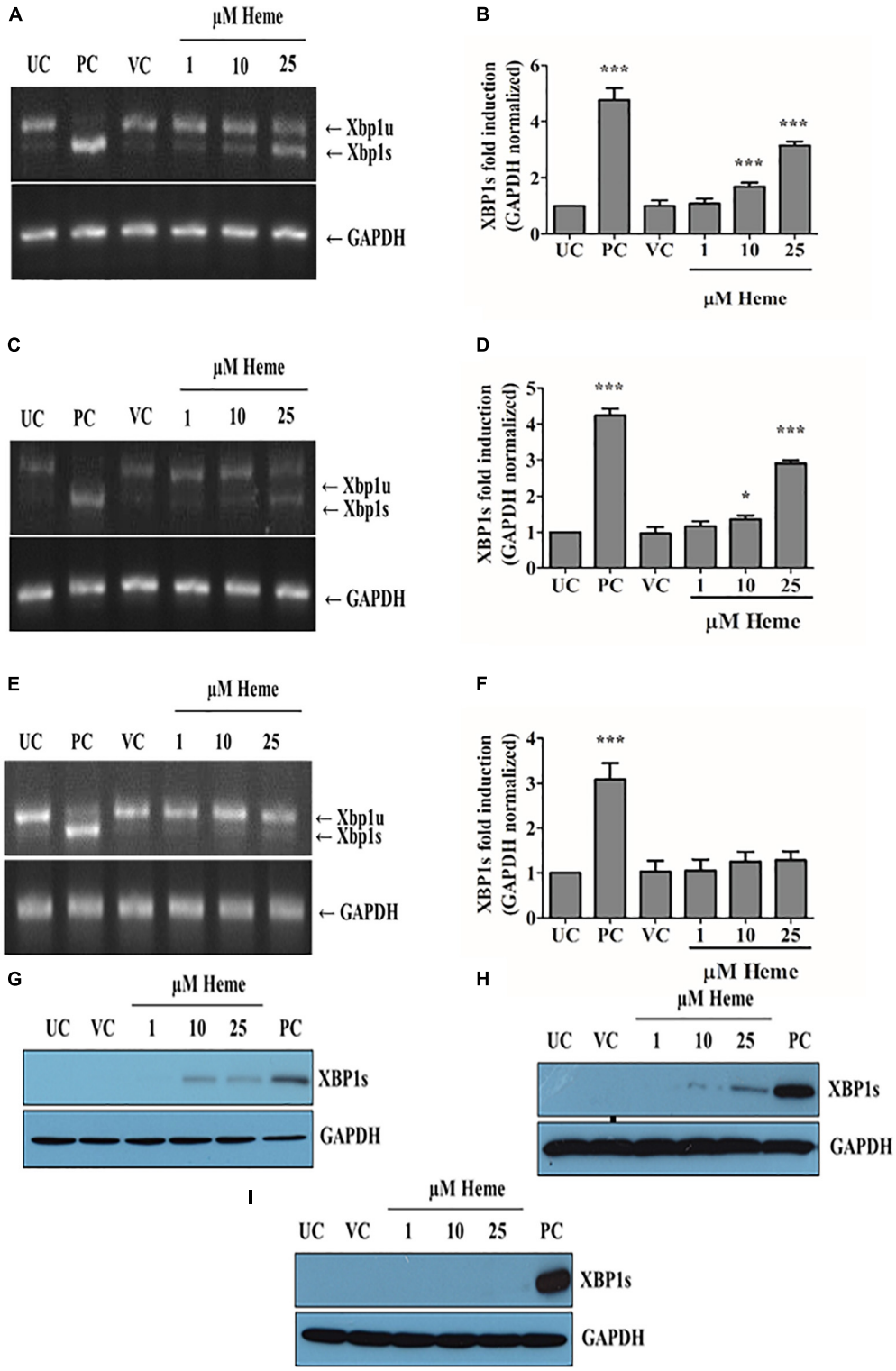

FIGURE 4 | Heme activates the IRE1/XBP1 arm of ER stress in a time- and dose-dependent manner. Expression of spliced XBP1 (XBP1s) was measured with RT-PCR followed by densitometric analysis of XBP1s after running PCR products on 2% agarose gel. XBP1s levels were normalized to GAPDH. **(A,B)** XBP1s levels measured 3, **(C,D)** 6, and **(E,F)** 16 h after the heme challenge. Agarose gels **A,C,E** show one representative image of five independent experiments performed in duplicates. Graphs **B,D,F** are presented as mean ± SD of five independent experiments performed in duplicates. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to untreated cells. **(G–I)** Representative immunoblots of five independent experiments showing XBP1s expression **(A)** 3, **(B)** 6, and **(C)** 16 h after the heme exposure. UC: untreated control; VC: vehicle control; PC: positive control, thapsigargin treated cells.

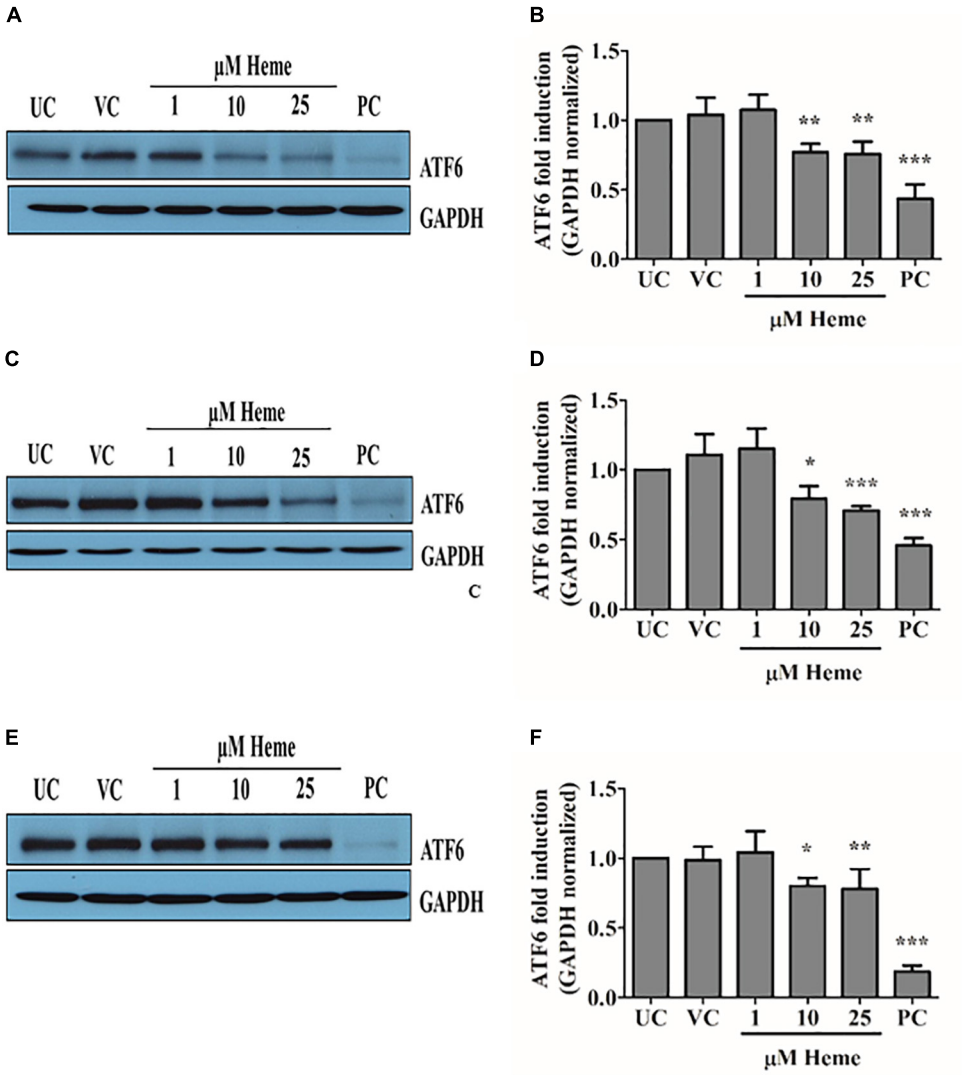

FIGURE 5 | Heme activates ATF6 arm of ER stress in a time- and dose-dependent manner. Proteolytic activation of ATF6 was investigated using Western blot and quantified by densitometry **(A–F)**. **(A,B)** ATF6 levels measured 3, **(C,D)** 6, and **(E,F)** 16 h after the heme challenge. Western blots **(A,C,E)** show one representative image of five independent experiments. Graphs **(B,D,F)** are presented as mean ± SD of five independent experiments performed in duplicates. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to untreated cells.

were detected 6 and 16 h after HAoSMCs were exposed to either 10 or 25 μM of heme (**Figures 6B–D**). Our results support that the ER chaperone Grp78 responds to heme stress. Thapsigargin-treated cells showed a more robust induction Grp78 (data no shown).

Heme Markedly Increase Heme Oxygenase 1 (HO1) and Ferritin Heavy Chain Expression in HAoSMCs

To confirm and monitor the intracellular effect of heme and its catabolism, we assessed the expression of HO1 and the translation of ferritin heavy chain. As expected, heme markedly increased HO1 mRNA as well as protein levels and also the iron release from heme catabolism by HO1 induced ferritin heavy chain levels (**Figures 7A–G**). The extent of HO1 induction is highly correlated with intracellular heme levels in a concentration-dependent fashion, and was apparent in response to 1 μM heme. HO1 and ferritin heavy chain were still elevated 16 h after heme treatment indicating high levels of HO1 and ferritin heavy chain.

The Heme Binding Proteins A1M and Hpx Attenuate the Activation of PERK, IRE1/XBP1, and ATF6 Pathways as Well as Grp78 Induction Protecting Against Heme Induced ER Stress

A1M and Hpx are proteins that protect cells from heme toxicity by acting as extracellular anti-oxidants, sequestering

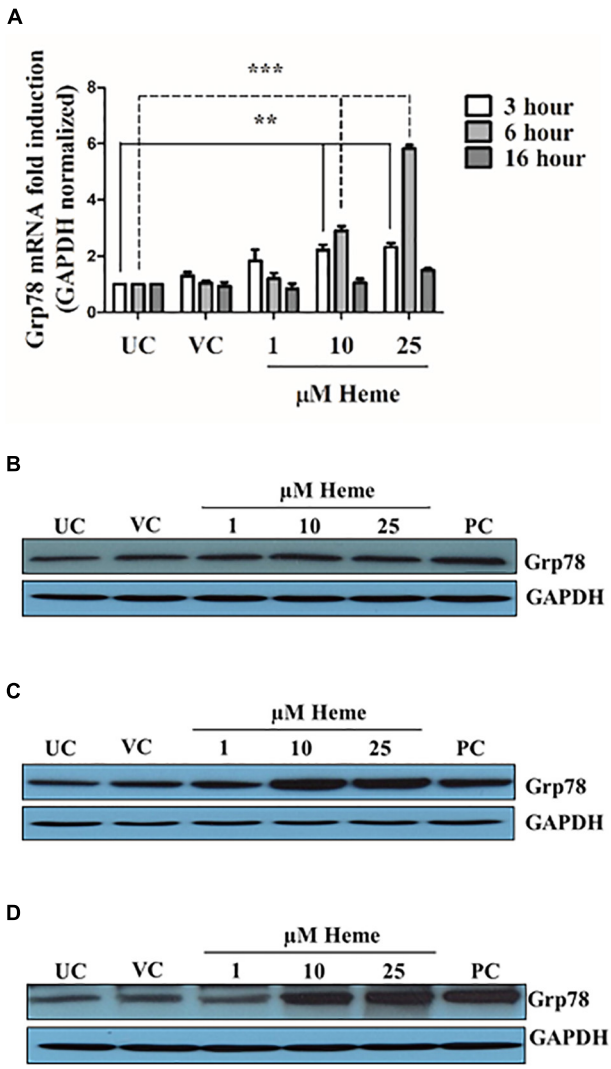

FIGURE 6 | Heme induces Grp78 expression in a time- and dose-dependent manner. Cells were treated either with heme or vehicle as mentioned above. Grp78 expression was measured using qRT-PCR and immunoblot **(A–D)**. **(A)** Relative expressions of Grp78 was determined by qRT-PCR, normalized to GAPDH and compared to the untreated controls at each time points. Results are presented as mean ± SD of five independent experiments performed in duplicates. $^{**}p < 0.01$, $^{***}p < 0.001$. **(B–D)** Representative Western blots of whole cell lysates from five independent experiments are shown representing Grp78 protein levels **(B)** 3, **(C)** 6, and **(D)** 16 h after the heme treatment.

heme and limiting its chemical reactivity. Our next question was whether recombinant A1M (rA1M) and Hpx inhibit the ER stress and UPR pathways induced by heme. For these experiments HAoSMCs were incubated with heme-protein complexes produced by incubating heme (25 μM) with either 12.5 μM of rA1M (a stoichiometric molar ratio of heme: protein of 2:1) or 25 μM of Hpx (a stoichiometric molar ratio of heme: protein of 1:1). We analyzed ATF4, CHOP, Grp78 and HO1 mRNA expression as well as IRE1-mediated XBP1 splicing 3 or 16 h after challenging cells with either heme or the heme-protein complexes. When presented as heme-protein complexes, both rA1M and Hpx completely attenuated ATF4, CHOP, and Grp78 mRNA expression as well as inhibited XBP1 splicing 3 h after the treatment (**Figures 8A–H**, **9A,B**) and markedly decreased HO1 mRNA expression at 3 h (**Figures 9C,D**).

Both rA1M and Hpx prevented the phosphorylation of two early markers of ER stress eIF2a and ATF4 at the protein level (**Figure 10A**). Accordingly, rA1M and Hpx completely inhibited the expression and nuclear translocation of CHOP and proteolysis of ATF6 as demonstrated by immunofluorescence staining of CHOP and densitometric analysis of ATF6 immunoblots, respectively (**Figures 10A,B** and **Supplementary Figure 6**). Furthermore, rA1M as well as Hpx markedly reduced the expression of late ER stress marker Grp78 as shown in **Figure 10C**. At the protein level, when rA1M was in the medium, HO1 and ferritin did not appear to be induced. Surprisingly, the levels HO1 and ferritin heavy chain protein were increased when Hpx was present in the medium. This contrasts with our previous data on endothelial cells, when Hpx prevented HO1 induction (Balla G. et al., 1991). In an earlier *in vivo* work, heme-Hpx is targeted to the liver after intravenous injection in rats (Smith and Morgan, 1979). The scavenger receptor termed low density related lipoprotein receptor (LRP1), that binds heme-Hpx complexes very tightly (Kd ∼4 nM, Hvidberg et al., 2005) *in vitro,* has been reported to be expressed on vascular smooth muscle cells (Okada et al., 1996; Swertfeger et al., 2002; Lillis et al., 2005). Thus, LRP1 on these HAoSMCs may account for some uptake of heme-HPX or, under these experimental conditions, some heme was not bound to Hpx and entered the cells.

Overall, these data show that both rA1M and Hpx attenuate the activation of all ER stress pathways provoked by heme.

N-Acetyl Cysteine Attenuates ROS Generation and ER Stress in Heme-Exposed Cells

It was previously described that heme induces ROS in a number of cell types (Porto et al., 2007; Fortes et al., 2012; Barcellos-de-Souza et al., 2013; Erdei et al., 2018). Therefore, we tested whether heme triggers ROS generation in HAoSMCs (**Figures 11A–F**). As expected, heme (10 and 25 μM) induced ROS in a dose-dependent manner measured by the CM-H2DCFDA assay (**Figure 11A**). Importantly, heme-induced ROS generation was effectively inhibited by either the ROS scavenger NAC or the extracellular heme binding protein hemopexin and A1M (**Figure 11B**). Due to its potent antioxidant properties, NAC was reported to attenuate ROS-mediated ER stress in a number of pathologies (Porto et al., 2007; Fortes et al., 2012; Barcellos-de-Souza et al., 2013; Erdei et al., 2018). Thus, we investigated whether NAC could prevent heme-induced ER stress as well. Our results showed that NAC inhibited ATF4 mRNA expression, significantly but not completely prevented heme-provoked elevation of CHOP mRNA, and decreased the expression of HO1 (**Figures 11C–E**). The ER stress moderating effect

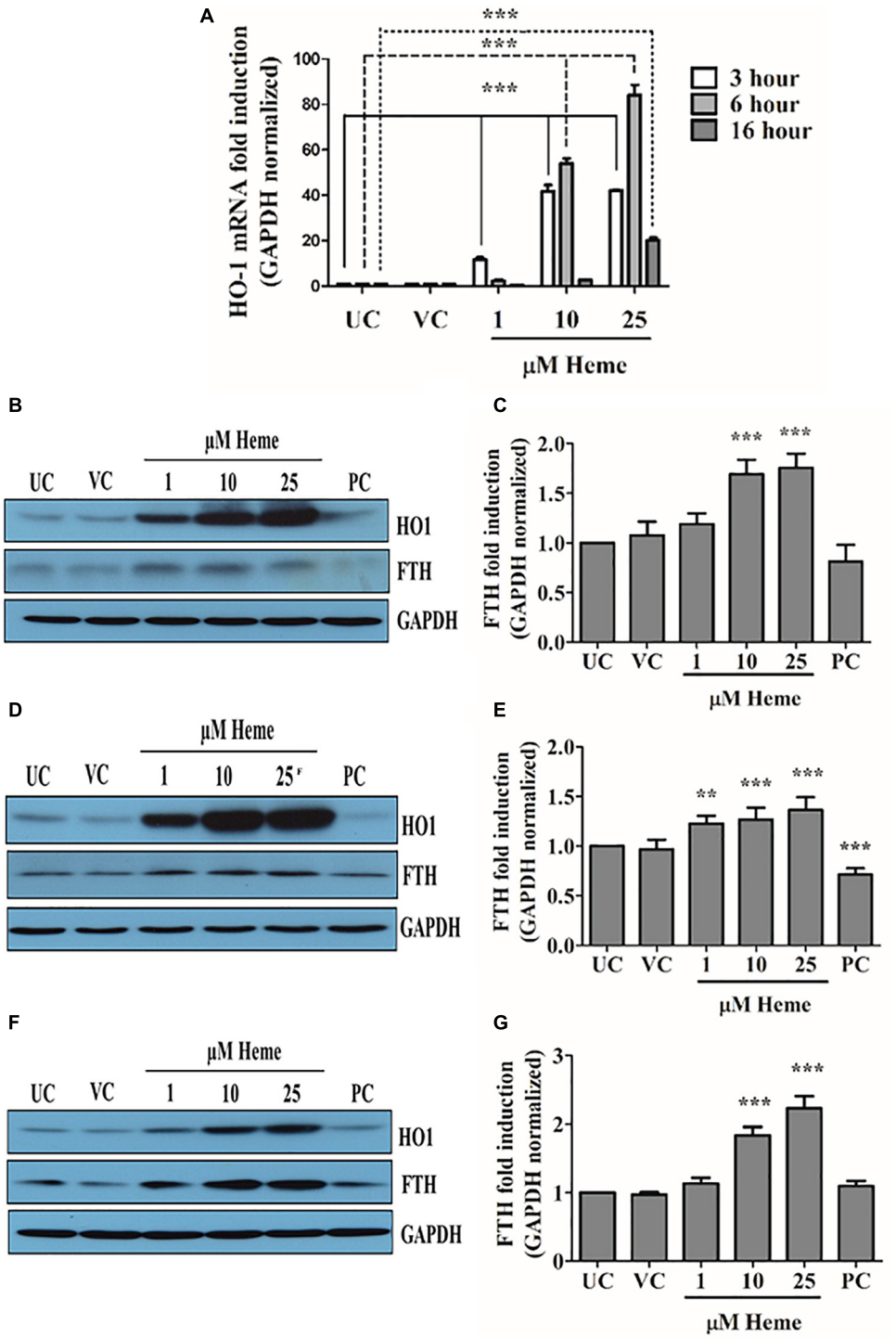

FIGURE 7 | Heme induces marked HO1 and ferritin heavy chain expression in a concentration- and time-dependent manner. Cells were treated either with heme or vehicle as mentioned above. HO1 expression was measured using qRT-PCR and immunoblot, while ferritin heavy chain by immunoblot **(A–G)**. **(A)** Relative expressions of HO1 was determined by qRT-PCR, normalized to GAPDH and compared to the untreated controls at each time points. Results are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.001$. **(B,D,F)** Representative Western blots of whole cell lysates from five independent experiments are shown representing HO1 and H-ferritin (FTH) protein levels **(B)** 3, **(D)** 6, and **(E)** 16 h after the heme treatment. **(C,E,G)** Densitometric analysis of FTH expression **(C)** 3, **(E)** 6, and **(G)** 16 h after the heme exposure. Results are presented as mean ± SD of five independent experiments. FTH expressions are normalized to GAPDH and compared to the untreated controls at each time points. UC, untreated control; VC, vehicle control; PC, positive control, thapsigargin treated. **$p < 0.01$, ***$p < 0.001$.

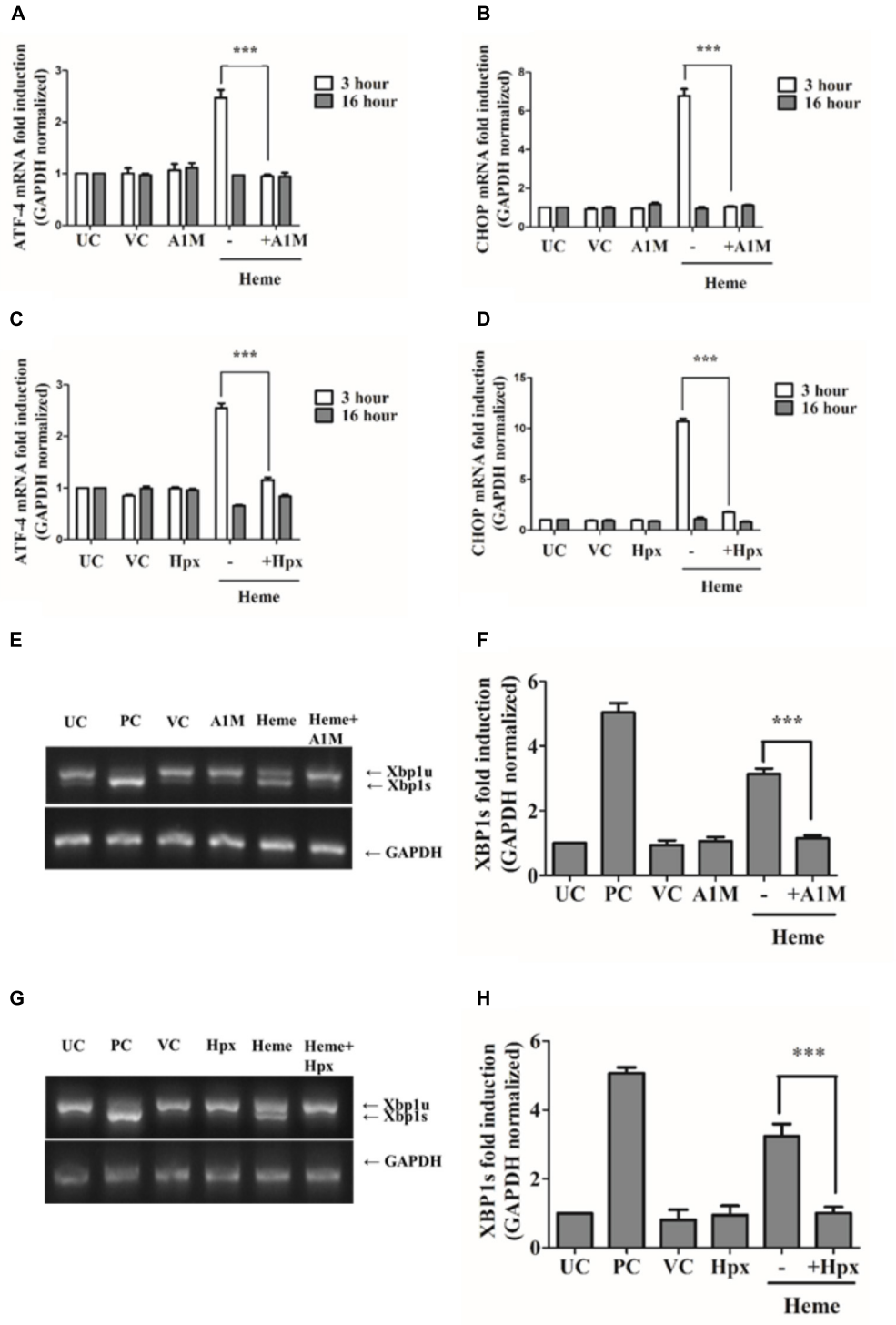

FIGURE 8 | A1M and Hpx attenuates ATF4 and CHOP gene induction as well as IRE1/XBP1s activation. Cells were treated with heme (25 μM) or heme + rA1M (25 μM heme + 12.5 μM rA1M) or equimolar amonts of heme + Hpx (25 μM heme + 25 μM Hpx) for 60 min in serum-free DMEM. Medium was then replaced with DMEM + 10% FCS and antibiotics. ATF4 and CHOP gene induction in response to heme were measured using qRT-PCR 3 and 16 h after the heme challenge **(A–D)**, while XBP1 splicing was assessed 3 h after the heme treatment by RT-PCR followed agarose gel electrophoresis and quantified by densitometry **(E–H)**. **(A–B)** Relative expressions of ATF4 and CHOP in response to heme and heme + rA1M complex were determined by qRT-PCR, normalized to GAPDH. ATF4 and CHOP levels of rA1M + heme treated cells were compared to those detected in heme challenged cells. Results are presented as mean ± SD of five independent

(Continued)

FIGURE 8 | Continued
experiments performed in duplicates. ***$p < 0.0001$ using unpaired *t*-test **(C–D)** Relative expressions of ATF4 and CHOP in response to heme and heme + Hpx complex were determined by qRT-PCR, normalized to GAPDH. ATF4 and CHOP levels of Hpx + heme treted cells were compared to those detected in heme challenged cells. Results are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.0001$ using unpaired *t*-test. **(E–F)** Cells were treated with heme or heme + rA1M as described above. Expression of spliced XBP1 (XBP1s) was measured 3 h after the treatment with RT-PCR followed by densitometric analysis of XBP1s after running PCR products on 2% agarose gel. XBP1s levels were normalized to GAPDH. Results are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.0001$ using unpaired *t*-test. **(G,H)** Cells were treated with heme or heme + Hpx as described above. Expression of spliced XBP1 (XBP1s) was measured 3 h after the treatment with RT-PCR followed by densitometric analysis of XBP1s after running PCR products on 2% agarose gel. XBP1s levels were normalized to GAPDH. Results are presented as mean ± SD of five independent experiments. ***$p < 0.0001$ using unpaired *t*-test.

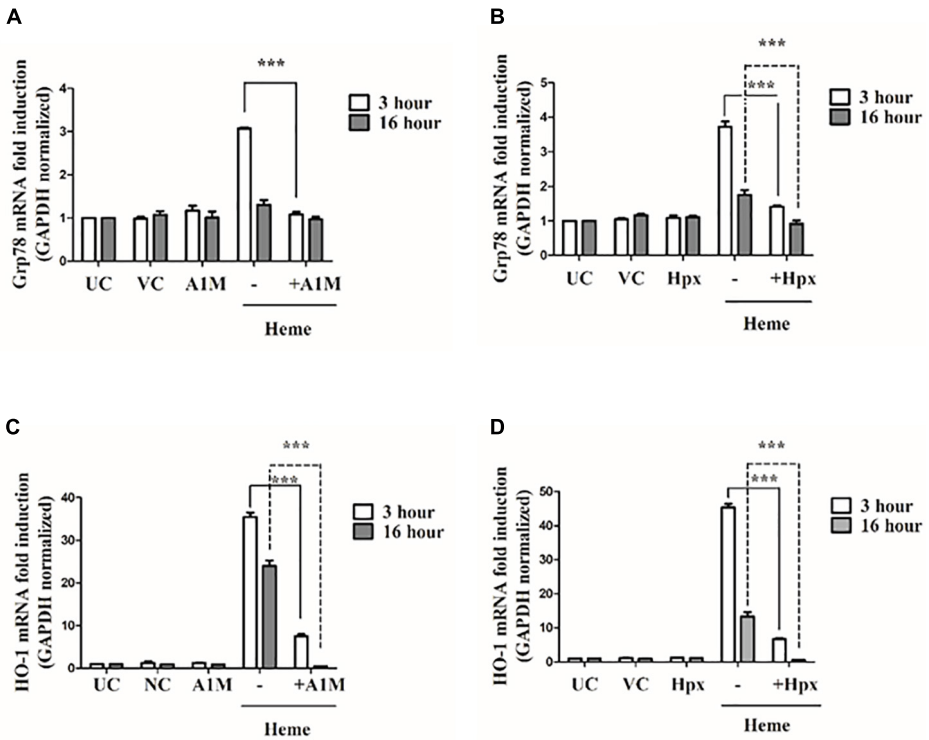

FIGURE 9 | A1M and Hpx attenuates Grp78 and HO1 gene induction. Cells were treated with heme or heme + rA1M or equimolar amonts of heme + Hpx as described in the legend to **Figure 8**. Grp78 and HO1 expression was measured using qRT-PCR 3 and 16 h after the treatment **(A–D)**. **(A)** Relative expressions of Grp78 in response to heme and heme + rA1M complex were determined by qRT-PCR, normalized to GAPDH. Grp78 levels of rA1M + heme treated cells were compared to those detected in heme challenged cells. Results are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.0001$ using unpaired *t*-test **(B)** Relative expressions of Grp78 in response to heme and heme + Hpx complex were determined by qRT-PCR, normalized to GAPDH. Grp78 levels of Hpx + heme treted cells were compared to those detected in heme challenged cells. Results are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.0001$ using unpaired *t*-test. **(C)** Relative expressions of HO1 in response to heme and heme + rA1M complex were determined by qRT-PCR, normalized to GAPDH. HO1 levels of rA1M + heme treated cells were compared to those detected in heme challenged cells. Results are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.0001$ using unpaired *t*-test. **(D)** Relative expressions of HO1 in response to heme and heme + Hpx complex were determined by qRT-PCR, normalized to GAPDH. HO1 levels of Hpx + heme treted cells were compared to those detected in heme challenged cells. Results are presented as mean ± SD of five independent experiments. ***$p < 0.0001$ using unpaired *t*-test.

of NAC was pronounced at protein level as well, since NAC inhibited the heme-induced activation of ER stress pathways analyzed by immunoblot (**Figure 11F**). In addition, NAC decreased HO1 protein levels in heme-treated cells as well and was able to significantly, but not completely reduce the expression and nuclear translocation of CHOP by heme (**Supplementary Figure 7**). These data suggest that heme induced ROS generation can be effectively prevented either by capturing heme in the extracellular space by rA1M or Hpx or by the antioxidant NAC. By lowering ROS generation, NAC was able to inhibit heme-induced ER stress as well.

Determination of Cell Viability of HAoSMCs in Response to Heme

Next, we analyzed whether heme induced ER stress triggers cell death in HAoSMCs. Cells were treated with various doses of heme (1, 10, and 25 μM, respectively) and cell viability was tested by MTT assay (**Supplementary Figure 8**). Thapsigargin (1 μM)

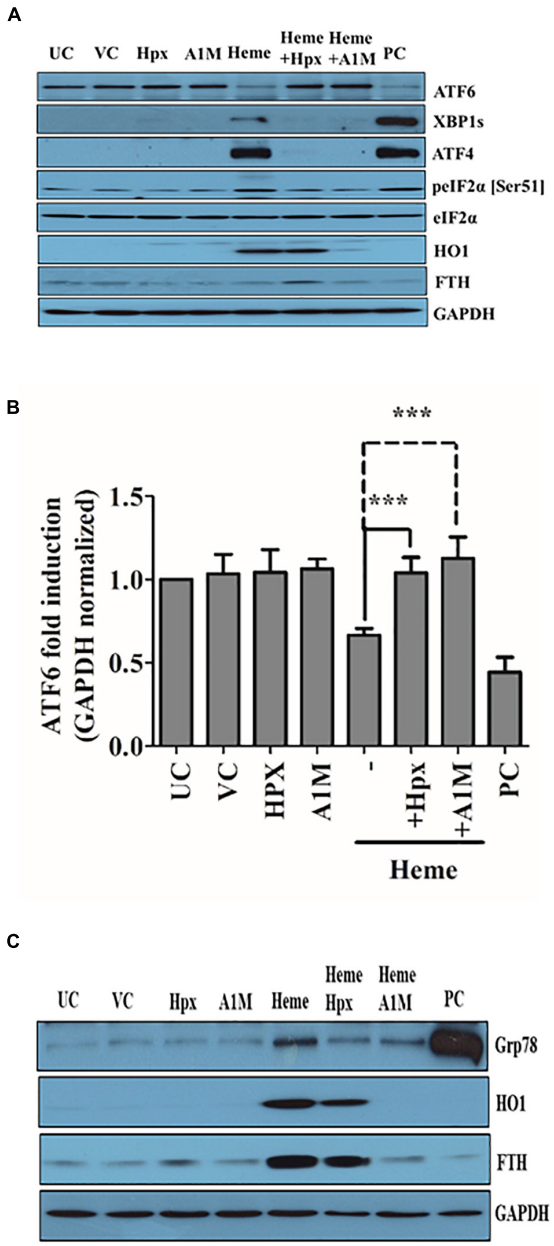

FIGURE 10 | A1M and Hpx attenuates the activation of PERK and ATF6 pathways. Cells were treated with heme or heme + rA1M or equimolar amonts of heme + Hpx as described above. Early ER stress markers (phosphorylated eIF2α, ATF4, and ATF6) were measured with immunoblot, while CHOP was assessed by immunofluorescence 3 h after the treatments **(A–C)**. Heme stress marker HO1 and FTH were measured 3 and 16 h after the treatment. **(A)** Representative Western blots of whole cell lysates from five independent experiments are shown representing ATF6 proteolysis, eIF2α phosphorylation and subsequent ATF4 induction as well as HO1 and FTH expression. **(A)** Analysis of CHOP expression and nuclear translocation in response to heme and heme + rA1M or heme + Hpx treatment. **(B)** Graph shows ATF6 proteolysis induced by heme or heme + rA1M or heme + Hpx assessed by densitometric analysis of ATF6 immunoblots. Results are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.0001$ using unpaired *t*-test. **(C)** Representative Western blots of whole cell lysates from five independent experiments are shown representing Grp78, HO1, and FTH inductions 16 h after the treatment.

treated cells were used as positive control for ER stress. Our data showed that neither heme nor the ER stressor thapsigargin induced cell death in our experimental conditions.

Heme Decreases the Expression of Transforming Growth Factor β (TGFβ) and Collagen Type I in HAoSMCs

TGFβ and its downstream product collagen type I plays a pivotal role in the maintenance and repair of the protective fibrous cap in atherosclerotic plaques. Therefore, we next investigated whether heme induced ER stress affects TGFβ and subsequent collagen type I expression. Cells were treated with heme (25 μM) or thapsigargin (1 μM) then TGFβ and collagen type I mRNA expressions were quantified *via* qPCR 3, 6, and 16 h after the treatments. Our results showed that heme (25 μM) as well as thapsigargin (1 μM) significantly inhibited both TGFβ and collagen type I mRNA expressions at 6 and 16 h after treatments (**Figures 12A,B**). Both thapsigargin and heme increased collagen type I mRNA expression at 3 h after heme or thapsigargin challenge (**Figure 12B**). These data suggest that heme effect on fibrotic gene expression are biphasic, and both heme and thapsigargin markedly reduce fibrotic gene expression after an initial induction phase.

DISCUSSION

There is increasing evidence for a link between vascular diseases and ER stress as well as between atherosclerosis and oxidative stress (Ivanova and Orekhov, 2016; Hong et al., 2017; Kattoor et al., 2017; Yang et al., 2017). Several reports have demonstrated that ER stress and UPR are involved in cardiovascular diseases and multiple risk factors of atherosclerosis such as free cholesterol, 7-ketocholesterol, phospholipolyzed LDL or oxidized LDL have been described to trigger ER stress in macrophages, vascular endothelial cells, and HAoSMCs (Pedruzzi et al., 2004; Kedi et al., 2009; Sanson et al., 2009; Gora et al., 2010; Yao et al., 2010; Chistiakov et al., 2014).

The purpose of our research was to assess how heme, a known risk factor for atherosclerosis, contributes to the pathology of this complex and prevalent cardiovascular disease.

Heme is an amphipathic iron-protoporphyrin IX molecule that is, in part, responsible for human pathologies including atherosclerosis (Andrade et al., 2010; Larsen et al., 2010; Nagy et al., 2010; Nath and Katusic, 2012; Frimat et al., 2013; Elphinstone et al., 2016). It was previously reported that heme may mediate the oxidative degradation of proteins (Aft and Mueller, 1984; Vincent, 1989). A recent study published by our research group showed that the protein titin, responsible for the passive elasticity of muscle and in the contraction of striated muscle tissue, is sensitive to heme driven oxidation, can be involved in the increase of the passive force of myocardium driven by heme triggered oxidative protein modifications (Alvarado et al., 2015). Heme, which is also capable of sensitizing vascular endothelial cells toward ROS, and moreover, catalyzes the oxidative modification of LDL to oxLDL, represents an important risk factor of vessel wall injury (Balla G. et al., 1991;

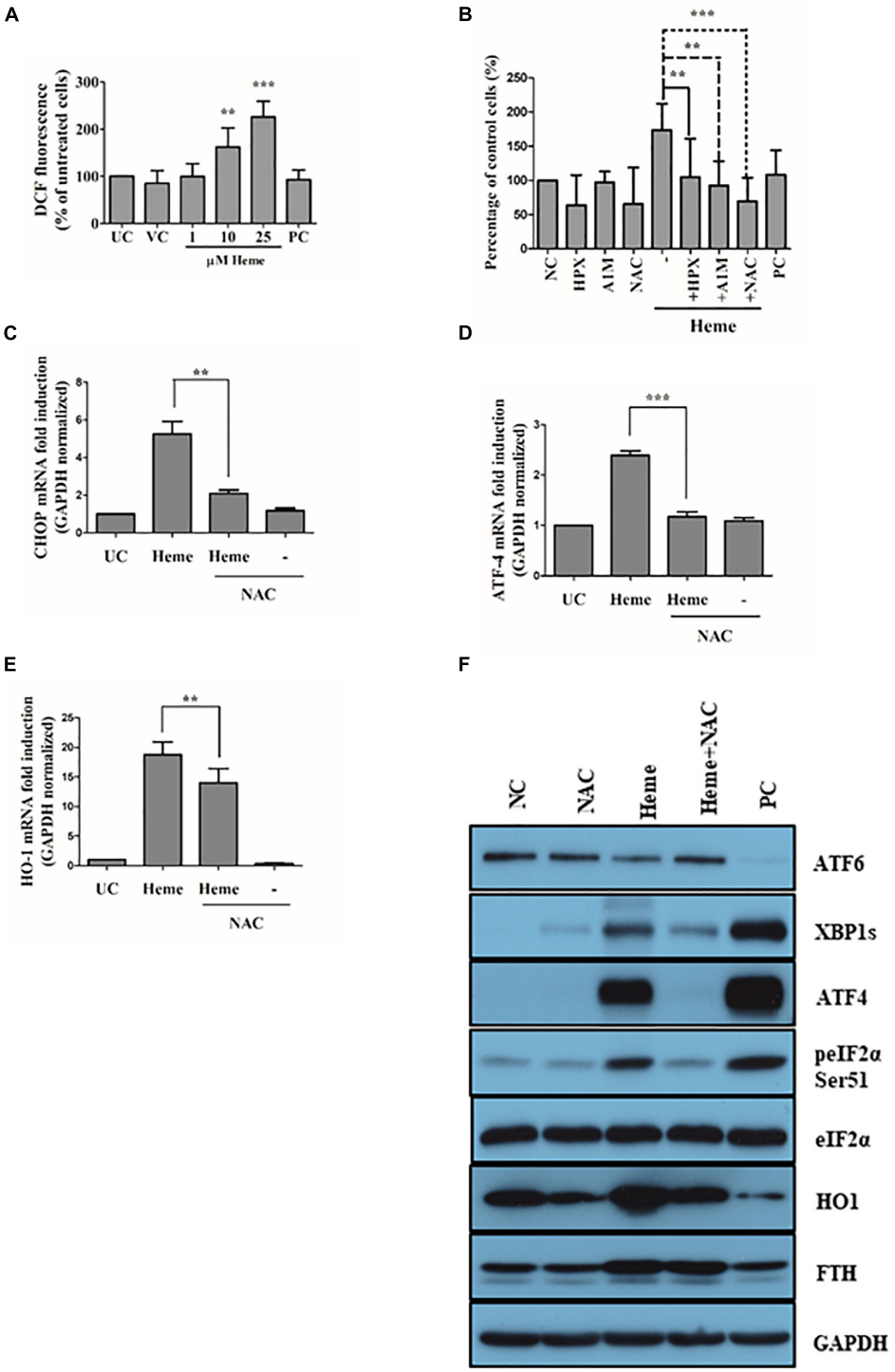

FIGURE 11 | Heme-generated ROS is involved in heme-induced endoplasmic reticulum (ER) stress. Cells were treated with heme (25 μM) alone or heme (25 μM) + rA1M (12.5 μM) or heme (25 μM) + Hpx (25 μM) or heme (25 μM) + N-acetyl cysteine (NAC; 10 mM) as described for **Figure 8** followed by the analysis of ROS generation and ER stress marker expression **(A–F)**. **(A)** Heme induced ROS generation was assessed by CM-H2DCFDA assay. Results are presented as mean ± SD of five independent experiments. ***$p < 0.0001$. **(B)** Inhibition of heme-induced ROS generation by Hpx, rA1M and NAC using CM-H2DCFDA assay. Results are presented as mean ± SD of five independent experiments. ***$p < 0.0001$ **(C–E)** Relative expressions of CHOP, ATF4 and HO1 in response to heme and heme + NAC were determined by qRT-PCR, normalized to GAPDH. mRNA levels of heme + NAC treted cells were compared to those detected in heme challenged cells. Results are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.0001$ using unpaired *t*-test. **(F)** Representative Western blots of whole cell lysates from five independent experiments are shown representing ATF6 proteolysis, XBP1s expression, eIF2α phosphorylation and subsequent ATF4 induction as well as HO1 and FTH expression.

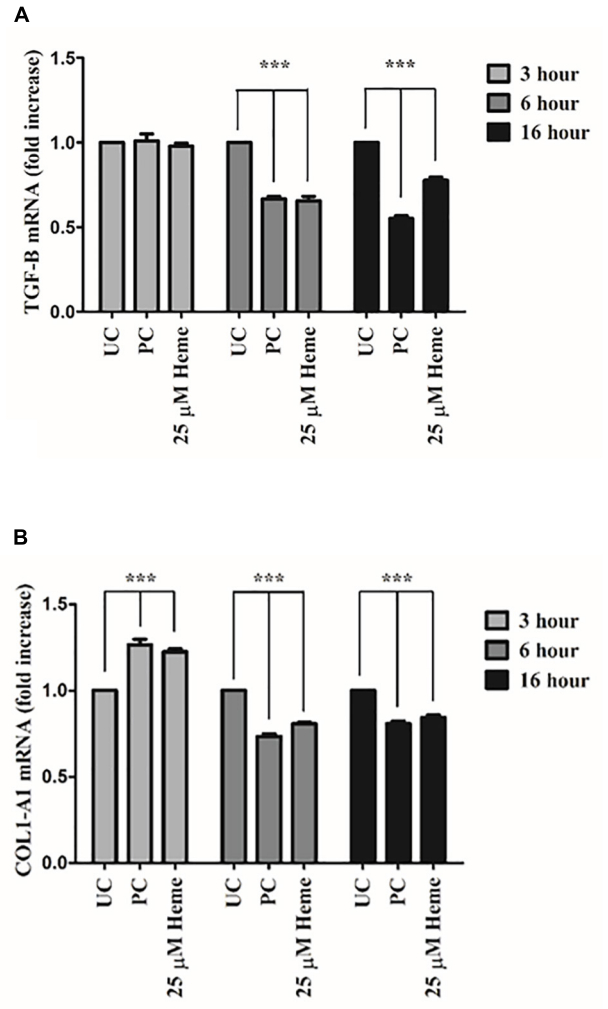

FIGURE 12 | Heme attenuates TGFβ and collagen type I expression in HAoSMCs. Cells were treated with heme (25 μM) or thapsigargin (1 μM) as described for **Figure 3** and TGFβ and collagen type I mRNAs were quantified by qRT-PCR **(A,B)**. Relative expressions of TGFβ **(A)** and collagen type I **(B)** mRNA levels were determined by qRT-PCR, normalized to GAPDH and compared to the untreated controls at each time points. UC: untreated control; PC: positive control, thapsigargin treated. Graphs **(A,B)** are presented as mean ± SD of five independent experiments performed in duplicates. ***$p < 0.001$ compared to untreated cells.

Jeney et al., 2002). Great number of observations confirmed the presence of ROS injuries in atherosclerotic plaques, and intriguingly, heme together with cell-free heme proteins are also present in human vascular wall samples (Jeney et al., 2002, 2014; Balla et al., 2005; Nagy et al., 2010). Hemoglobin interacts with lipid particles triggering further lipoprotein modifications and hemoglobin oxidation. The main hemoglobin derivative is methemoglobin readily capable to release its heme moieties to the surrounding extracellular space (Nagy et al., 2010; Jeney et al., 2014).

The focus of this study was to explore the role of heme in ER stress in HAoSMCs, since these cells are central players of vascular diseases. They mainly exist in a contractile phenotype regulating vascular tone and express a variety of HAoSMC-specific contractile markers, including α-smooth muscle action (SMA) and calponin (Owens, 1995). However, in response to injury, HAoSMCs can undergo a phenotypic switch that is characterized by reduced contractile marker expression and increased proliferation (Rensen et al., 2007). This conversion plays a pathophysiologic role in the development of atherosclerosis (Rzucidlo et al., 2007). However and alternatively, it might be beneficial during atherogenesis to compensate for the loss of HAoSMC due to apoptosis and subsequent inflammation (Bennett et al., 2016). Thus, a considerable body of evidence suggests that HAoSMCs are key players in vascular diseases, which makes them an ideal target to examine the potential role of heme-induced ER stress in atherosclerosis.

Here, we show for the first time that heme induces ER stress and the UPR in HAoSMCs. The different arms of ER stress were followed after heme treatment in a series of cell culture experiments. Our results support that heme activated all three ER stress responsive branches: PERK-eIF2α, IRE1α-XBP1, and ATF6 pathways. Specifically, HAoSMC cultures responded to heme by the activation of ATF6 and IRE1α-XBP1 pathways as well as through an increase in eIF2α phosphorylation and subsequent ATF4 induction *via* the PERK pathway. Increased levels of Grp78 as well as ATF6 activation in response to heme suggest a triggering of the adaptive phases of the cellular stress response. No cytotoxicity was detected in response to heme via MTT assay.

Phosphorylation of eiF2α and ATF4 expression in response to heme possibly indicate that heme triggered oxidative stress together with ER stress in HAOSMCs. ATF4 is a Janus faced factor, since ATF4-mediated integrated stress response, initiated by eIF2α phosphorylation, has been shown to protect cells against oxidative stress (Harding et al., 2003). These observations suggest that ATF4, a marker of ER stress and UPR, might be directly involved in the heme stress response. These results support our hypothesis that heme-induced ROS and oxidative stress are involved in heme-induced ER stress. Heme was reported to enhance the generation of ROS in a variety of cell types which might be involved in heme-related pathologies (Porto et al., 2007; Fortes et al., 2012; Barcellos-de-Souza et al., 2013; Erdei et al., 2018). One of the major targets of ROS are proteins leading to excessive protein modifications with often detrimental consequences for protein structure and hence function (Berlett and Stadtman, 1997; Ehrenshaft et al., 2015; Davies, 2016). We heve demonstrated that heme does induce ROS formation in a concentration-dependent manner in HAoSMCs. Furthermore, by decreasing the ROS level, NAC markedly inhibited heme-induced ER stress in HAoSMCs showing that ROS formation contributed to ER stress triggered by heme.

ATF4 subsequently activates CHOP, a pro-apoptotic protein (Oyadomari and Mori, 2004). Our results on CHOP induction in response to heme correlates well with studies reporting activation of PERK/eIF2α/ATF4/CHOP pathway in a response to oxidized LDL, which, like heme, is considered a pathological factor of atherosclerosis (Hong et al., 2014; Tao et al., 2016). In addition, a strongly positive correlations between CHOP expression and

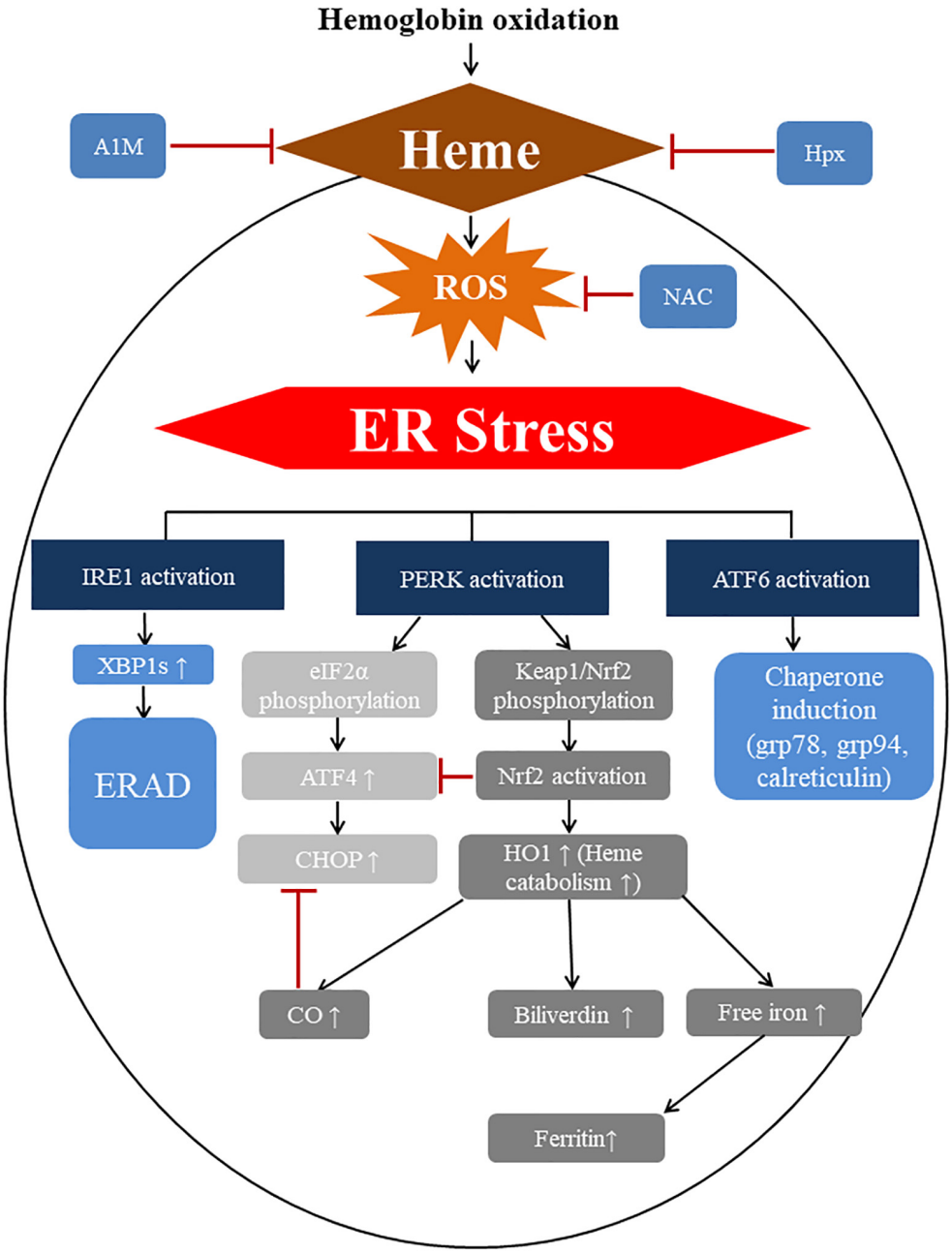

FIGURE 13 | Mechanism of heme induced ER stress with possible therapeutic interventions. Heme released from oxidized hemoglobin enters into cells across the plasma membrane (by unknown mechanisms) possibly *via* passive diffusion or transporters (remains to be defined) and induces ROS intracellularly with subsequent protein oxidative damage that leads PERK activation, phosphorylation of eIF2α with subsequent ATF4 as well as CHOP induction and nuclear translocation. Oxidation of Keap1 releases Nrf2, which PERK is known to phosphorylate Keap1/Nrf2 followed by nuclear translocation of Nrf2, which interacts with various targets and possibly influences CHOP expression directly as well as indirectly. For example, Nrf2 might directly inhibit ATF4 binding to CHOP promoter thereby inhibiting ATF4-mediated CHOP expression. Nrf2 also induces HO1, one of the key heme responsive genes. HO1 decreases the heme burden of cells and releases heme catabolites such as carbon-monoxide (CO), biliverdin (which is converted to the antioxidant bilirubin by biliverdin reductase in the cytosol), and iron (Fe^{2+}). CO might inhibit CHOP expression, which suggests that Nrf2 may negatively influence CHOP in an indirect, HO1-mediated way as well. Activation of XBP1s through IRE1 in response to heme possibly induces ER-associated protein degradation (ERAD), which decreases misfolded protein loads and reduces unfolded protein burden of ER. Heme-induced activation of ATF6 also facilitates chaperone expression like Grp78 increasing the folding capacity of ER. Thus, heme potentially activates both pro-apoptotic and pro-survival ER responses, however, our data support that the pro-apoptotic response is only transitory, while pro-survival ones (ATF6, Grp78) are permanently activated. The observed marked induction of heme responsive genes as well as ER stress responsive genes suggest that these pathways interact with each other to cope with heme-induced stress. Finally, capturing heme in the extracellular space by the heme binding proteins alpha-1-microglobulin and hemopexin or decreasing reactive oxygen species level by NAC attenuate heme-induced ER stress.

the progression of atherosclerosis in human coronary arteries and carotid lesions as well as ApoE$^{-/-}$ mice have been reported during the past few years (Myoishi et al., 2007; Thorp et al., 2009; Tsukano et al., 2010). Several lines of evidence suggested that IRE1/XBP1 activation are likely involved in vascular diseases (Zeng et al., 2009; Liberman et al., 2011). We observed after HAoSMCs were incubated with heme that the IRE1/XBP1 pathway was activated, but only transiently. We propose that XBP1 activation might act as a pro-survival signal for HAoSMCs through the activation of ER-associated degradation (ERAD) to reduce misfolded protein loads. In addition, the adaptive ER stress response involves initiation of the ATF6 arm, which induces a number of chaperones such as Grp78, Grp94, and calreticulin (Okada et al., 2002). One potential beneficial effect of ATF6 activation is supported by a recent study showing that ATF6 decreases myocardial ischemia/reperfusion damage (Jin et al., 2017). The sustained activation of ATF6 pathway observed in response to heme suggests that ATF6 might facilitate the recovery from ER stress. Grp78, the master regulator of ER stress, is a pro-survival factor and a representative chaperone of adaptive ER stress response (Rutkowski et al., 2006). Under oxidative stress Grp78 could be protective (Yu et al., 1999; Jiang et al., 2017). Here, we revealed that heme induces the sustained and robust up-regulation of Grp78. Therefore, our data support that Grp78 together with other chaperones, possibly induced by ATF6, could potentially contribute to cell survival during heme-induced ER stress, depending upon the levels of heme.

The protective HO1/ferritin system is induced for heme catabolism that releases Fe(II) in cells in response to heme and hemoglobin (Balla J. et al., 1991; Vercellotti et al., 1994). Thus, as expected we detected a robust induction of HO1 and ferritin in HAoSMCs after incubation with heme. Significantly, our data show that high levels of heme can occur in localized hemolytic conditions in atherosclerosis. ER stress develops in spite of the normally protective response of proteins involved in heme trafficking to HO1 in the ER. Intriguingly, several lines of evidence support that there is a direct interaction between HO1, the key player of heme stress, and PERK, a representative kinase of canonical in ER stress. In the cytosol, Nrf2, which is a key transcriptional activator of the HO1 gene, is a direct substrate of PERK. Thus, upon ER stress Nrf2 is released from Keap1 in the cytosol and phosphorylated by PERK. Phospho-Nrf2 then translocates into the nucleus where it acts to induce a number of stress responsive genes in addition to HO1, such as glutathione S-transferase and the rate limiting enzyme in glutathione biosynthesis, glutamylcysteine synthetase (Venugopal and Jaiswal, 1996; Wild et al., 1999; Nguyen et al., 2000; Cullinan et al., 2003; Loboda et al., 2016). Activation of this Nrf2 pathway is independent of eIF2α phosphorylation and promotes cell survival upon, and also after, ER stress (Cullinan et al., 2003; Cullinan and Diehl, 2004). These findings demonstrate that PERK initiates two parallel ways to cope with ER stress, one of which (HO1/ferritin) is also at least part of the traditional pathway of heme stress. Interestingly, there are multiple links between Keap1/Nrf2/HO1 and PERK pathways, since Nrf2 and the downstream protein of PERK arm, ATF4, share certain gene targets (Thimmulappa et al., 2002; Kwak et al., 2003; Lee et al., 2003) including CHOP, a key regulator of ER stress. Nrf2 inhibits CHOP expression, whereas ATF4 promotes it (Harding et al., 2000; Ma et al., 2002; Cullinan and Diehl, 2004). Certain regulatory effects may be cell type specific. Zong and collaborators demonstrated that Nrf2 affected CHOP expression by modulating the binding of ATF4 to the CHOP promoter in thyroid cancer cells, and the accumulation of Nrf2 negatively regulated CHOP induction (Zong et al., 2012). These authors concluded that Nrf2 might inhibit the binding of ATF4 to CHOP promoter.

Another possible interaction between the effects of raising intracellular heme levels/heme stress and ER stress is the key enzyme that lowers intracellular heme, HO1, which catalyzes heme degradation to carbon monoxide, biliverdin (which is converted to bilirubin) and free Fe(II) iron. Liu et al. (2005) suggested that HO1 may inhibit CHOP transcription by modulating the level of carbon monoxide. HO1 is known to be induced during ER stress triggered by various perturbants such as brefeldin A, thapsigargin, and homocysteine. Apoptosis provoked by these stressors/stresses was potentiated by HO1 inhibition, which shows that HO1 contributes to cell survival during ER stress (Liu et al., 2005). Significantly, these published data support a mechanism whereby heme-induced ER stress can rescue cells from CHOP-mediated cells death. This would take place by a direct inhibition of ATF4 binding to CHOP promoter and by an HO1-related carbon monoxide mediated way. Our current understanding on HIER stress in summarized in **Figure 13**.

There are extracellular protective agents against heme-induced ER stress. For example, the potential therapeutic efficacy of the heme binding protein Hpx against heme toxicity has been shown in patients (Janz et al., 2013; Schaer et al., 2013; Jung et al., 2015). Also, there are animal models of various human diseases such as severe sepsis as well as hemolytic mice models where heme mediated damages are pronounced (Larsen et al., 2010; Vinchi et al., 2013; Smith and McCulloh, 2015). Moreover, Hpx prevents macrophage inflammatory activation which can contribute to chronic tissue injury in hemolytic disorders (Vinchi et al., 2016). In our present study Hpx completely prevented ER stress induced by heme. Also, the protective effect of Hpx against heme-catalyzed cytotoxicity in human and bovine endothelial cells was previously demonstrated by us when heme uptake and heme potentiated H_2O_2- and polymorphonuclear leukocyte-mediated cytotoxicity were completely blocked when Hpx was added simultaneously and levels that stoichiometrically bound heme (Balla G. et al., 1991). Here, we found that Hpx acting as an extracellular antioxidant significantly inhibited heme induced ER stress but not HO1/ferritin expressions, which may be due in part to expression of the heme-Hpx binding protein LRP1 The cytoprotective effect of Hpx is reinforced by a study on neuronal cells, where free heme decreased cell survival, while heme complexed with Hpx did not trigger cell death even at supraphysiological concentrations (Li et al., 2009). After endocytosis, heme-Hpx complexes induce HO1 expression in mouse hepatoma, in HL60 cells as well as in mouse primary neurons, where data showed that HO1 expression is required

for Hpx-mediated cytoprotection (Alam and Smith, 1989; Alam et al., 1999). *In vivo* studies in rats implicated the liver as the main organ for uptake of heme-hemopexin complexes (Smith and Morgan, 1978). However, it is worth mention that the Hpx bound heme in phosphate buffered saline (PBS pH 7.4) *in vitro* to ∼ 100% after addition of 1 molar equivalent of stock mesoheme in DMSO. The efficiency of heme binding generally the same for both proto- and meso-heme. Thus, it is unexpected to see HO1 and ferritin induction that would indicate and excess of heme in the mixture in the cell experimental medium of heme and Hpx. Because the Hpx preparation was determined to bind heme in PBS *in vitro* to ∼ 100% after addition of 1 molar equivalent of stock mesoheme in DMSO to 1 molar equivalent of Hpx, the potential free heme in the experimental medium causing unexpected HO1 and ferritin expression is attributed to the experimental medium and conditions which are different from the *in vitro* efficacy of heme binding potential of Hpx. On the other hand, HAoSMCs are thought to express the scavenger receptor LRP1 that binds heme-Hpx (Okada et al., 1996; Swertfeger et al., 2002; Hvidberg et al., 2005; Lillis et al., 2005) so some endocytosis of heme-Hpx may have taken place, leading to the unexpected HO1 and H-Ferritin expression. Moreover, it might be beneficial that Hpx allowed the activation of the endogenous, protective HO1/ferritin heavy chain system to cope with further stress stimuli. In our present research, we have also shown that A1M inhibited heme induced ER stress. Overall, our research further bolster up the feasibility of Hpx-based therapeutics in diverse pathologies in which heme-mediated tissue damage plays an etiopathogenetic role and the potential therapeutic role of A1M in diseases where free heme/Hb is present. In other *in vitro* models, A1M hindered intracellular oxidation and upregulation of HO1 induced by heme in primary keratinocytes also in erythroid cell line, K562 (Olsson et al., 2008, 2011). A1M prevented cell lysis of K562 cells caused by heme and cleared the cells from bound heme. Similar effects have been described in heme-treated skin explants (Olsson et al., 2008, 2011).

We have also confirmed that significant amounts of heme accumulate in complicated atherosclerotic plaques. It has been demonstrated that cell-free Hb is present in ruptured atheromas and exists in oxidized forms. We observed that HAoSMCs, even those at quite a distance from the border of intraplaque bleeding, showed the characteristics of ER stress with marked expression of ER stress/UPR marker Grp78 and CHOP. Therefore, our data underline the importance of a new phenomenon; the presence of unique sanctuary areas in complicated lesions, where excess free hemoglobin and heme most likely interact with cells leading to excessive protein modifications with hallmarks of ER stress and UPR. These data indicate that excess heme present in complicated lesions might trigger ER stress *in vivo*.

A body of evidence suggest that TGFβ promotes plaque stability and reduces atherosclerotic plaque size (Bobik, 2006; Chen et al., 2006, 2016; Bot et al., 2009; Reifenberg et al., 2012; Hassan et al., 2018). In addition, inhibition of TGFβ signaling accelerates atherosclerosis, decreases collagen synthesis and induces hemorrhages and iron deposition (Mallat et al., 2001; Lutgens et al., 2002). Overall, factors that decrease collagen synthesis in HAoSMCs greatly affect their ability to maintain the protective fibrous cap in atherosclerotic lesions. TGFβ signaling is negatively influenced by HO1, the key effector protein of heme catabolism in hepatocellular carcinoma cells (Park et al., 2018). In NIH3T3 and human lung fibroblasts, TGFβ-induced collagen synthesis is inhibited by quercetin in a HO1-dependent fashion (Nakamura et al., 2011). Furthermore, TGFβ-mediated collagen synthesis is impaired by HO1 in atrial fibroblast (Yeh et al., 2016). Heme impairs TGFβ production in human mononuclear cells in Zn/Cu superoxide dismutase-dependent manner (Andrade et al., 2010). These data show that heme can inhibit both TGFβ production and the signaling cascade downstream of TGFβ, thereby regulating TGFβ responsive genes such as collagen. In addition, TGFβ signaling was markedly inhibited by the ER stress inducer thapsigargin in NIH3T3 and oligodendroglial OLI-neu cell lines (Alevizopoulos et al., 1997; Ming et al., 2010). ER stressor thapsigargin inhibited collagen synthesis as well in chick embryo chondrocytes (Clark et al., 1994). The modulatory effect of heme on TGFβ synthesis and signaling including collagen production can be mediated either by the induction of HO1, SOD, or, similar to thapsigargin, in an ER stress catalyzed way. Active TGFβ and its receptors are limitedly expressed in isolated areas of advanced atherosclerotic lesions together with lower serum levels in atherosclerosis (Grainger et al., 1995; Bobik et al., 1999; McCaffrey et al., 1999). These findings suggest that heme induced ER stress might contribute to lower expression of TGFβ and collagen type I resulting in plaque instability. We may conclude that our new observation related to fibrogenesis and heme effects are strongly supported by other models.

We demonstrate for the first time that heme induces ER stress and UPR in HAoSMCs. Heme is such a robust stress for cells, that it is able to induce CHOP, a pro-apoptotic protein. At the same time we were able to show, that heme induced only a transient increase in ATF4 with a concomitant sustained expression of Grp78 and ATF6 activation indicating an adaptive response to ER stress. Parallel with that, sustained activation of HO1 and ferritin heavy chain were observed in HAoSMCs in response to heme.

CONCLUSION

Our findings provide *in vivo* as well as *in vitro* evidence that heme triggers ER stress that might be a novel coordinated program of responses to heme: "heme induced endoplasmic reticulum" stress (HIER stress). We demonstrated, that the cytotoxic and harmful effects of heme were attenuated by Hpx and A1M through their heme binding capability. Our results support those efforts where Hpx and A1M could serve as therapeutic agents in various diseases where excess heme is present.

AUTHOR CONTRIBUTIONS

TG, DP, and AN designed and performed the experiments, analyzed the data. TG wrote the manuscript and contributed to study design. ZH performed immunohistochemistry. ZH and GM conducted pathological analyses of carotid arteries

and advised histology. LP performed oxidized hemoglobin and heme measurements from human samples. PN provided human carotid artery specimens. BÅ and MG provided recombinant alpha-1-microglobulin and edited the manuscript. AS provided rabbit hemopexin, contributed to interpretation of the data and revised the manuscript. GB and JB contributed to the study design and edited the manuscript. GT and DP share first authorship.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01595/full#supplementary-material

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MINI REVIEW
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The Extracellular Protein, Transthyretin Is an Oxidative Stress Biomarker

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The extracellular protein, transthyretin is responsible for the transport of thyroxin and retinol binding protein complex to the various parts of the body. In addition to this transport function, transthyretin has also been involved in cardiovascular malfunctions, polyneuropathy, psychological disorders, obesity and diabetes, etc. Recent developments have evidenced that transthyretin has been associated with many other biological functions that are directly or indirectly associated with the oxidative stress, the common hallmark for many human diseases. In this review, we have attempted to address that transthyretin is associated with oxidative stress and could be an important biomarker. Potential future perspectives have also been discussed.

Keywords: transthyretin, oxidative stress, cryptic protease activity, thyroxin, retinol binding protein, biomarker

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INTRODUCTION

Transthyretin (TTR), also known as prealbumin is a 55 kDa homo-tetrameric protein found in plasma and cerebrospinal fluid. It consists of four identical subunits, with each subunit consisting of 127 amino acid residues (Gonzalez and Offord, 1971; Kanda et al., 1974). TTR structure is rich in β-sheets with four binding sites; two for thyroxine and two for retinol-RBP complex (Ingbar, 1958; Naylor and Newcomer, 1999). It is encoded by a 7 kb gene (comprising of four exon and three introns) located at chromosome 18q11.2– q12.1 (Tsuzuki et al., 1985; Sparkes et al., 1987). It is primarily responsible for the transport of thyroxin and retinol-retinol binding complex (RBP-complex) to different parts of the body and brain (Raz and Goodman, 1969; Power et al., 2000). The major site of serum TTR synthesis is liver with normal concentration in the range of 0.2–0.4 mg/ml and half-life of 2 days. In central nervous system, TTR is expressed in choroid plexus and is released into the cerebrospinal fluid with concentration in the range of 0.02–0.04 mg/ml (Soprano et al., 1985). In addition to plasma and cerebrospinal fluid, it is also expressed in the endothelial cells of Islets of Langerhans, retinal and ciliary pigment epithelia in trace amounts (Cavallaro et al., 1990; Kawaji et al., 2005; Westermark and Westermark, 2008). TTR may also undergo oligomerization and such TTR oligomers are specifically picked up by cardiomyocytes, neuronal and kidney cells leading to organ malfunctions (Colon and Kelly, 1992). Deficiency of the normal function of TTR has been known to be associated with obesity and diabetes (Yang et al., 2005). The roles of TTR in the central nervous system, especially in cognition and memory, psychological health and emotion have also been widely understood (Fleming et al., 2007; Brouillette and Quirion, 2008). The oligomeric form of the TTR has been found to be involved in the pathophysiology of various diseases including atherosclerosis, familial amyloidosis polyneuropathy (Costa et al., 1978), senile

systemic amyloidosis (Westermark et al., 1990), familial amyloidosis cardiomyopathy (Jacobson et al., 1997; Yokoyama et al., 2015; Sant'anna et al., 2017) etc. Although main function of transthyretin is the transport of thyroxine and retinol bound to retinol binding protein (RBP), there are many other biological roles of TTR that are directly or indirectly related to anti-oxidant and oxidant properties and could be an important oxidative stress biomarker or therapeutic target. For instance, (i) TTR level correlates well with reactive oxygen species (ROS) or reactive nitrogen species (RNS) (Saito et al., 2005; Fong and Vieira, 2013); (ii) TTR gene expression is regulated by stress hormone, glucocorticoid and sex hormone, estradiol (Li et al., 2011; Martinho et al., 2012); (iii) Even though TTR is an extracellular protein, it can induce oxidative stress in endoplasmic stress (ER) and hence involved in unfolded protein response (UPR) (Teixeira et al., 2006; Genereux et al., 2015; Chen et al., 2016); (iv) The oligomeric forms of TTR also plays an important role in inducing oxidative stress and could be involved in different pathophysiologies (Hammarström et al., 2002; Zhao et al., 2013). In the light of these observations, this review article has been designed to discuss that TTR is associated with oxidative stress and has implications for potential disease specific biomarker.

TTR IS A NEURONAL STRESS BIOMARKER

It has already been understood that oxidative stress is one primary cause of Alzheimer's disease (AD) and many other neurodegenerative diseases (Marques et al., 2003). Recent advances have unveiled that such a cause of oxidative stress has a good correlation with the role of TTR. This is evident from various studies. First, TTR level is upregulated in patients with neurodegenerative disorders (Li et al., 2011) wherein oxidative stress is the common cause of the pathophysiology. Because quantitative real time PCR of TTR mRNA and western blot analysis, have shown that primary neurons from AD mice exhibit upregulation of TTR level as compared to non-demented age-matched individuals or control mice (Li et al., 2011). Second, TTR expression is directly regulated by sex hormones (e.g., estradiol) or stress hormones (e.g., glucocorticoids) in neuronal cells (Martinho et al., 2012). For instance, when rat choroid plexus and choroid plexus epithelial cell lines were incubated with varying concentration of hydrocortisone and estradiol (E2) (0, 10, 100, or 1,000 nM) for 6, 12, 18, 24 and 36 h (Martinho et al., 2012), there was increase in TTR protein and TTR mRNA levels in a concentration dependent manner of the hormones. Similarly, incubation of the cells with respective receptor antagonists results in the suppression of TTR induction. In another experiment, they also analyzed the level of corticosterone in liver, choroid plexus and cerebrospinal fluid of adult rats in response to chronic and acute stress. Stress was induced by increasing the animal density. It was observed that the given treatments drive the upregulation of expression of TTR. In another development, based on *in silico* study, Wakasugi et al. (1986) demonstrated that rat TTR gene contains a glucocorticoid-responsive element in its 3′ region of the first intron (Wakasugi et al., 1986) and this element is conserved in humans as well (Sasaki et al., 1985). Thus, it was concluded that upregulation of TTR expression by glucocorticoid treatments is *via* glucocorticoid-responsive element. Taken together, the results indicate that TTR has a close association with the level of oxidative stress and hence might consequently contribute to the pathogenicity of neurodegeneration.

Third, other studies also reported that TTR has the ability to suppress or remove β-amyloid deposits from neuronal tissues (Buxbaum et al., 2008) making TTR a crucial target for the therapeutic intervention of AD. In fact, direct evidence of the involvement of TTR in AD stems from the identification of physical interaction between TTR and Aβ (Gimeno et al., 2017). Mechanistically, TTR present in the cerebrospinal fluid could sequesters β-amyloid and inhibits the oligomerization and plaque formation (Schwarzman et al., 1994). It is believed that TTR uses its cryptic protease activity to proteolyze Aβ into smaller non-amyloidogenic fragments (Costa et al., 2008; Silva et al., 2017). In another development, recent study further revealed that TTR has higher affinity to Aβ aggregates rather than the fibrils and bind to these pre-toxic aggregates in a chaperon-like manner in both the extracellular and intracellular environment (Buxbaum et al., 2008). It has also been understood the higher the binding affinity between TTR and Aβ, the higher is the inhibitory potential because stabilizers that increase TTR tetramer stability augments the inhibitory effect (Costa et al., 2008; Ribeiro et al., 2012). Similarly, few TTR mutants that is more stable than the Wt TTR has been shown to exhibit more disaggregating potential than Wt TTR (Costa et al., 2008).

It has been known that major cytotoxicity of deposition of β-amyloid is oxidative stress (Butterfield et al., 2001). Since there exists a good correlation between oxidative stress and TTR expression, we speculate that oxidative stress induces glucocorticoids which in turn increase TTR expression via its action on the glucocorticoid receptors. The increased level of TTR will further help to deal with the β-amyloid deposits bringing about its role in preventing AD (Nilsson et al., 2018). In addition to AD, there are a large number of neuronal disorders due to oxidative stresses. These include psychological (e.g., depression), movement disorder (e.g., Parkinson), cognitive disorders etc. Therefore, possibility of the association between these diseases and TTR level may be exploited as a potential biomarker (or therapeutic target) for such disorders.

CRYPTIC PROTEASE ACTIVITY OF TRANSTHYRETIN INDUCES OXIDATIVE STRESS BY CLEAVING APO A-1

High-density lipoprotein (HDL) complex is responsible for reverse cholesterol efflux and cholesterol transport from cells and tissues back to liver (Gordon et al., 1989). Besides cholesterol efflux, HDL also exhibit anti-oxidant activity by forming complex with many anti-oxidant enzymes like paraoxonase, platelet-activating factor acetylhydrolase, glutathione peroxidase, lipid transfer proteins like lecithin: cholesterol acyl transferase, cholesterol ester transfer protein, Apolipoprotein A-I (ApoA-I)

and 1-palmitoyl-2-oleoyl-phosphatidylcholine. Among these anti-oxidant enzymes, Apo A-I is the major anti-oxidant and anti-inflammatory component associated with HDL (Navab et al., 2000). It employs anti-oxidant activity by eliminating lipid hydroperoxides from low-density lipoproteins (LDL) and anti-inflammatory properties by shutting down the expression of adhesion molecules (Navab et al., 2000).

One important protein that affects the anti-oxidant property of HDL is the serum protein, TTR 1-2% of serum TTR is associated with HDL molecules (Sousa et al., 2000). As mentioned above, TTR transports thyroxine and retinol bound to RBP. In the absence of retinol-RBP complex, TTR occasionally exhibit its cryptic protease function (Liz et al., 2004). This activity of TTR brings about specific cleavage of Apo A-I resulting in the loss of anti-oxidant function of HDL (Liz et al., 2007; Podrez, 2010). **Figure 1** illustrates the mechanism of how TTR acts to cleave the Apo A-1. Immunologically, the proteolyzed product of Apo A-I acts as a pro-inflammatory molecule that further adds in oxidative stress (Navab et al., 2000). In another development, both the proteolyzed product of apo A-I i.e. N-terminal and C-terminal domains are observed to be amyloidogenic (de Sousa et al., 2000). Since, amyloids or proteins aggregates are one important basic cause of oxidative stress (Abramov et al., 2004), the formation of the amyloidogenic species will further augment the magnitude of the oxidative stress.

Interestingly, Sousa and his group (2004) recently reported that the cryptic protease activity of TTR was seen only when RBP-retinol complex was not bound to TTR (Liz et al., 2004) (**Figure 1**). This indicates that such type of oxidative stress (due to cryptic protease activity of TTR) may perhaps be related to the retinol deficiency and hence diseases associated with it (Basu et al., 1989). Therefore, the protease activity of TTR can be a potential biomarker for oxidative stress as well as for diseases associated with retinol deficiency. Furthermore Apo-A I deficiency (due to the cleavage by TTR) make HDL unable to remove cholesterol from the tissues. This will eventually result in the atherosclerotic plaque formation. Thus TTR cryptic protease activity and associated oxidative stress may further by employed as a biomarker for cardiovascular disorders.

TTR IS GLUTATHIONYLATED

Glutathiol (GSH) is a low molecular weight thiol group present in all the cells and serum. GSH also acts as a good indicator of cellular redox state and anti-oxidant defence (Dalle-Donne et al., 2009). The antioxidant property of GSH is mediated by glutathione peroxidase as it oxidizes GSH to GSSG thereby reducing hydrogen peroxide and lipid hydroperoxides (Toborek and Hennig, 1994). Protein-glutathionylation is a redox-mediated post-translational modification, which involves conjugation of a glutathione with a cysteine thiol group on the proteins (Ghezzi, 2005). Glutathionylation not only plays a critical role in many important biological functions (regulation of metabolic pathways, calcium homeostasis, signal transduction, cytoskeleton remodeling, inflammation and protein folding) but also is involved in oxidative stress (Kaplowitz, 1981). The involvement in oxidative stress may stem from at least in two viewpoints. Large scale adduct formation with cysteine group in proteins will eventually lead to deficiency of GSH levels making the system difficult to handle the oxidative stress. Alternatively, once GSH has been bound to cysteine residues in proteins, there is release of free electron (Ghezzi, 2005) that consequently help to generate free radicals thereby inducing oxidative stress. Interestingly, TTR has been reported to be glutathionylated under certain conditions. This was revealed from a study conducted by Ando and his group (1998) to determine the *in vivo* behavior of transthyretin in blood using electrospray ionization mass spectrometry analysis coupled with high-pressure liquid chromatography (HPLC) (Terazaki et al., 1998). Purified TTR from normal subjects was injected into the rats and after 3 h, blood and the urine were analyzed by measuring free or modified TTR. Lower level of free TTR in blood and no TTR secretion into the urine were observed (Terazaki et al., 1998) indicating that major fraction of TTR have been modified by glutathione. Inside the cell, glutathionylation is not restricted to TTR alone but also occurs to many other proteins, the results indicate that TTR indeed contributes to the oxidative stress generated due to protein glutathionylation.

Escher et al. (2007) reported that the levels of glutathionylated form of TTR are inversely correlated in patients with Mycosis fungoides (MF) or non-Hodgkin's lymphoma (Escher et al., 2007). It is worth noting that MF is associated with the genetic polymorphism in genes involved in the regulation of oxidative stress (Lightfoot et al., 2006). The results hint that the development of MF is because of oxidative stress originated through genetic or post-translational modifications. Therefore, the glutathionylated forms of TTR may be a potential biomarker for early diagnosis or therapeutic target for MF.

TTR OLIGOMERS AS MULTIPLE BIOMARKERS

It has been well understood that dissociation of TTR oligomer is the rate-limiting step to TTR amyloidosis because dissociation results in the exposition of important sites for oligomerization (Colon and Kelly, 1992; Sousa et al., 2001). Similarly, mutations in TTR disrupt its tetramer and thus form toxic oligomers (Hammarström et al., 2002). The toxic TTR oligomers are believed to preferentially deposit in the extracellular matrix (ECM) of hepatocytes or neuronal cells leading to the development of familial amyloidosis, which encompass FAP (Familial Amyloid Polyneuropathy) (Benson and Kincaid, 2007; Saraiva et al., 2012) and FAC (Familial Amyloid Cardiomyopathy) (Costa et al., 1978). Mechanistically, large deposition of such TTR oligomers in the cardiac and neuronal cells results in the tissue injury that ultimately lead to the increase in inflammatory response (Ton et al., 2014). Since oligomers are known to induce oxidative stress in cells and inflammatory response is going to add more impact on oxidative stress, accumulation of the oligomers eventually results in organ failure or tissue damage due to massive oxidative stress. Although in general oligomers are the exact cause or consequences of such

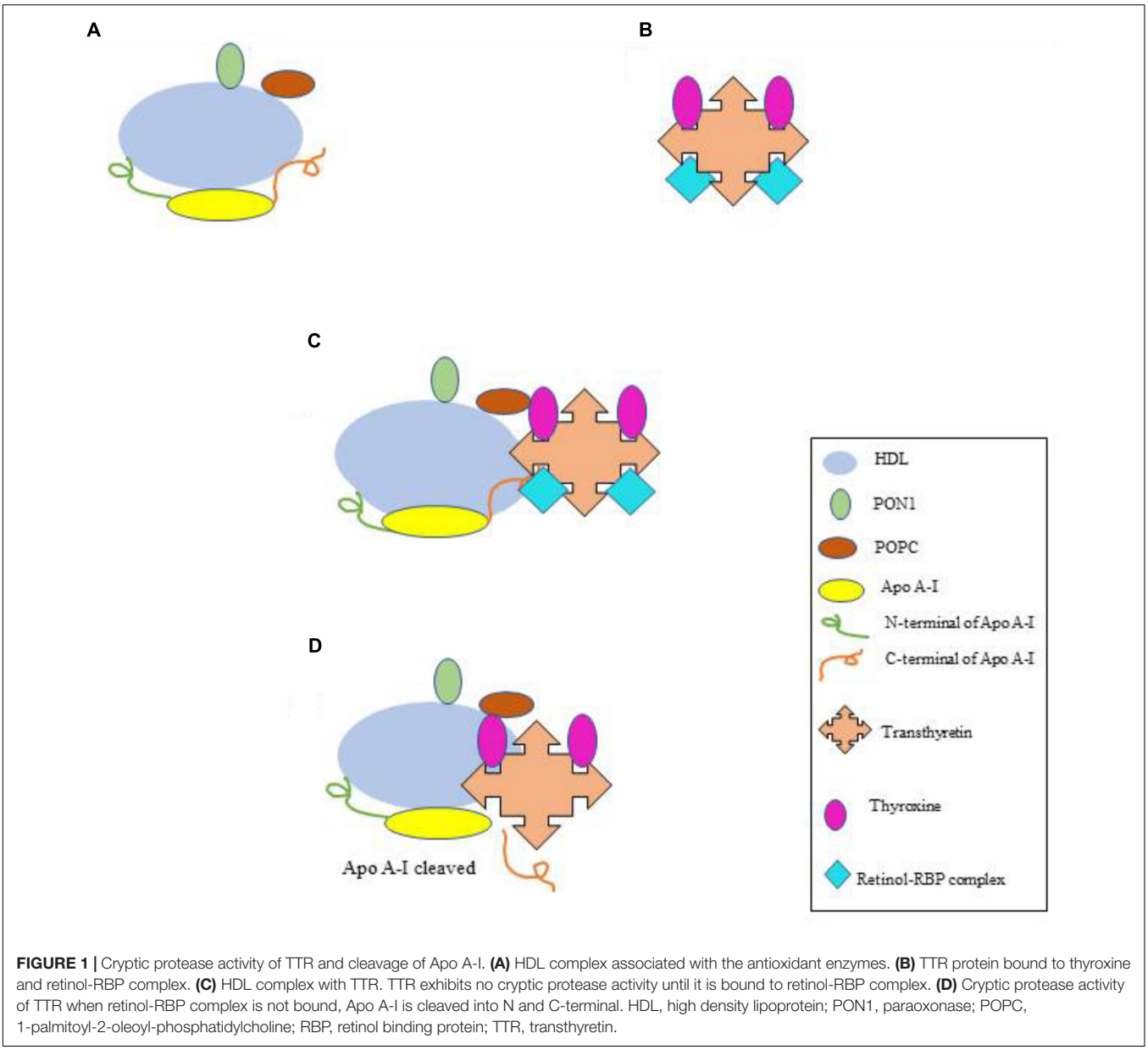

FIGURE 1 | Cryptic protease activity of TTR and cleavage of Apo A-I. **(A)** HDL complex associated with the antioxidant enzymes. **(B)** TTR protein bound to thyroxine and retinol-RBP complex. **(C)** HDL complex with TTR. TTR exhibits no cryptic protease activity until it is bound to retinol-RBP complex. **(D)** Cryptic protease activity of TTR when retinol-RBP complex is not bound, Apo A-I is cleaved into N and C-terminal. HDL, high density lipoprotein; PON1, paraoxonase; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; RBP, retinol binding protein; TTR, transthyretin.

oxidative stress is not clearly understood, it is certainly possible that a positive feedback loop is formed wherein oxidation causes more oligomerization of TTR, which in turn causes more TTR oxidation. In addition to cardiomyopathy and polyneuropathy, this feedback loop so formed (in case of TTR oligomers), may affect following consequences as outlined in **Figure 2** leading to involvement of TTR in different pathophysiologies or various biological processes. Following sections will describe the involvement of TTR oligomers in each of the consequences.

TTR Oligomer Is Related to Reactive Nitrogen Species

In addition to the ROS like superoxide radicals, hydrogen peroxide etc., (RNS) including nitrate and nitrite ions also plays a major role in oxidative stress. Fong and Vieira (2013) gave the first evidence for the increased production of RNS in presence of TTR aggregates in two different human cell lines, epidermoid (A431) and schwannoma (sNF94.3) (Fong and Vieira, 2013). Moreover, the authors also observed that the cells treated with TTR aggregates showed decreased metabolic activities as compared to TTR non-treated cells (Fong and Vieira, 2013). This indicates that the RNS-induced pro-oxidative effects could also hamper the metabolic activity of the cells. In a previous study by Saito et al. (2005), it was established that Wt and V30M (amyloidogenic variant) undergoes S-nitrosylation and due to which the proteins become amyloidogenic (Saito et al., 2005). Nitric oxide is generated in vessels from endothelial cells and smooth muscle cells. Vessels are the primary site for deposition of protein oligomers, therefore the nitrosylation of the TTR

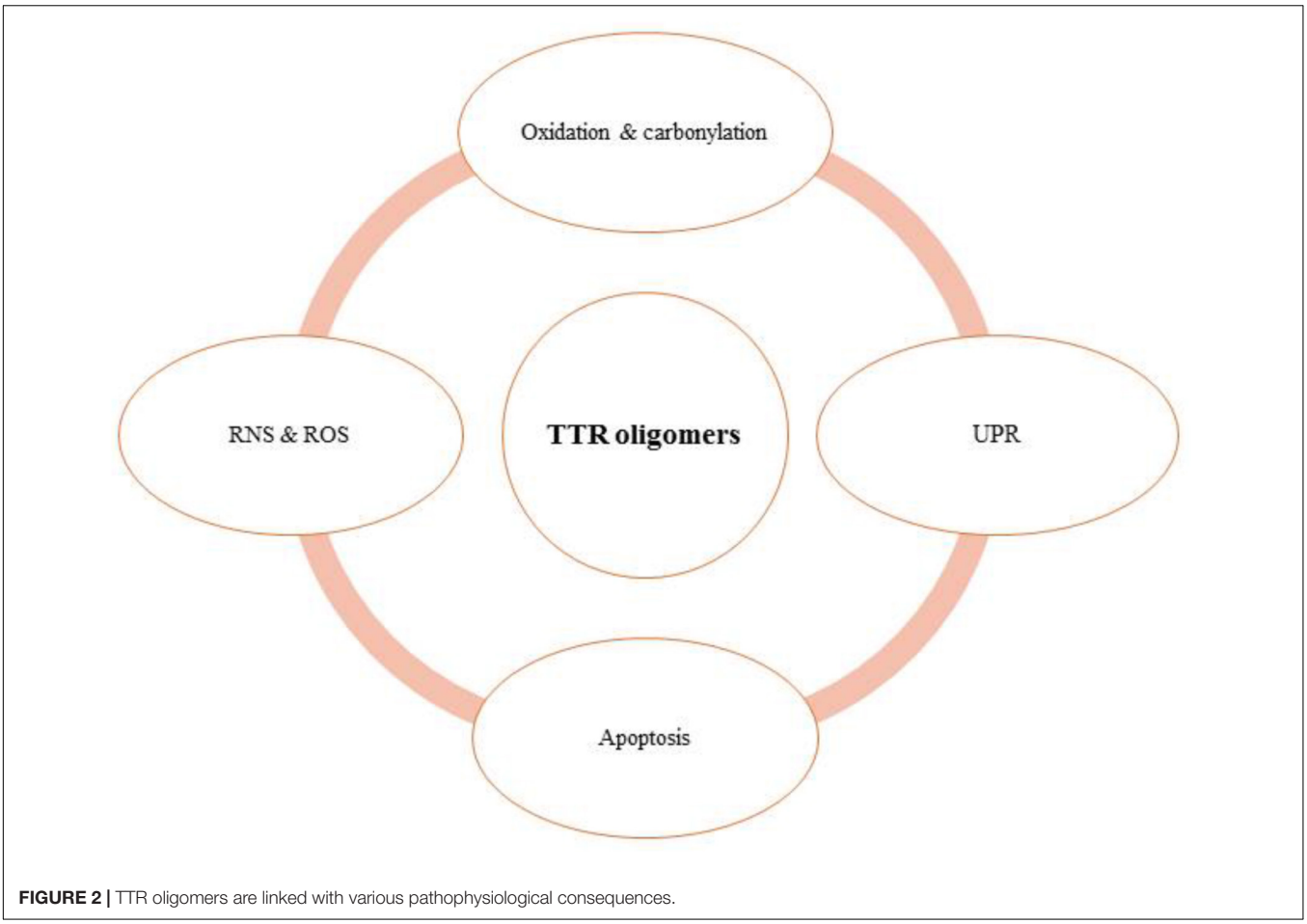

FIGURE 2 | TTR oligomers are linked with various pathophysiological consequences.

oligomers further enhances the ability to form amyloid fibrils which further contributes to the increased oxidative damage and *vice versa*. Since, there is a close correlation between TTR level and RNS or ROS, determination of TTR oligomeric level may help to evaluate the extent/magnitude of oxidative stress.

TTR Oligomers Can Be Used to Detect Endoplasmic Reticulum (ER) Stress

When there is increased generation of misfolded or unfolded proteins in the ER, UPR initiates (Teixeira et al., 2006; Walter and Ron, 2011). This UPR is mediated by three signal pathways which involves inositol-requiring enzyme 1, activating transcription factor 6 and double-stranded RNA-activated protein kinase-like ER kinase (Walter and Ron, 2011; Minakshi et al., 2017; Rahman et al., 2017, 2018). Since TTR is an extracellular protein and it is unlikely that it may be involved in UPR generation in the ER. However, when TTR oligomers are deposited in neuronal cells, cardiomyocytes and kidney cells, there are chances that the misfolded species or TTR oligomers reach ER and cause oxidative stress. In a systemic study by Teixeira et al. (2006) the role of TTR oligomers in ER stress response was investigated using TTR transgenic mouse, and cell lines models (Teixeira et al., 2006). It was established that there was increased levels of BiP-immunoglobulin binding protein (an ER-resident chaperone) with extracellular TTR amyloid deposits in the brain of transgenic mouse. Furthermore using mouse neuronal ND7 cell line, it was also observed that oligomer-induced increased expression of BiP was mediated by the release of calcium from ER to cytosol (Teixeira et al., 2006). Because the activity of Caspases-3 was observed in cells treated with only TTR oligomers and not in cells treated with dantrolene and xestospongin (inhibitors of Ca^{2+} release). The involvement of other secondary messengers like D-myo inositol 1,4,5-triphosphate which receives signals at plasma membrane suggests that extracellular TTR oligomers have the potential to trigger ER stress in the surrounding cells. Taken together, the results convincingly supported the premise that the deposited TTR oligomers in tissues helps to induce UPR in the ER (Sekijima et al., 2005). Since TTR is a serum protein and is not present in ER, the study links the possibility of invoking UPR in ER by other extracellular proteins.

Level of TTR Aggregates Negatively Correlates With the Activity of Catalase

Amyloid deposits consist of proteinaceous fibers which keep on depositing in tissues and form plaques. In case of AD, it was observed that these amyloid deposits had a toxic effect on cells as the cells showed apoptosis both *in vitro* and *in vivo*. Andersson et al. (2002) performed an *in vitro* study with different

cell lines like neuroblastoma cell lines, PC12 cells, HeLa cells and some haematopoietic cell lines to investigate for the toxic effect of aggregated mutant TTR (Andersson et al., 2002). It was observed that the toxic effect was not cell specific and further it triggered the signaling cascade which ultimately led to apoptosis (Macedo et al., 2008). Interestingly, it has been shown that TTR induced apoptosis was inhibited by catalase in a concentration-dependent manner. Since catalase is an enzyme responsible for catalyzing H_2O_2 which is a predominant ROS, the results suggest that TTR oligomer-induced apoptosis is via production of H_2O_2 or other ROS.

Oligomers of Different TTR Variants Exhibit Different Magnitude of Oxidative Stress

Protein oligomerization or amyloidogenesis has been considered to be one common hallmark of oxidative stress (Dobson, 1999). In fact, these protein oligomers are really toxic to the cells and can affect the integrity and hence function of various cell organelles (Zampagni et al., 2011). It is also believed that protein oligomers elevate the production of ROS which causes the oxidative stress and *vice versa* causing cell damage (Zempel et al., 2010). Protein oligomers can also force the release and normal function of Cytochrome C by directly affecting mitochondrial potential (Caroppi et al., 2009) or by affecting other pro-apoptotic molecules (Ott et al., 2007). Despite these developments, how different protein oligomers (or generated by different variants) could associate with the magnitude of proteotoxicity has not been explored yet. In this connection, TTR oligomers represents an emblemic signature as oligomers generated by different variants, is related to time of onset of disease pathology and hence determines the nature of proteotoxicity or oxidative stress (Quintas et al., 2001; Taguchi et al., 2013). For instance, Wt TTR oligomerization that leads to systemic senile amyloidosis was apparent in older individuals at the age of around 60–70 (Zhao et al., 2013). On the other hand, the pathogenic symptoms due to oligomers of the mutant variant V122I appeared early in age and patients die generally 10 years before the onset of senile systemic cardiomyopathy caused by Wt aggregation. The onset of familial amyloid polyneuropathy caused by the variant, V30M is around 25–33 years and death occurs 10 years after the onset (Koike et al., 2012; Takahashi et al., 2014; Arvidsson et al., 2015). L55P is considered to be most pathogenic variant of TTR and starts oligomerization at the physiological pH *in vitro* (as compared to the other mutants) and patients die at a very young age (Lashuel et al., 1999; Hammarström et al., 2002). Not only mutant variants, but Wt TTR has also been reported to undergo oxidation and carbonylation whose proteotoxicities (and hence oxidative stress) matches with the age of individuals. Thus, identification of different variants of TTR may be employed as a biomarker for the age related oxidative stress. Future research should focus on identification of newer TTR variants and their related onset of diseases or magnitude of oxidative stress.

SUMMARY AND FUTURE PERSPECTIVES

It is clearly evident from this review that different activity or post-translational modification of TTR is linked to specific disease pathologies *via* oxidative stress. The potential of TTR to cause oxidative stress is not only confined to serum, but also in ER. Therefore, in-depth insights to the various mechanism of oxidative stress induced by TTR and its oligomers will eventually lead to appropriate therapeutic strategies for these specific diseases. It is also understood that TTR oligomers can invoke different signaling cascades leading to different biological consequences (e.g., apoptosis, ROS and RNS generation, UPR and redox mediated oxidation) resulting in oxidative stress. It would therefore be important to explore the signaling cascade in detail by which oligomers help to induce such multiple consequences. Nevertheless TTR would be a potential biomarker of several human diseases linked with oxidative stress.

AUTHOR CONTRIBUTIONS

LS conceived the idea. MS, SK, and SR contributed to writing of the manuscript.

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REVIEW
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Crosstalk Between Oxidative Stress and Endoplasmic Reticulum (ER) Stress in Endothelial Dysfunction and Aberrant Angiogenesis Associated With Diabetes: A Focus on the Protective Roles of Heme Oxygenase (HO)-1

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Type-2 diabetes prevalence is continuing to rise worldwide due to physical inactivity and obesity epidemic. Diabetes and fluctuations of blood sugar are related to multiple micro- and macrovascular complications, that are attributed to oxidative stress, endoplasmic reticulum (ER) activation and inflammatory processes, which lead to endothelial dysfunction characterized, among other features, by reduced availability of nitric oxide (NO) and aberrant angiogenic capacity. Several enzymatic anti-oxidant and anti-inflammatory agents have been found to play protective roles against oxidative stress and its downstream signaling pathways. Of particular interest, heme oxygenase (HO) isoforms, specifically HO-1, have attracted much attention as major cytoprotective players in conditions associated with inflammation and oxidative stress. HO operates as a key rate-limiting enzyme in the process of degradation of the iron-containing molecule, heme, yielding the following byproducts: carbon monoxide (CO), iron, and biliverdin. Because HO-1 induction was linked to pro-oxidant states, it has been regarded as a marker of oxidative stress; however, accumulating evidence has established multiple cytoprotective roles of the enzyme in metabolic and cardiovascular disorders. The cytoprotective effects of HO-1 depend on several cellular mechanisms including the generation of bilirubin, an anti-oxidant molecule, from the degradation of heme; the induction of ferritin, a strong chelator of free iron; and the release of CO, that displays multiple anti-inflammatory and anti-apoptotic actions. The current review article describes the major molecular mechanisms contributing to endothelial dysfunction and altered angiogenesis in diabetes with a special focus on the interplay between

oxidative stress and ER stress response. The review summarizes the key cytoprotective roles of HO-1 against hyperglycemia-induced endothelial dysfunction and aberrant angiogenesis and discusses the major underlying cellular mechanisms associated with its protective effects.

Keywords: heme oxygenase-1 (Ho-1), endothelial dysfunction, angiogenesis, oxidative stress, ER stress, diabetes, hyperglycemia

INTRODUCTION

Endothelial dysfunction is considered as the structural alteration and functional impairment of vascular endothelial layer, characterized by a reduction in NO bioavailability and aberrant angiogenesis (Lerman and Burnett, 1992; Creager et al., 2003; Jamwal and Sharma, 2018). Endothelial dysfunction is a key player in the pathological onset of various cardiovascular complications associated with diabetes, whether affecting microvasculature including retinopathy, nephropathy, and neuropathy or macrovasculature such as ischemic heart disease and ischemic stroke (Ebrahimian et al., 2006). There is much scientific evidence showing that hyperglycemia, a characteristic manifestation of diabetes, is implicated in the development of endothelial dysfunction. Other molecular mechanisms involved in vascular endothelial perturbations that ensue in such a metabolic disorder include disruption of a large array of metabolic pathways within the endothelial cell leading to endoplasmic reticulum (ER) stress, oxidative stress, inflammation and apoptosis (Cimellaro et al., 2016; Incalza et al., 2018).

Heme oxygenase (HO) is a cytoprotective enzyme, which contributes to maintaining a healthy vascular endothelium. HO operates as a key rate-limiting enzyme in the process of degradation of the iron-containing molecule, heme, yielding the following byproducts: carbon monoxide (CO), iron, and biliverdin (Kikuchi et al., 2005; Ryter et al., 2006; Rochette et al., 2018). There are two main HO isoforms known (HO-1, HO-2), in addition to a putative third one (HO-3) (Nitti et al., 2017). Among the three isoforms, the role of HO-1 as a major protective enzyme is best documented. Its anti-oxidant, anti-apoptotic (Wang et al., 2010; Mishra and Ndisang, 2014; Tiwari and Ndisang, 2014), and anti-inflammatory (Hayashi et al., 1999; Lee and Chau, 2002; Morse et al., 2003; Ndisang and Jadhav, 2009) effects have attracted much interest in literature. The cytoprotective effects of HO-1 depend on multiple cellular processes including the generation of bilirubin, an anti-oxidant molecule, from the degradation of heme; the induction of ferritin, a strong chelator of free molecular iron; and the liberation of CO, responsible for HO-1 major anti-inflammatory and anti-apoptotic effects (Durante, 2003; Tiwari and Ndisang, 2014). However, the anti-inflammatory and anti-apoptotic effects of CO are only observed if CO is generated in low levels. Large amounts of CO can have lethal consequences because of the strong affinity of CO binding to the heme in hemoglobin and mitochondrial proteins. CO is a stable non-radical small gas molecule that is weakly soluble in water. It belongs to the family of gasotransmitters which emerged as important signaling molecules. CO signals inside the cell in several manners. For instance, CO stimulates guanylyl cyclase to form cGMP and regulates the action of some transcription factors (Mustafa et al., 2009; Garcia-Mata and Lamattina, 2013).

HO-1 is induced by a variety of pro-oxidant agents and stimuli, such as ultraviolet rays, heavy metals, inflammatory cytokines and iron-containing molecule, heme (Li et al., 2011). HO-1 is now recognized for playing anti-oxidative and cytoprotective roles both *in vitro* and *in vivo*. For example, mice deficient for HO-1 were found to spontaneously develop iron depots in kidneys and livers, tissue damage, chronic inflammation and oxidative structure modification of macromolecules (e.g., proteins, DNA) (Poss and Tonegawa, 1997a,b). Similar to animal models, the first reported human case of HO-1 deficiency involved significant iron tissue depots, growth deficiency, anemia and high susceptibility to reactive oxygen species (ROS)-mediated damage (Yachie et al., 1999). At the molecular level, it is believed that HO-1 protects vessel wall from pathological remodeling and endothelial cell dysfunction (Duckers et al., 2001; Durante, 2003; Awede et al., 2010; Hyvelin et al., 2010). Several methodologies were employed to induce HO-1 in the vessels with the use of pharmacological inducers being one of the most promising approaches (Awede et al., 2010; Hyvelin et al., 2010). Heme itself and its man-made analogs are strong pharmacological inducers of HO-1 and were found to protect against the development of cardiovascular diseases in several studies both *in vitro* and *in vivo* (Awede et al., 2010; Hyvelin et al., 2010; Li et al., 2011). A study conducted by Yang et al. (2015) where the serum of rats exposed to cigarette smoke was used to induce oxidative stress in human umbilical vein endothelial cells (HUVECs), has shown a significant decrease in endogenous production of ROS following the induction of HO-1 by hemin (Yang et al., 2015). Maamoun et al. (2017) found that the pharmacological induction of HO-1 using Cobalt-protoporphyrin (CoPP) reduced ROS production in HUVECs exposed to intermittent high glucose.

With regards to the anti-inflammatory effects of HO-1, Chang et al. (2014) have shown that the treatment of HUVECs with iodine contrast medium caused anti-proliferative and inflammatory reactions, and enhanced the expression of intercellular adhesion molecule (ICAM)-1 and adhesion molecules receptors while cells co-incubated with the HO-1 inducer were completely protected (Chang et al., 2014). The cytoprotective role of HO-1 has also been illustrated in cancer cells, where one study has demonstrated that the upregulation of HO-1 in renal cancer cells promoted their survival capacity via the induction of the expression of pro-survival molecule

Bcl-xL and decreased expression of Beclin-1 and LC3B-II, that are involved in the process of autophagy, an effect that has been reversed by HO-1 knockdown (Banerjee et al., 2012). Furthermore, in vascular cells, it has been found that HO-1 induction protected HUVECs from high glucose mediated cell death through the reduction of caspases 3 and 7 activation (Maamoun et al., 2017).

In the current article, we have reviewed the major mechanisms contributing to endothelial dysfunction, the key initial step in the onset of atherosclerotic process, in the context of diabetes and hyperglycemia. Furthermore, we have reviewed the cytoprotective roles of HO-1 against diabetes- and hyperglycemia-induced endothelial dysfunction and aberrant angiogenesis and discussed the major underlying molecular mechanisms associated with these protective effects with special emphasis on signaling pathways related to oxidative stress and ER stress response.

ENDOTHELIAL DYSFUNCTION AND HYPERGLYCEMIA: KEY MOLECULAR DISTURBANCES

The endothelium is a single cell layer that forms the interface between blood stream and adjacent tissues. Over the recent decades the complexity of this selectively permeable barrier and its important contribution to controlling vascular homeostasis have been established (Michiels, 2003; Khazaei et al., 2008; Jamwal and Sharma, 2018). The endothelium allows the selective passage of certain substances such as nutrients through the vessel wall to the adjacent tissues. The endothelium is recognized as an endocrine organ that is able to produce and secrete many hormones and mediators which are crucial for the optimal functioning of the vasculature such as factors regulating vascular tone, coagulation, immune response and growth of adjacent vascular cells (Khazaei et al., 2008; Jamwal and Sharma, 2018). In normal physiological conditions, vascular endothelial cells are exposed to plasma blood sugar levels between 3.8 and 6.1 mmol/L. Exposure of endothelial cells to glucose levels of 11.1 mmol/L and above is regarded as a diabetic condition [the national institute for health and care excellence (NICE) guidelines, 2015]. However, unlike *in vivo* conditions, in cell culture, the definition of high glucose varies considerably according to the cell model used, depending on glucose levels in culture medium where the cells are being selected and harbored. Two examples that demonstrate such variability are the endothelial cell line EA.hy926 and HUVECs, where the former is propagated in culture medium containing 25 mM glucose, whereas the latter is routinely grown in a culture medium containing 5.5 mM of glucose (Maamoun et al., 2017). As a result, 25 mM glucose would be considered to be a high concentration of glucose for HUVECs, but is a standard level in EA.hy626 endothelial cell culture medium. Alterations occur in both the micro- and macrovasculature in response to hyperglycemia. An abnormally high glucose concentration can disrupt the balance within the endothelial cell and a state of 'endothelial cell dysfunction' results. Endothelial cell dysfunction is defined by the presence of one or more of the following characteristics: (I) low bioavailability of NO, (II) impaired endothelium-dependent relaxation, (III) weak fibrinolytic capability, (IV) excess of production of growth factors, adhesion molecules and pro-inflammatory molecules (e.g., cytokines), oxidative stress, and aberrant angiogenesis (Cade, 2008).

Hyperglycemia-Mediated Oxidative Stress and Endothelial Injury

Macrovascular and microvascular complications have been shown to be mainly or partly dependent on hyperglycemia (Zoungas et al., 2012). Hyperglycemia can induce vascular endothelial damage through different pathways: (I) enhanced polyol activity, causing sorbitol and fructose accumulation; (II) enhanced production of advanced glycation end products (AGEs); (III) activation of mitogenic protein kinase C (PKC); (IV) heightened hexosamine flux pathway and (V) a decrease of body anti-oxidant defenses (Brownlee, 2001; Kornfeld et al., 2015). There is considerable evidence that these biochemical pathways can be induced by excessive generation of ROS, leading to increased oxidative stress in a positive feedback loop (Son, 2012). Oxidative stress occurs when the concentrations of ROS exceed those of anti-oxidant neutralizing species, such as glutathione (GSH) and HO. ROS are a heterogeneous population of molecules including free radicals, such as hydroxyl radical (OH^-), superoxide anion (O_2^-), peroxyl (RO_2), and hydroperoxyl (HRO_2^-), and non-charged species, such as hydrogen peroxide (H_2O_2) and hydrochloric acid (HCl) (Pieper et al., 1997). Under intracellular hyperglycemic conditions, excessive production of ROS develops through several mechanisms, the most important of which is excessive activation of mitochondrial electron transport chain. Other sources of ROS include glucose-induced activation of NADPH oxidase (NOX) and xanthine oxidase (Nishikawa et al., 2000). This excessive ROS production inflicts DNA damage resulting in poly ADP ribose polymerase (PARP)-1 activation, which in turn inhibits the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (G3PDH) resulting in the pile-up of upstream glycolytic metabolites, with the resulting influx into polyol pathway, hexosamine pathway, diacylglycerol (DAG) and PKC pathway, as well as generation of AGEs (Du et al., 2003) (**Figure 1**).

Glucose, through the polyol pathway, is converted into polyalcohol sorbitol by aldose reductase utilizing NADPH as a cofactor, which in turn reduces intracellular concentrations of NADPH resulting in the inhibition of glutathione/peroxidase anti-oxidant system, which is dependent on NADPH as a cofactor, and consequently induces overproduction of H_2O_2 and ROS in general. Moreover, ROS accumulation inhibits glucose-6-phosphate dehydrogenase (G6PDH), which is the rate limiting enzyme for pentose shunt pathway that is fundamental to the maintenance of reducing equivalents for the glutathione/peroxidase system anti-oxidant system, amplifying the oxidative stress (**Figure 2**). Furthermore, sorbitol is oxidized to fructose by sorbitol dehydrogenase using NAD as a co-factor, increasing the intracellular ratio of $NADH/NAD^+$,

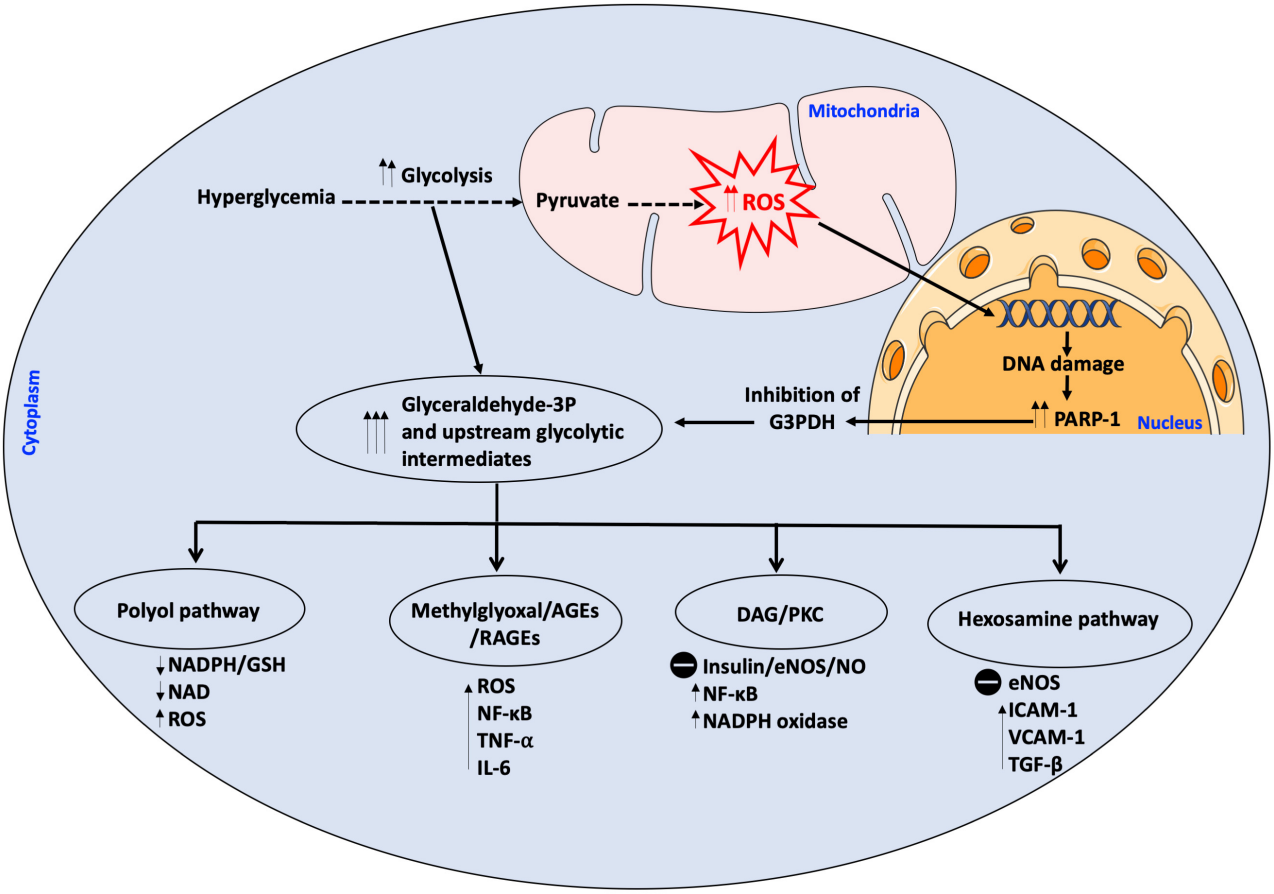

FIGURE 1 | Intracellular hyperglycemia results in increased production of mitochondrial ROS. ROS cause DNA damage releasing PARP-1 which inhibits G3PDH. This results in the accumulation of upstream glycolytic intermediates. Other pathways are activated as a result of the accumulation of glycolytic intermediates upstream of G3P which have detrimental effects on the cell; these pathways are; (1) Polyol pathway which depletes the cell of its antioxidant GSH system due to scavenging of NADPH, thereby augmenting ROS production, (2) Methylglyoxal which is a precursor to AGEs/RAGEs which activate pro-inflammatory and pro-oxidant molecules, (3) DAG/PKC which impairs insulin signaling and eNOS activity, activates pro-inflammatory and pro-oxidant molecules, (4) Hexosamine pathway with the resultant activation of pro-inflammatory molecules, inhibition of eNOS at the Akt activation site and activation of TGF-β with increase in ROS. All these pathways increase ROS in a positive feedback loop. AGEs, advanced glycation end products; DAG, diacylglycerol; eNOS, endothelial NO synthase; G3PDH, Glyceraldehyde 3-phosphate dehydrogenase; GSH, Glutathione; ICAM-1, intracellular adhesion molecule-I; IL-6, interleukin 6; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate; NF-κB, nuclear factor kappa B; NO, nitric oxide; PARP-1, poly ADP ribose polymerase 1; PKC, protein kinase C; RAGE, AGEs receptor; ROS, reactive oxygen species; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule 1. The symbol ⊖ means inhibition.

exacerbating the inhibition of G3PDH which is dependent on NAD as a cofactor; thus amplifying the accumulation of glyceraldehyde-3-phosphate (G3P) with the consequent flux into triose phosphate pathway via triose phosphate isomerase (TPI) yielding dihydroxyacetone phosphate (DHAP). Higher levels of triose phosphate promote the formation methylglyoxal and DAG. Methylglyoxal is a precursor of AGEs, which are formed by binding of methylglyoxal to the free amino groups of intracellular and extracellular proteins (Allaman et al., 2015; Maessen et al., 2015) (**Figure 3**).

DAG activates certain PKC isoforms by binding to their membrane-bound receptors. Consequently, active PKC isoforms may contribute to hyperglycemia-induced vascular endothelial damage by inhibiting insulin-stimulated endothelial NO synthase (eNOS) expression and NO production in endothelial cells, increasing thus the activity of nuclear factor kappa B (NF-κB) and the pro-oxidant enzyme NOX (Giri et al., 2018). Under hyperglycemic conditions, PKC-α and PKC-γ, were reported to be able to enhance NOX activity (Venugopal et al., 2002; Dasu et al., 2008). PKC-β, a molecular mediator of hypertrophic responses, was also found to be selectively over-activated in vascular and cardiac cells of animal models of diabetes. Liu et al. (2012) observed that the selective inhibition of PKC-β, improved left ventricular function and structure in a diabetes animal model of streptozotocin-injected rats. More recently, it was reported that the activation of PKC-α in the intestine of streptozotocin-induced diabetic mice contributed to the enhanced uptake of iron leading to iron loading that contributes to diabetic complications (Zhao et al., 2018). Furthermore, the inhibition of PKC-ζ was shown recently to improve insulin sensitivity and

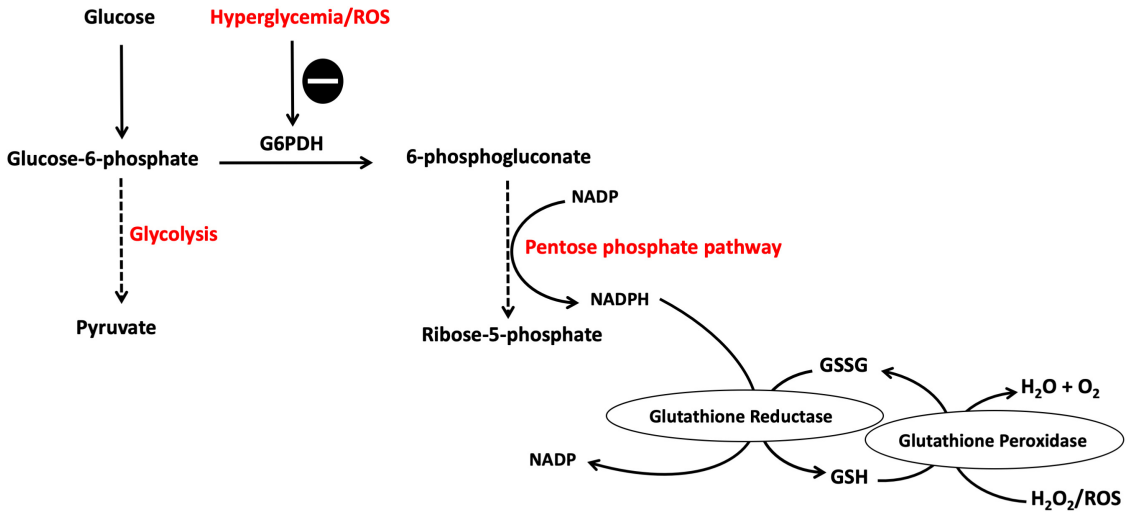

FIGURE 2 | Pentose phosphate pathway (PPP) is inhibited by hyperglycemia and ROS. Hyperglycemia-induced ROS cause the inhibition of G6PDH which is the rate limiting enzyme for this pathway. This results in reduced NADPH production, a cofactor fundamental in the GSH/peroxidase antioxidant system, with the consequent buildup of more ROS causing a vicious circle of oxidative stress induction. G6PDH, glucose-6-phosphate dehydrogenase; GSH, glutathione; NADPH, reduced NAD phosphate. The symbol ⛔ means inhibition.

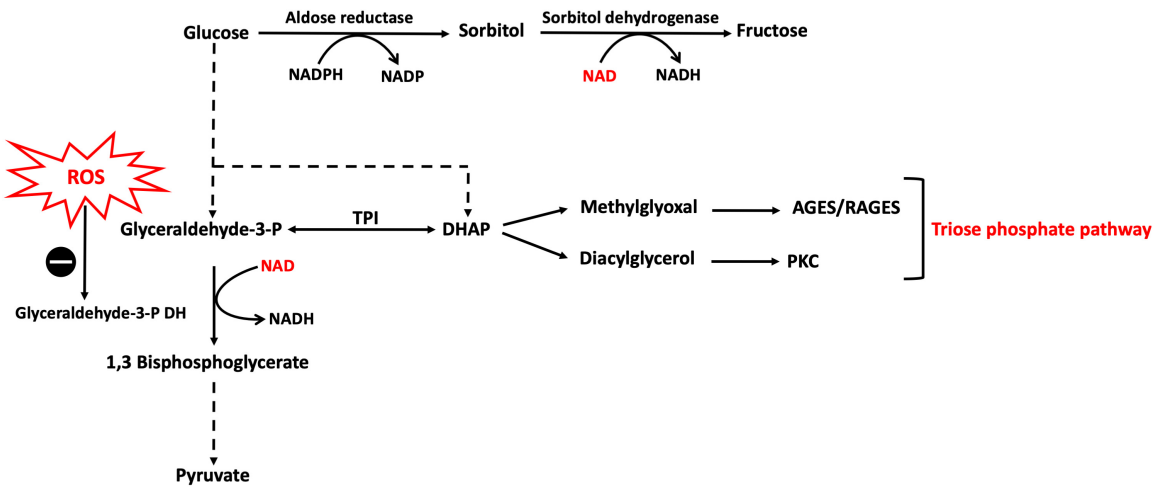

FIGURE 3 | Polyol and triose phosphate pathways activation caused by hyperglycemia-induced ROS. Hyperglycemia results in an excess of ROS production which inhibits G3PDH with the consequent accumulation of glycolytic intermediates upstream to G3P leading to increase flux into other pathways, namely polyol and triose phosphate. In polyol pathway, NAD reducing equivalent is consumed by sorbitol dehydrogenase reaction leading to reductions in NAD levels inside the cell which further confounds the inhibition of G3PDH. DHAP, dihydroxyacetone phosphate; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; NAD, nicotinamide adenine dinucleotide; TPI, triose phosphate isomerase. The symbo ⛔ means inhibition.

uptake of glucose in rat adipocytes rendered insulin-resistant by incubating them in high glucose and high insulin concentrations (Lu et al., 2018).

Hyperglycemia-induced ROS overproduction is also caused by the interaction of AGEs with their receptors (RAGEs) (Rochette et al., 2014). This interaction generates intracellular ROS and activates NF-κB, which modulates the expression of a many genes associated with inflammation and vascular remodeling, including interleukin (IL)-6, tumor necrosis factor (TNF)-α, ICAM-1, vascular cell adhesion molecule (VCAM)-1 and monocyte chemotactic protein-1 (MCP-1) (Bierhaus et al., 2001; Younce et al., 2010). Furthermore, AGEs which are present in the extracellular matrix can decrease the availability of NO, reducing thereby endothelium-dependent vasodilation (Xu B. et al., 2005). Finally, the activation of the hexosamine pathway contributes to the vascular damage induced by hyperglycemia owing to its capacity to disturb multiple cellular processes, including signal transduction, gene transcription, cell survival, and proteasome-mediated degradation (Love and Hanover, 2005). The activation of the hexosamine pathway can inhibit eNOS activity by

impairing insulin receptor activation and signal transduction and increasing ROS production (Rajapakse et al., 2009a,b).

ER Stress and Endothelial Dysfunction in Diabetes

In addition to hyperglycemia-induced oxidative stress, ER stress was also linked to various features of endothelial dysfunction in diabetics with decreased NO bioavailability and aberrant angiogenesis being two prominent features (Sheikh-Ali et al., 2010). Aberrant angiogenesis may be either an excessive angiogenic response (e.g., retinopathy, nephropathy) or a deficiency in angiogenesis (e.g., disturbed wound healing, reduced sprouting of collateral vessels, neuropathy, ischemia and peripheral vascular disease) (Kota et al., 2012). Under normal physiological conditions, the ER plays a central role in protein synthesis and co-translational protein modification through the folding of newly synthesized proteins by native disulfide bond formation to attain the final stable conformational state prior to being directed to their final destination whether it is to be secretory or membrane bound proteins. The homeostasis of ER is put at stress when the influx of the newly synthesized misfolded or unfolded polypeptide chains exceeds the repair and refolding capacity of the ER; this condition is commonly referred to as ER stress response (Walter and Ron, 2011).

Endoplasmic reticulum stress response develops both in type-1 or type-2 diabetes mellitus due to the presence of hyperglycemia that increases the demand for the synthesis of enzymatic machinery necessary to carry out complete glucose oxidation through glycolysis, tricarboxylic acid cycle (TCA) and oxidative phosphorylation by mitochondrial electron transport chain (Flamment et al., 2012; Hu et al., 2017). This increase in protein load on the ER increases the chances of accumulation of unfolded or poorly folded proteins inside the ER by the formation of incorrect or non-native disulfide bonds due to mispairing of cysteine residues in the protein being synthesized (Lisa et al., 2012). When this occurs, the cell triggers the unfolded protein response (UPR), which aims to resolve the problem by three different mechanisms working synergistically together: (I) Enhancement of gene expression of molecular chaperones to support and improve the correct folding of proteins; (II) Activation of ER-associated degradation machinery (ERAD) to get rid of aberrant proteins; and (III) Attenuation of translation rate to slow down the entry of new proteins to the ER lumen (Flamment et al., 2012). The molecular system involved in such response includes three membrane-bound proteins that orchestrate the whole process, namely inositol requiring enzyme (IRE)-1α, protein kinase RNA-like ER kinase (PERK) and activating transcription factor (ATF)-6, which under basal conditions are kept in an inactive state by binding to 78 kDa glucose-regulated protein (GRP)-78 or binding immunoglobulin protein (BiP); however, when there is an increase of misfolded proteins, GRP78/BiP preferentially binds to them hence relieving those ER effectors from their inhibition state. The dissociation of GRP78 from the three effectors is followed by the dimerization and hence activation of both IRE-1α and PERK, and the transfer of ATF-6 to the Golgi body for proteolytic processing activation before the final active ATF-6 translocates to the nucleus and stimulates the transcription of target genes. The UPR is a physiological pro-survival process which attempts to restore normal homeostatic state of the ER within the cell; however, if the stress condition remains unresolved, these three effectors involved in the UPR can also activate multiple inflammatory responses and eventually may lead to cell death through the activation of several pro-apoptotic sub-pathways (Gardner and Walter, 2011; Walter and Ron, 2011; Hassler et al., 2012; Gardner et al., 2013) (**Figure 4**).

Previous studies have demonstrated the role of ER stress response in endothelial cell inflammation and cell death in vascular diseases associated with diabetes. Work by Chen et al. (2012) demonstrated that the ATF-4-mediated activation of signal transducer and activator of transcription 3 (STAT3) is responsible for inflammation, vascular endothelial damage and loss of angiogenic capacity in streptozotocin-induced wild-type mouse model of type-1 diabetes, while ATF-4 knockout mice were protected. Other *in vitro* studies have also shown that the blockade of STAT3 reduced high glucose-mediated ER stress response activation, suggesting a crosstalk between ER stress and inflammatory signaling pathways. Moreover, another study of diabetic cardiovascular complications in streptozotocin-induced mouse model of diabetes found that the activation of the epidermal growth factor receptor (EGFR) tyrosine kinase signaling pathway participated in the induction of ER stress response and microvascular dysfunction in these mice. Enhanced EGFR phosphorylation was associated with the activation of the PERK/eukaryotic initiation factor (eIF)-2α/ATF-4 axis. The pharmacologic blockade of the kinase activity of EGFR ameliorated endothelium-dependent relaxation and increased NO production (Galan et al., 2012). Previously, Agouni et al. (2011) investigated in a mouse model of diet-induced obesity the impact of whole-body alleviation of ER stress response through the specific liver-deletion of protein tyrosine phosphatase (PTP)-1B, a protein located on the luminal surface of the ER that was shown to be implicated in the activation of ER stress (Owen et al., 2013), on endothelial cell function. Authors reported that the alleviation of ER stress response improved endothelium-dependent vasodilation in mouse aortas and improved vascular NO bioavailability (Agouni et al., 2014).

The role of ER stress in ischemia-induced neovascularization was demonstrated in type-2 diabetes genetic mouse model (*db/db*). The chemical chaperones, tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid (4-PBA), known to alleviate ER stress, improved hind-limb ischemia after femoral artery ligation. These small molecules reduced the expression of ATF-4 and C/EBP homologous protein (CHOP), enhanced blood flow recovery following ligation, and increased the expression of pro-angiogenic factors (Amin et al., 2012). There is also growing evidence that ER stress response might play an important role in promoting angiogenesis, and therefore may act as a novel mediator of the angiogenic process. This evidence implicates these pathways in the upregulation of angiogenic factors such as vascular endothelial growth factor (VEGF)-A, fibroblast growth factor (FGF), IL-8, and endothelin (ET)-1 (Paridaens et al., 2014). Notably, it was shown that ER stress-mediated

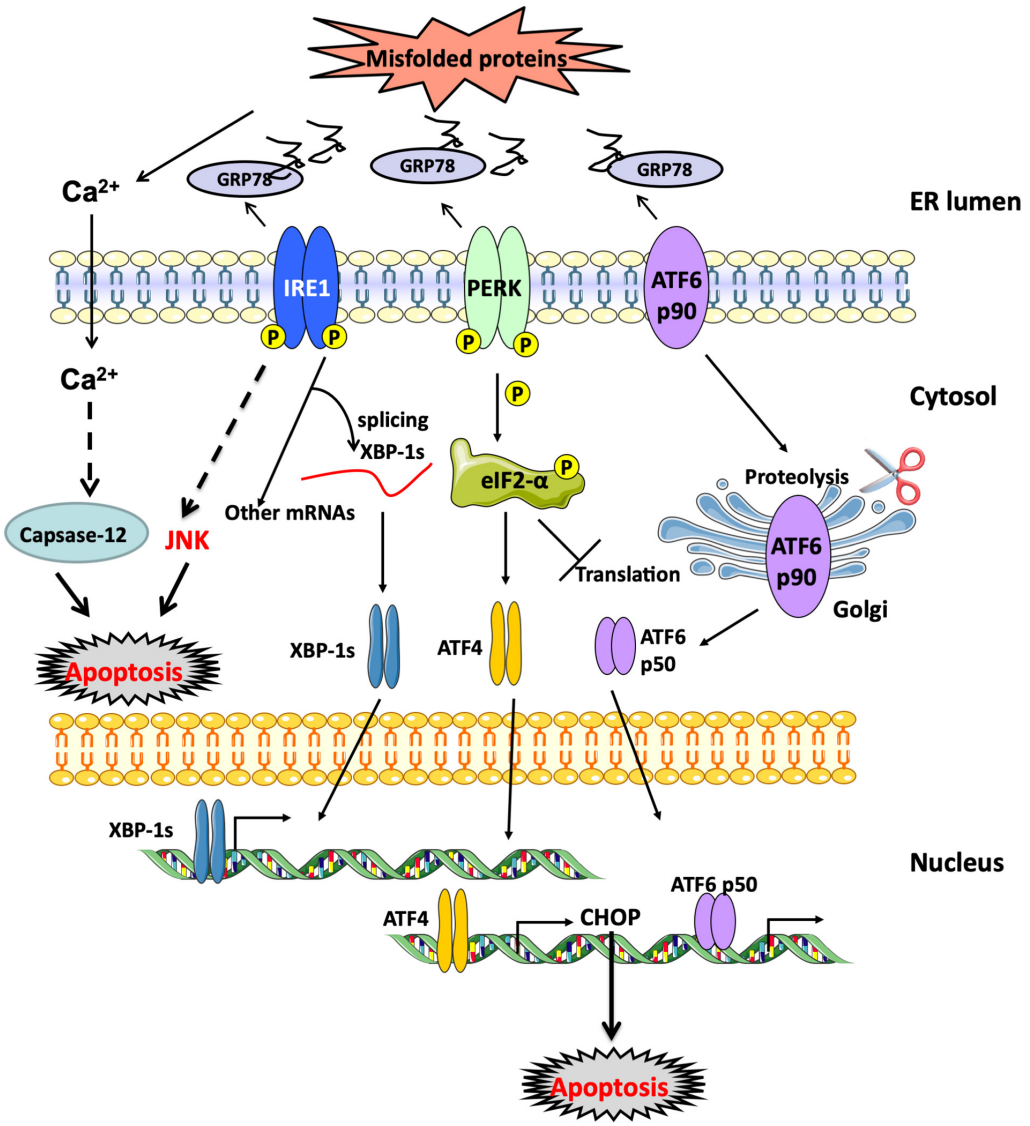

FIGURE 4 | UPR response activation. ER membrane-bound proteins, PERK, ATF-6 and IRE-1α, are activated when unfolded proteins accumulate within the ER lumen and GRP78/BiP dissociates from them. Downstream effects of PERK involve the phosphorylation of eIF-2α resulting in the attenuation of protein translation with the preferential increase in ATF-4 expression. ATF-6 (p90) translocates to the Golgi body where it undergoes proteolytic cleavage. Active cleaved ATF-6 (p50) translocates then to the nucleus and stimulates the expression of molecular chaperones to aid in the folding process. Finally, active IRE-1α through its endoribonuclease activity undergoes intron splicing of *XBP-1* mRNA to synthesize the active spliced XBP-1s which binds to the promoter regions of several genes involved in the synthesis of chemical chaperones and ERAD proteins to restore normal protein homeostasis as part of the UPR rescue response. ATF-6, activating transcription factor 6; BiP, binding immunoglobulin protein; eIF-2α, Eukaryotic translation initiation factor 2α; ERAD, endoplasmic-reticulum-associated protein degradation; GRP78, 78-kDa glucose regulated protein; IRE-1α, inositol-requiring enzyme 1α; PERK, PKR-like ER kinase; XBP-1, X-box binding protein 1.

overexpression of VEGF-A was dependent on the IRE-1α/X-box binding protein (XBP)-1, PERK/ATF-4, as well as ATF-6 arms of ER stress response. The upregulation of VEGF-A expression by UPR effectors was also reported in diabetic nephropathies and retinopathies, indicating the involvement of UPR and ER stress responses in the enhancement of angiogenesis beyond the physiological conditions (Ghosh et al., 2010). However, Maamoun et al. (2017) have reported more recently that the chemical chaperone, 4-PBA prevented intermittent high glucose-induced impaired angiogenic capacity in HUVECs characterized by a weaker ability to form tube-like structures on a matrigel matrix and a reduced the expression of VEGF-A. Nonetheless, the exact molecular mechanisms involved in the switch between pro- and anti-angiogenic stimulation by ER stress response are not fully understood.

Endoplasmic reticulum stress also plays a key role in the onset of atherosclerosis in diabetes, a major consequence of endothelial dysfunction. Several independent risk factors for cardiovascular diseases, including hyperglycemia (Werstuck et al., 2006), hyperhomocysteinemia (Werstuck et al., 2001), obesity (Ozcan et al.,

2004; Agouni et al., 2011), and dyslipidemia have been associated with ER stress, indicating that it may be a converging molecular link for atherogenesis (Cunha et al., 2008). The activation of UPR response has been observed at all stages of atherogenesis (Zhou et al., 2005). ER stress inducers can promote lipid tissue accumulation by activating the sterol regulatory element binding proteins (SREBP), which control the transcriptional regulation of lipid synthesis and transport in endothelial cells (Colgan et al., 2007). ER stress inducers also activate NF-κB, which promotes the expression of pro-inflammatory and pro-coagulant genes contributing thus to atherogenesis (Jiang et al., 2003). Moreover, ER stress effector IRE-1α causes the activation of c-Jun N-terminal kinases (JNK) which impairs insulin signaling reducing thus insulin-stimulated eNOS activity with subsequent reduction of NO production (Andreozzi et al., 2007). ER stress has also been shown *in vivo* and *in vitro* to activate pro-apoptotic caspases and cell death of human endothelial cells (Xu C. et al., 2005; Maamoun et al., 2017).

There is also evidence suggesting a crosstalk between ER stress and oxidative stress. Maintaining ER balance is intimately associated with oxidative state of the cell. In contrast to cytosol, the ER lumen is an oxidizing environment characterized by an elevated ratio of oxidized to reduced glutathione (GSSG/GSH) which encourages the proper native disulfide bond formation (van der Vlies et al., 2003). During disulfide bond-dependent protein folding, proteins are oxidized to form disulfide bonds by protein disulfide isomerase which allows proper polypeptide rearrangement to reach their final native conformational state. Any mispairing of cysteine residues would result in the formation of non-native disulfide bonds that prevent proteins from achieving their native optimal conformation, a condition that results when there is an abnormally high request for protein synthesis as in the case of hyperglycemia. In addition, hyperglycemia-induced AGEs accumulation as well as hexosamine and polyol pathways activation can impair the correct protein folding, leading to the activation of UPR and ER stress responses (Chakravarthi and Bulleid, 2004).

In normal conditions, during protein folding, electrons are transferred from targeted cysteine residues in the polypeptide chain being synthesized forming native disulfide bonds to bring the protein structure to its final conformational state. This reaction is catalyzed by two crucial enzymes, namely protein disulfide isomerase (PDI) and ER oxidoreductase (ERO)-1α, which aid in this electron transfer from the target cysteine residues to molecular oxygen, generating H_2O_2; however, in the presence of a high protein folding load, as in hyperglycemia, there is an increase in non-native disulfide bond formation, which results in GSH consumption as a protective mechanism. This results in GSH depletion contributing thus to excessive ROS generation and consequent development of oxidative stress (van der Vlies et al., 2003) (**Figure 5**). Moreover, hyperglycemia-induced oxidative stress is suggested to contribute to the inactivation of disulfide isomerases resulting in the buildup of unfolded proteins in addition to the misfolded ones (Tu and Weissman, 2004). Since protein folding is a highly energy-dependent process, protein misfolding-induced ATP depletion leads to more glucose consumption to promote mitochondrial oxidative phosphorylation to increase ATP synthesis and consequently increasing ROS production to pathological levels. As a result of unfolded/misfolded protein accumulation, Ca^{2+} leak ensues from the ER lumen into the cytosol and increased intracellular Ca^{2+} levels further stimulate mitochondrial ROS

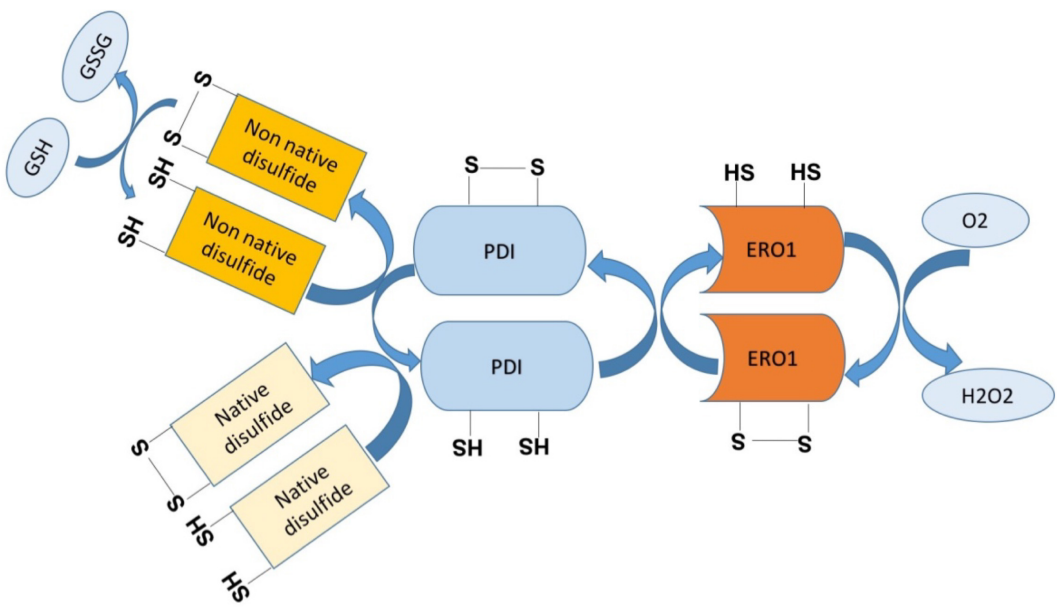

FIGURE 5 | Schematic representation showing ER protein folding as a source of ROS. During ER stress response, there is accumulation of misfolded proteins within the ER where non-native disulfide bonds form resulting in the depletion of glutathione (GSH) that is trying to correct the aberrant bonds. IRE-1α, inositol-requiring enzyme 1 α; PDI, protein disulfide isomerase.

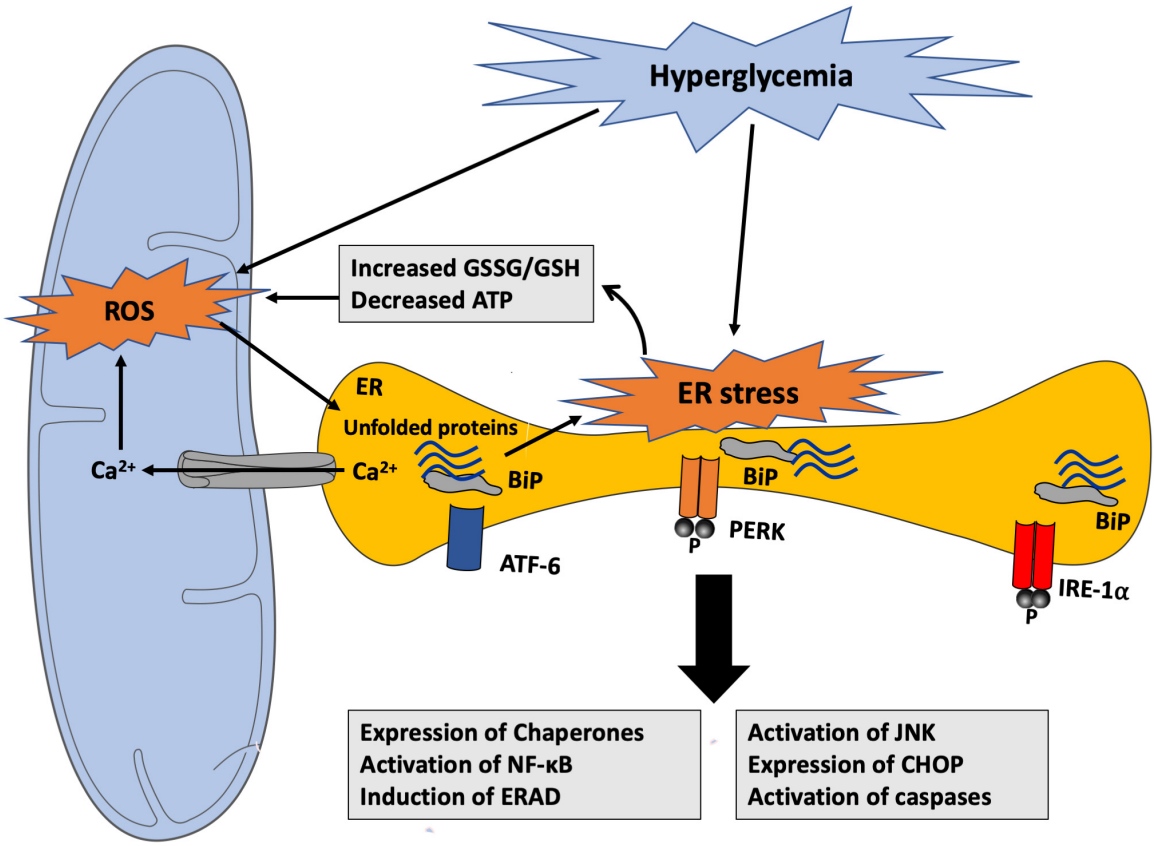

FIGURE 6 | Hyperglycemia-induced ROS generation and activation of ER stress response. Hyperglycemia-generated ROS can promote the accumulation of unfolded proteins within the ER contributing to ER stress response through different mechanisms. ER dysfunction causes GSH depletion, Ca^{2+} leak from the ER lumen into the cytosol and into the mitochondria, and ATP depletion that can impair its structural and functional integrity and stimulate mitochondrial ROS production. ROS, in turn, can alter protein folding, thereby inducing unfolded protein accumulation within the ER lumen. ATF-6, activating transcription factor 6; BiP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; ERAD, endoplasmic-reticulum-associated protein degradation; GSH, Glutathione; GSSG, oxidized Glutathione; IRE-1α, inositol-requiring enzyme 1α; JNK, c-Jun N-terminal kinases; NF-κB, nuclear factor kappa B; PERK, PKR-like ER kinase; ROS, reactive oxygen species.

production. In fact, Ca^{2+} increases the permeability of inner mitochondrial membrane and blocks the electron transport chain at the level of complex III, thereby causing electron leak to molecular O_2, thus exacerbating the ongoing ROS production (Gorlach et al., 2006) (**Figure 6**). Taken together, there is substantial evidence that both ER stress and oxidative stress are concomitantly induced in hyperglycemic conditions and they play an important role in the perturbations that ensue in the vascular endothelium leading to the development of endothelial dysfunction in diabetes.

Mitochondrial Dysfunction and Endothelial Dysfunction in Diabetes

Much evidence suggests that mitochondrial dysfunction is a major source for hyperglycemia-mediated generation of ROS through the electron transport chain, which contributes to oxidative damage pathways as discussed above, namely the polyol, triose phosphate, hexosamine, and AGEs pathways (Nishikawa et al., 2000). Normally, NADH and $FADH_2$ released from the TCA cycle translocate from the mitochondrial matrix to the inner mitochondrial membrane, where they will donate free electrons to the series of mitochondrial membrane-bound complexes. Free electrons are transferred through mitochondrial complexes I, II, III and cytochrome c (IV), until they are finally transferred to molecular oxygen to form water. The electron transfer across these complexes generates a gradient of protons in the mitochondrial intermembrane space, generating thus a proton potential gradient across the inner mitochondrial membrane, which activates ATP synthesis. When electrons are moving from complex III to complex IV, ROS radicals are generated in small amounts during normal oxidative phosphorylation process; these ROS radicals are then captured by enzymatic anti-oxidants such as glutathione (GSH) and superoxide dismutase (SOD) isoforms. Under hyperglycemic conditions, higher levels of glucose-derived pyruvate and acetyl-CoA enter the TCA cycle increasing thus the generation of NADH and $FADH_2$ that, in turn, transfer free electrons into the electron transport chain and consequently massively increase proton gradient through the mitochondrial membrane. This

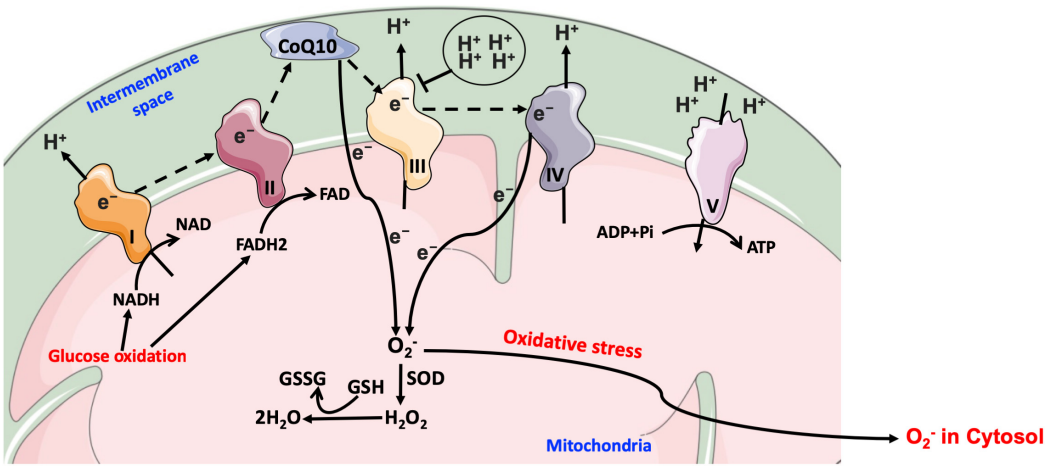

FIGURE 7 | Mitochondrial electron transport chain in hyperglycemia. Under hyperglycemic conditions, higher levels of glucose-derived pyruvate increase the flux of NADH and $FADH_2$ into the electron transport chain and consequently increase the proton gradient across the mitochondrial membrane. The transfer of free electrons to complex III is blocked, leading the electrons to be donated to coenzyme Q10, which then transfers electrons to molecular oxygen, thereby generating O_2^-. CoQ10, coenzyme Q10; e^-, electrons; O_2^-, superoxide; GSH, glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; Pi, inorganic phosphate; SOD, O_2^- dismutase.

abnormally high proton gradient blocks complex III, forcing free electrons to move back to coenzyme Q10, which then transfers these electrons to molecular oxygen in a process known as electron leak, thereby generating O_2^- anions (Rolo and Palmeira, 2006). As mentioned above, in normal physiological conditions there is still some mitochondrial ROS being generated during the electron migration at complex IV; however, it is within the capacity of the glutathione/peroxidase system and SOD to scavenge it and prevent buildup of oxidative stress (**Figure 7**).

NADPH Oxidase (NOX) Activation and Endothelial Dysfunction in Diabetes

In addition to the mitochondrial dysfunction being a major source of hyperglycemia-induced ROS overproduction, studies have shown there are other principle sources that include: NOX and uncoupled eNOS (Inoguchi et al., 2000; Volpe et al., 2018). The NOX family of enzymes were identified first in phagocytic cells, neutrophils and macrophages. They are the only family of enzymes with a primary function to generate ROS (H_2O_2, O_2^-) by catalyzing the electron transfer from cytosolic NADPH to molecular O_2 across plasma and lysosomal membranes (Thannickal and Fanburg, 2000; Salazar, 2018). There is a total of seven isoforms identified so far which are abundantly expressed in phagocytic cells. These isoforms include, NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2. Four of these are expressed by endothelial cells, NOX1, NOX2, NOX4, and NOX5 (Salazar, 2018). In endothelial cells, NOXs are believed to produce moderate levels of ROS which are needed for redox signaling during normal cell metabolism process. However, in certain pathological states, NOX expression and activity in the vasculature was found to be increased, thus contributing to excess release of ROS.

NOX isoforms consist of a catalytic subunit "NOX," which catalyzes the transfer of free electrons from cytosolic NADPH to molecular oxygen. Specifically, for NOX-1, 2, and 4, additional regulatory subunits are also required for a complete activation of the complex. These subunits include $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$. By contrast to the other isoforms, NOX-5 is a unique polypeptide subunit which is regulated by Ca^{2+}. One additional difference between these isoforms is that while NOX-1, 2, and 5 generate superoxide O_2^-, NOX-4 generates the non-charged radical H_2O_2 (Drummond and Sobey, 2014). The subunit $p47^{phox}$ is pivotal for NOX activation and is frequently named the 'organizer subunit' since it is crucial for the formation of an active NOX complex. It resides within the cytosol in resting state; however, upon its phosphorylation at several serine residues, it undergoes conformational changes to expose its intramolecular SH-3 domain which interacts with the cytosolic activator $p67^{phox}$ subunit, and chaperones it to the membrane-bound subunits, NOX and $p22^{phox}$, to finally form the active NOX complex (Schulz et al., 2011; Drummond and Sobey, 2014).

Hyperglycemia, free fatty acids, and oxidized low-density lipoprotein (LDL) have been shown to enhance endothelial NOX activity (Shen, 2010). Vessels isolated from diabetic subjects exhibit increased O_2^- production and higher expression levels of several NOX subunits ($p22^{phox}$, $p47^{phox}$, and $p67^{phox}$), suggesting that NOX isoforms are more active in diabetes (Pitocco et al., 2010). The excess of O_2^- can rapidly react with NO to form $ONOO^-$ which, in turn, oxidizes tetrahydrobiopterin (BH_4), thus making it unavailable for interaction with eNOS. In the presence of low amounts of BH_4, eNOS becomes uncoupled and donates free electrons to O_2 instead of L-arginine, producing therefore O_2^- instead of NO. Clinical studies demonstrated that BH_4 supplementation improved endothelium-dependent vasodilation in diabetic patients, highlighting the important role of uncoupled eNOS in endothelial cell dysfunction in

diabetes (Son, 2012). Collectively, hyperglycemia is tightly associated with NOX activation, mitochondrial dysfunction, and ER stress response. These cell responses are unequivocally major mechanisms underpinning ROS overproduction and oxidative stress-mediated endothelial cell damage in diabetes.

ENDOTHELIAL DYSFUNCTION AND ALTERED ANGIOGENIC CAPACITY IN DIABETES: A FOCAL ROLE FOR NO AND ROS

Blunted eNOS activity and/or protein expression and subsequent decreased NO bioavailability is a hallmark of endothelial dysfunction associated with diabetes (Hirata et al., 2010). Protein expression of eNOS is tightly controlled by several molecular processes, which include transcriptional and post-transcriptional regulation of mRNA expression (Searles, 2006), and post-translational regulation of the protein structure and function where the role of Akt activity is crucial in enhancing the phosphorylation of eNOS at Ser1177 causing its activation (Shen et al., 2006). In early stages of hyperglycemia-induced cellular stress, high glucose-induced ROS production alters Ca^{2+} homeostasis leading to Ca^{2+} leaking from ER stores into the cytosol (Ding and Triggle, 2010; Triggle and Ding, 2010). As a result, intra-ER Ca^{2+} levels fall below a critical level interfering thus with the activity of many ER chaperones which depend on Ca^{2+} availability inside the ER lumen and hence interfere with proper protein folding leading to activation of UPR and eventually apoptosis (Morgan et al., 2008). Furthermore, the resulting increase in cytosolic Ca^{2+} induces the activation of eNOS which can produce high amounts of NO that can then quickly react with O_2^- to form $ONOO^-$, thereby oxidizing BH_4 and eventually leading to eNOS uncoupling and a reduction in the bioavailability of NO (Ding and Triggle, 2010). In advanced stages, when ER stress reaches a point where it cannot be resolved, the cell must activate pro-apoptotic signals. Endothelial cells incubated in high glucose, activate IRE-1α -mediated cell death response following a disruption Ca^{2+} homeostasis due to an increase in the levels of ROS (Bishara and Ding, 2010).

The activation IRE-1α stimulates JNK and p38 mitogen-activated protein kinases (MAPK) pathways triggering thus the downstream pro-apoptotic mediators, apoptosis signal-regulating kinase (ASK)-1 and caspase-12, via CHOP activation (Xu C. et al., 2005). ASK-1 can cause NO deficiency by inhibiting eNOS through reduced phosphorylation at Ser1177 site (Yamashita et al., 2007). A study conducted by Galan et al. (2014) demonstrated in coronary artery endothelial cells that were subjected to high glucose or ER stress inducer, tunicamycin, the role of NOX in the suppression of eNOS promotor region and decreased phosphorylation. These effects were restored by a chemical chaperone, 4-PBA, that helped resolve ER stress. They also reproduced similar results *in vivo* by showing a significant reduction in endothelium-dependent and independent relaxation in control mice compared with $p47^{phox}$-deficient mice, indicating the involvement of $p47^{phox}$ subunit of NOX complex as a molecular link for ER stress-induced reduction in NO production and the consequent vascular endothelial dysfunction (Galan et al., 2014). More recently, it has been reported that high glucose-induced ER stress caused an increase in the phosphorylation of $p47^{phox}$ at Ser345 (Maamoun et al., 2017). This critical phosphorylation was found to precede the activation of NOX (Perisic et al., 2004). Nevertheless, regardless of the exact mechanisms involved in the inhibition of eNOS, it is generally accepted that high glucose induces a reduction in NO production and bioavailability and hence contributes to endothelial dysfunction.

Micro- and macrovascular beds are altered in diabetes by various changes in angiogenic mechanisms (Simons, 2005). However, from a vascular standpoint, diabetes is a paradoxical disease (Costa and Soares, 2013). Excessive angiogenesis contributes to the onset of both diabetic retinopathy (Wilkinson-Berka, 2004) and nephropathy (Osterby and Nyberg, 1987). Insufficient angiogenesis, however, plays a critical role in several pathological states including impaired wound healing process, weak neovascularization of coronary collaterals, embryonic vasculopathy during gestational diabetes, and acute transplant rejection in recipients suffering from diabetes (Galiano et al., 2004). Furthermore, diabetic neuropathy is a complication linked with reduced nutritive blood flow secondary to diabetes. Altered arteriogenesis, which is referred to as the process of remodeling (such as increase in diameter) of arteries, has also been widely observed in diabetics (Abaci et al., 1999). The weak mobilization of endothelial progenitor cells (EPC) from bone marrow and their defective function are also key features of diabetes that can cause aberrant neovascularization and hence contribute to the increase of cardiovascular risk (Tepper et al., 2002).

Angiogenesis is a process of formation of new capillary networks in response to reduced oxygen concentration (hypoxia) or pro-angiogenic molecular signals. This mechanism implicates the release of pro-angiogenic factors from both hypoxic endothelial cells and the their surrounding pericytes, which stimulate endothelial cell proliferation and sprouting of new vessels from existing ones (for review see Stapor et al., 2014). This is different from arteriogenesis, a mechanism involving the growth and development of existing arterioles following an acute vascular occlusion (Folkman and Shing, 1992). There are several equally important hypotheses for the mechanisms underpinning disturbances in angiogenesis in diabetes, including a downregulation of VEGF-A signaling response, alterations in inflammatory signals, activation of ER stress response, and accumulation of AGEs initiating oxidative stress and over-production of ROS (Tamarat et al., 2003). Accumulating data indicate that the impact of ROS on the vasculature depends essentially on the oxidative level of the environment. However, the precise amount of ROS needed to cause vascular dysfunction is difficult to predict. Under basal conditions, low ROS levels, especially hydrogen peroxide, can work as intracellular secondary messengers to modulate the action of pro-angiogenic factors such as VEGF-A; however, higher levels of ROS can conversely disturb neovascularization process (Griendling and FitzGerald, 2003a,b).

Several ROS species, including O_2^-, H_2O_2, OH^- radicals, lipid peroxides, and $ONOO^-$, have been recognized to play

major roles in vascular biology controlling the angiogenic process (Tojo et al., 2005). Ebrahimian et al. (2006) reported that diabetes-mediated oxidative stress impaired post-ischemic neovascularization. Authors found that the reduction of ROS production restored key pro-angiogenic signaling pathways (e.g., VEGF-A). Nishikawa et al. (2000) proposed that high glucose-mediated mitochondrial production of ROS can stimulate several molecular mechanisms involved in aberrant angiogenesis. Other studies indicated that O_2^- release by NOX complex plays a key role in VEGF expression and angiogenesis in a mouse model for diabetic ischemic vascular disease. In addition, increased expression of NADPH subunit NOX2 ($gp91^{phox}$) has been found to correlate with increases in ROS and decreased VEGF-A in ischemic diabetic retinopathy (Al-Shabrawey et al., 2008). Furthermore, high levels of O_2^- production by NOX are likely to scavenge NO or cause eNOS uncoupling and hence reduce NO bioavailability (Guzik et al., 2002). These changes in NO signaling pathway may also contribute to the regulation of post-ischemic angiogenic process because of the well-documented role of NO as a key modulator of bone marrow mononuclear cell (MNC) mobilization, differentiation and homing (Aicher et al., 2003). Tepper et al. (2002) reported that the phosphorylation and activation of p38 MAPK by diabetes-induced ROS production leads to impaired differentiation of bone marrow MNC into EPC *in vitro* and impaired their pro-angiogenic capacity *in vivo*. Diabetes has also been shown to activate p38 MAPK in vascular wall via signaling pathways that may involve PKC (Igarashi et al., 1999). The activation of p38 MAPK is known to downregulate EPC proliferation and differentiation which may further contribute to impaired angiogenesis in diabetes. Not only has development of oxidative stress with increased production of ROS been reported in *in vitro* and *in vivo* diabetic models of impaired angiogenesis, but also the anti-oxidant defense capacity is reduced which can also contribute to diabetes-induced oxidative stress (Wohaieb and Godin, 1987). Previous reports showed that chronic exposure of HUVECs to high glucose caused suppression of kelch-like ECH-associated protein (Keap)-1/erythroid 2–related factor (NrF)-2 pathway which is the major modulator of cytoprotective responses to stresses mediated by ROS including the HO system. The suppression of Keap1/NrF-2 correlated with impaired migration and tube-like formation capacity of HUVECs (Chen et al., 2015) (**Figure 8**).

HO-1 AND ITS PROTECTIVE EFFECTS AGAINST DIABETES-MEDIATED ENDOTHELIAL DISTURBANCES

Heme oxygenases are a group of anti-oxidant enzymes that play a pivotal role controlling intracellular levels of heme by mediating the biodegradation of heme to release the following byproducts, Fe^{2+}, CO, and biliverdin in mammalian cells (Kikuchi et al., 2005; Ryter et al., 2006; Abraham and Kappas, 2008; Rochette et al., 2018). Biliverdin, a water-soluble molecule, is then metabolized by biliverdin reductase into bilirubin. In diabetes and other cardiovascular disorders such as hypertension, the excess of free heme leads to the formation ROS species, which in turn contribute to the onset of endothelial dysfunction. The HO system, by metabolizing the excess heme, can generate various byproducts, which may initiate different cytoprotective cardiovascular effects: (I) Pro-survival effect on endothelial cells; (II) Reduced inflammation and oxidative stress in vasculature; (III) Improved vascular homeostasis and control of the vascular tone regulation; and (IV) Enhanced neovascularization. Bilirubin and biliverdin are the two main active byproducts of HO action and are considered as the strongest scavengers of ROS in the body; however, CO can have anti-apoptotic and anti-inflammatory roles via the activation of guanylyl cyclase (Stocker et al., 1987; Nitti et al., 2017). CO is capable of suppressing the release of key inflammatory molecules such as of TNF-α and IL-1β and stimulating the synthesis of the human cytokine-synthesis inhibitory factor (CSIF or IL-10) (Otterbein et al., 2000). The last byproduct of heme degradation is free iron, which despite participating in Fenton reaction, which generates the very reactive OH^- radical, also activates Fe/ATPase pump, which pumps free iron outside of the cell and enhances protein expression of ferritin which can then scavenge free iron and exert therefore beneficial effects (Crichton et al., 2002).

Three HO isoforms have been reported in humans: HO-1 (32 KDa), which is the inducible form, and two other isoforms with constitutive expression [HO-2 (36 KDa) and a putative HO-3 (33 KDa)] (Nitti et al., 2017). The inducible HO-1, is expressed at very small amounts in normal tissues, except the liver and spleen, where it plays a key role in the elimination process of damaged and aged red blood cells (Gottlieb et al., 2012). Protein expression of HO-1 can be enhanced by many stimuli, which can induce ROS production. Among the pharmacological and chemical stimuli known to induce HO-1, there is heme itself [a byproduct of HO-1 action), heavy metals (e.g., cobalt], inflammatory cytokines, ultraviolet radiation, bacterial membrane component lipopolysaccharide (LPS), hyperglycemia, H_2O_2, cell growth factors, NO, and CO (Ryter et al., 2006). On the other hand, the expression of HO-2 is not induced by the factors inducing HO-1. However, HO-2 is constitutively expressed in high levels in certain tissues such as the brain, testis, smooth muscle cells and endothelial cells (Chen et al., 2003). In addition to maintaining heme homeostasis, basal amounts of HO-2 also play a key role in anti-oxidant cellular responses by regulating the activation of extracellular SOD, Akt, and ASK-1, through the modulation of heme degradation rate into its biological byproducts. Furthermore, HO-2 plays a pivotal role in sensing levels of oxygen and in cellular response to hypoxia (Munoz-Sanchez and Chanez-Cardenas, 2014). The protective role of HO-2 in hypoxia was demonstrated in endothelial cells. HUVECs and aortic endothelial cells exposed to hypoxia were found to exhibit a stable HO-2 protein expression despite a significant decline in its mRNA expression and a decrease in general protein translation. An increased interaction of HO-2 transcript with polysomes was observed during hypoxic events, leading to a selective activation of HO-2 protein translation, which maintained protein expression levels of HO-2. The maintenance of steady levels of HO-2 protected endothelial cells from apoptosis during hypoxic episodes (He et al., 2010). Finally, HO-3 is encoded by a pseudogene derived

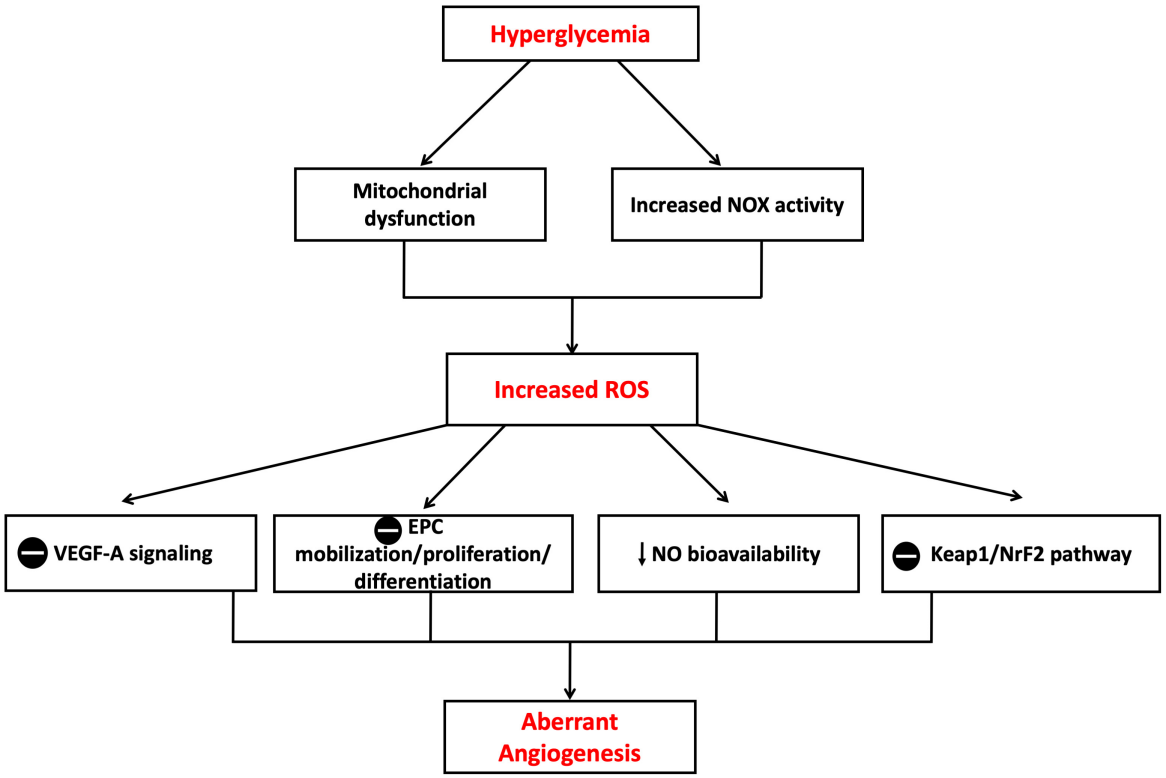

FIGURE 8 | Mechanisms of aberrant angiogenesis induced in hyperglycemia. Increased ROS production in hyperglycemic environment both *in vitro* and *in vivo* is believed to cause insult on the angiogenic process. Theories proposed include suppression of VEGF-A signaling, suppression of EPC mobilization, proliferation and differentiation, reduction in NO bioavailability and suppression of Keap1/NrF-2 pathway with resultant suppression in HO-1 expression. EPC, Endothelial progenitor cell; Keap1, Kelch-like ECH-associated protein 1; NrF-2, nuclear factor erythroid 2–related factor 2; VEGF-A, Vascular endothelial growth factor A. The symbol ⛔ means inhibition.

from the transcript of HO-2, but so far has only been identified in rats (McCoubrey et al., 1997).

The modulation of HO-1 expression involves a complex molecular interplay between different regulatory mechanisms. MAPK, phosphatidyl inositol 3-kinase (PI-3K)/Akt, protein kinases (PKA, PKC, and PKG) (Stocker and Perrella, 2006), NrF-2, activator protein (AP)-1, NF-κB, cyclic adenosine monophosphate-response element-binding (CREB) protein, and activation transcription factor (ATF)-2 are signaling pathways that have been found to play a role in gene regulation of HO-1 expression (Kim H.P. et al., 2011; Kim Y.M. et al., 2011). Of particular interest, in response to oxidative damage, NrF-2 controls the transcriptional induction of HO-1. NrF-2 is maintained in inactive form in the cytosol by interacting with protein Keap1, which prevents the nuclear translocation of NrF-2 and enhances proteasome-mediated degradation of NrF-2. Following induction of an oxidative stress state, NrF-2 is activated after its dissociation from Keap1; active NrF-2 translocates then to the nucleus and triggers the transcription of HO-1 gene. In support of the role of NrF-2 in controlling HO-1 expression, previous reports have found that fibroblasts from mice deficient in NrF-2 express low levels of HO-1 (Leung et al., 2003; Liu et al., 2007).

Several heavy metal protoporphyrins induce the expression of HO-1; however, Cobalt protoporphyrin (CoPP) is regarded as probably the most potent. CoPP indirectly modulates the production of two factors involved in regulating the expression of HO-1, NrF-2 and transcription regulator protein Bach1. Bach1 is a basic leucine zipper transcription factor that forms heterodimers with proteins from the Maf family and repress the transcription of the HO-1 gene. CoPP enhances the degradation of Bach1 protein. NrF-2 is also a member of the family of basic leucine zipper transcription factors, which is involved in protection against oxidative stress through its antioxidant response element (ARE)-directed induction of HO-1. CoPP also induces the overexpression of NrF-2 by decreasing its degradation, and being a basic leucine zipper transcription factor, the NrF-2 dimerizes with the Maf family transcription factors and together these heterodimers bind to ARE of the HO-1 promotor region resulting in enhancement in expression (Shan et al., 2006). Liu et al. (2013) also reported that CoPP increases the expression of the transcription factor forkhead box protein O1 (FOXO1), which results in transcriptional upregulation of HO-1 gene (Liu et al., 2013).

Besides this mRNA transcriptional activation of HO-1, it was found that HO-1 is also subjected to a post-transcriptional

regulatory process which aims at reducing the expression of HO-1 to prevent that its overexpression becomes constitutive. This negative regulation of HO-1 expression is under the control of two microRNA (miR) molecules, miR-217 and miR-377, which work together to reduce the mRNA levels of HO-1 and hence reduce its protein expression. Knocking down these two microRNA molecules abrogated the attenuation of HO-1 expression (Beckman et al., 2011). Lin P.H. et al. (2008) showed that HO-1 expression is also regulated by a post-translational mechanism under the action of the ubiquitin/proteasome system. These findings were further supported by the increased HO-1 expression in the presence of proteasome inhibitor, MG-132, in a model of heme-induced oxidative damage (Chen and Regan, 2005).

Role of HO-1 in Vascular Inflammation

It has been established that HO-1 is able to suppress inflammatory responses by the concomitant generation of CO and bilirubin, two anti-inflammatory molecules, and the removal of pro-inflammatory agent, heme. CO and bilirubin are reported to blunt both innate and adaptive immune responses by modulating the activation and functions of multiple cell types involved in inflammation such as immune cells, endothelial cells and platelets, which ultimately results in reduced recruitment and tissue infiltration of immune cells (Durante, 2011). The anti-inflammatory role of HO-1 was determined in HO-1 knockout mice. In these mice, HO-1 deficiency led to higher release of pro-inflammatory molecules (Kapturczak et al., 2004). In patients undergoing a surgery, higher levels of HO-1 were associated with lower levels of pro-inflammatory cytokine IL-6 (Taha et al., 2010). HO-1 was found to lower leukocyte rolling, adhesion, and transmigration to the sub-intimal space, by blunting the expression and function of surface adhesion molecules. In contrast, the inhibition of HO-1 caused the overexpression and over activation of adhesion molecules, activating thus leukocyte recruitment (Belcher et al., 2006). Multiple preclinical and clinical studies have been performed in many inflammatory vascular diseases to understand the impact of HO-1 overexpression on the pathogenesis and progression of the inflammatory process. Much evidence shows that the induction of HO-1 in the vessel wall inhibited the transmigration of monocytes to the sub-intimal space facilitated by oxidized low-density lipoproteins (LDL), leading to reduced atherosclerotic lesion buildup in mice lacking LDL receptor, a widely used model for atherosclerosis (Ishikawa et al., 2001). Circulating amounts of bilirubin in the general public were found to negatively correlate with the incidence of ischemic accidents (Mayer, 2000). This is not surprising knowing that bilirubin was found to decrease endothelial activation and dysfunction (Kawamura et al., 2005).

Role of HO-1 in Angiogenesis

HO-1 and its gas byproduct CO have been recognized for their strong pro-angiogenic roles. Several pro-angiogenic molecules, including VEGF, were found to mediate their effects via the induction of HO-1. However, depending on the situation, the pro-angiogenic properties of HO-1 could be considered as beneficial or deleterious. For instance, favoring angiogenesis is critical for many physiological responses such as wound healing and neovascularization following an ischemic episode. However, activation of angiogenesis driven by HO-1 may be deleterious in other conditions such neoplastic states (Dulak et al., 2008).

In investigating the role of HO-1 in promoting angiogenesis, Zhao et al. (2011) found that partial deficiency of maternal HO-1 caused defects in the feto-maternal interface, structural defects in placental vasculature and deficiency in spiral artery remodeling. These alterations were not related to the fetal genetic background and were only dependent on the maternal genetic status of HO-1. Moreover, restoring blood flow and the reestablishment of optimal oxygen concentrations to an injury site are also achieved by a proper angiogenic process. The wound-healing sequence of events consists of multiple steps that all require an intact angiogenesis process (Bauer et al., 2005). Growth factors (e.g., VEGF), chemokines, and hypoxia-inducible factors (HIF) all contribute to the orchestration of the multi-step wound healing procedure (Tepper et al., 2005). The deletion of HO-1 in mice led to an impaired wound healing process compared to wild-type littermates. This was partly because of an impaired EPC mobilization capacity linked to capillary formation at the site of injury (Deshane et al., 2007). Furthermore, it has been noted that the natural activation of HO-1 expression in wounded skin areas was weaker and slower in diabetic mice compared to controls. In these animal models of diabetes, the local adenoviral-mediated delivery of HO-1, improved the wound healing process (Grochot-Przeczek et al., 2009). Furthermore, HO-1 was found to confer a cytoprotective role in ischemic cardiac tissue through the activation of protein expression of pro-angiogenic factors in infarcted areas of cardiac tissue (Zeng et al., 2010). Lin H.H. et al. (2008) observed that the transfer of the HO-1 gene to infarcted heart tissue confers protection, at least partly, through the activation of VEGF-A expression and the subsequent activation of angiogenesis. In addition, the overexpression of HO-1 in mesenchymal stem cells implanted in infarcted hearts, markedly reduced apoptosis and fibrosis in cardiac tissue and improved cardiac function and remodeling (Jeon et al., 2007). Recently, Maamoun et al. (2017) have reported that the incubation of HUVECs in intermittent high glucose conditions for 5 consecutive days caused the activation of ER stress response and an impaired capacity of cells to form tube-like structures on a 3-dimensional matrigel matrix. This effect was associated with a decrease in the mRNA and protein expression of VEGF-A, indicating reduced angiogenesis capacity in these cells. Authors found that the pharmacological induction of HO-1 with CoPP reduced ER stress response and prevented the effects of high glucose-mediated ER stress on angiogenic capacity of cells, further stressing the pro-angiogenic effects of HO-1 in endothelial cells (Maamoun et al., 2017).

It is crucial to also highlight that despite the evidence in literature in support of the beneficial role of HO-1 in angiogenesis control, some reports identified a negative impact of HO-1 in tumoral neovascularization. Some human tumors, such as renal cell carcinoma and prostate cancer, exhibit elevated expression levels of HO-1 (Goodman et al., 1997). HO-1 could therefore contribute to tumor cell growth, survival and progression and ultimately participate in the resistance to drug therapy

(Nowis et al., 2006). In these conditions, the inhibition of HO-1 reduced tumor development in murine cancer models indicating a potential therapeutic role for HO-1 inhibition in certain cancer types (Dulak et al., 2008). However, there are also some reports that show HO-1 may play an anti-angiogenic role in certain cancer situations. In prostatic cancer cells (PC3), Ferrando et al. (2011) found that the overexpression of HO-1 caused the downregulation of several pro-inflammatory and angiogenic factors (NF-κB, VEGF-A, VEGF-C). To model the *in vivo* angiogenic process, the authors intradermally inoculated PC3 cells, that had been transfected with a stable HO-1 DNA construct, into immunodeficient mice and found that these cells produced tumors that were less richly vascularized than controls (Ferrando et al., 2011). However, in other conditions driven by excessive angiogenesis such as diabetic retinopathy (Wilkinson-Berka, 2004) and nephropathy (Osterby and Nyberg, 1987), HO-1 induction was still reported to be protective, indicating the complexity of the physiological actions of HO-1. For instance, Fan et al. (2012) observed in streptozotocin-injected diabetic rats pre-injected with hemin, a HO-1 inducer, that retinal ganglion cells were less prone to apoptosis compared to controls (not pre-treated with hemin), suggesting that HO-1 induction in rat retinas protected against diabetes-mediated neurodegeneration. Furthermore, the expression levels of VEGF, a strong pro-angiogenic factor which contributes to exaggerated angiogenesis, was also attenuated in retinas from streptozotocin-injected rats treated with hemin (Fan et al., 2012).

Taken together, data outlined here identify HO-1 as a key modulator of the angiogenic process in different pathophysiological conditions. Therefore, HO-1 induction could be a potential therapeutic target for the inhibition of angiogenesis in carcinogenic conditions, as opposed to being a therapeutic target for the induction of angiogenesis in diabetic cardiovascular complications. These studies also highlight the complexity of signaling pathways underpinning the action of HO-1 in angiogenesis.

Role of HO-1 in Oxidative Stress

The most well-known properties of HO-1 are its anti-oxidant functions (Hayashi et al., 1999). Of particular note, the work conducted by Ishikawa et al. (2012) demonstrated that mice lacking the gene of HO exhibited a weaker anti-oxidant capacity and an extensive lipid peroxidation. The anti-oxidant capacity of HO-1 is due to its byproducts biliverdin and CO. Bilirubin strongly scavenges several oxygen free radicals including singlet oxygen, O_2^-, $ONOO^-$ and RO_2 radicals. Moreover, unlike GSH, bilirubin, because of its lipophilic nature, is closely associated with cell membranes, and hence protects them against lipid damage by peroxidation. Bilirubin was found to work better as an O_2^- scavenger and inhibitor of $ONOO^-$-mediated protein nitration than biliverdin (Jansen et al., 2010). In addition to its ROS scavenging properties, some studies proposed that the protective actions of bilirubin are also partly related to its capacity to inhibit inducible NOS (iNOS), which eventually leads to less production of the highly reactive and potent $ONOO^-$ free radical (Wang et al., 2004). CO also mediates part of the global anti-oxidant actions of HO-1 (Srisook et al., 2006). CO was reported to block $ONOO^-$-mediated protein nitration in endothelial cells through heightened intracellular GSH levels (Li et al., 2007). The anti-oxidant role of HO-1 was further demonstrated recently in HUVECs treated with intermittent high glucose. Authors reported that the induction of HO-1 using CoPP prevented high glucose-mediated concomitant increase in superoxide production and reduction in NO release, indicating an improved NO bioavailability. These focal effects were associated with a decrease in ER stress response activation, reduced phosphorylation of NOX regulatory subunit $p47^{phox}$ at Ser345, and an enhanced phosphorylation of eNOS at its activatory site (Ser1177) (Maamoun et al., 2017).

There is accumulating evidence that oxidative stress contributes to the early stages as well as the maintenance and progression of diabetic cardiovascular complications. Therefore, HO-1, owing to its strong anti-oxidant properties, may be utilized as a viable and promising therapeutic target in these conditions. The protective actions of HO-1 induction have been demonstrated in animal models of diabetic cardiomyopathy and vasculopathy (Li et al., 2012). Moreover, *in vitro* studies have demonstrated the highly potent anti-oxidant effect of bilirubin with increased protection of vascular endothelial cells, where even concentrations of bilirubin in the nanomolar range protected cells against oxidative stress damage both through direct scavenging of ROS radicals and indirectly by blocking the activity of NOX complex (Abraham and Kappas, 2008).

Role of HO-1 in Apoptosis

A high percentage of apoptotic endothelial cells was observed in animal models of diabetes-mediated vascular injury (Negi et al., 2011). Clinical data also suggest the involvement of apoptosis in diabetic vascular diseases where the expression of pro-apoptotic molecules was high while in contrast the expression of anti-apoptotic ones was low (Arya et al., 2011). HO-1 mediates its anti-apoptotic action through multiple molecular mechanisms including the inhibition of oxidative stress, ER stress and inflammation. NF-κB was shown to control cellular apoptosis in addition to its well-known role in inflammation. HO-1 induction by CoPP was found to reduce the expression of IL-6, a gene regulated by NF-κB, and CHOP in response to stressful stimuli such as ischemia-reperfusion injury, with the consequent inhibition of apoptosis (Ben-Ari et al., 2013). Other mechanisms suggested for the anti-apoptotic effects of HO-1 include blunting the apoptotic signaling pathway mediated by the interaction of TNF-α with its receptor (Jaeschke and Woolbright, 2013). These anti-apoptotic actions of HO-1 may be driven by the release of CO, which was shown to protect against apoptosis in many cell types, including endothelial cells, fibroblasts, hepatocytes and pancreatic β-cells. Brouard et al. (2000) were able to show that HO-1 inhibits apoptosis, namely by generating CO. The inhibition of HO-1 activity by tin protoporphyrin (SnPPIX) failed to prevent endothelial cell apoptosis. The direct effect of CO on apoptosis was confirmed by showing that exposing endothelial cells to exogenous CO, while inhibiting HO-1 activity by SnPPIX, prevented apoptosis. Moreover, investigators have also explored

some of the signaling pathways behind the action of HO-1/CO, and showed that it is partially mediated by the activation of p38 MAPK signaling transduction pathway. This was further supported by the observation that the selective blockade of p38 MAPK by the over-expression of a dominant negative mutant resulted in the abolishment of the anti-apoptotic effect of HO-1 (Brouard et al., 2000). In addition, as previously discussed, in diabetic vascular disease, oxidative stress occurs in endothelial cells which subsequently leads to apoptosis. Hence, HO-1 by merely exerting its anti-oxidant effects is reported to protect these cells from apoptosis (Schmeichel et al., 2003). Other studies focused on the role of bilirubin in this pro-survival behavior of HO-1. It has been shown that bilirubin was found to mediate its anti-apoptotic role by upregulating extracellular-signal-regulated kinase (ERK) MAPK and PI-3K/Akt/eNOS pathways (Hung et al., 2010).

More recently, pharmacological induction of HO-1 using CoPP was reported to protect endothelial cells from high glucose-mediated apoptosis through the alleviation of ER stress response. Maamoun et al. (2017) observed that the exposure of HUVECs to intermittent high glucose for a duration of 5 days led to cell death, while induction of HO-1 prevented this deleterious manifestation. The cytoprotective effects of HO-1 were associated with a reduction in the activation of PARP-1 and caspases 3 and 7. Furthermore, HO-1 induction in HUVECs prevented the activation of NF-κB and JNK pathways caused by high glucose treatment, indicating reduced cellular inflammation in the presence of HO-1 (Maamoun et al., 2017). While the amount of scientific evidence is still relatively small, some studies suggest HO-1 activation as a novel and promising strategy to improve survival of endothelial cells through HO-1 byproducts, bilirubin and CO, which may delay the development of various diabetic cardiovascular complications.

CONCLUSION

Crosstalk between oxidative stress and ER stress significantly contributes to the onset and development of endothelial dysfunction and altered angiogenesis and therefore represent major therapeutic targets for alleviating micro- and macrovascular complications associated with this metabolic disturbance. HO-1 carries out, anti-oxidant, anti-inflammatory, anti-apoptotic and angiogenic actions, through its by-products CO and bilirubin, and can thus affect therefore multiple cellular pathways involved in endothelial dysfunction, including oxidative stress and ER stress response. As a multi-target molecule affecting major aspects of cardiovascular dysfunction, HO-1 induction represents a potential therapeutic approach for cardiovascular complications associated with diabetes.

AUTHOR CONTRIBUTIONS

HM and AA wrote the manuscript and constructed the figures. TB, GP, and SM contributed to selected sections and critically revised the manuscript. All authors approved the final version for submission.

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Plasma Heme Scavengers Alpha-1-Microglobulin and Hemopexin as Biomarkers in High-Risk Pregnancies

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Women with established preeclampsia (PE) have increased plasma concentration of free fetal hemoglobin. We measured two hemoglobin scavenger system proteins, hemopexin (Hpx) and alpha-1-microglobulin (A1M) in maternal plasma using enzyme-linked immunosorbent assay during the late second trimester of pregnancy in women with high and low risk of developing PE. In total 142 women were included in nested case-control study: 42 women diagnosed with PE and 100 controls (49 randomly selected high-risk and 51 low-risk controls). The concentration of plasma A1M in high-risk controls was higher compared to low-risk controls. Women with severe PE had higher plasma A1M levels compared to women with non-severe PE. In conclusion, the concentration of plasma A1M is increased in the late second trimester in high-risk controls, suggesting activation of endogenous protective system against oxidative stress.

Keywords: preeclampsia, heme, hemopexin, alpha-1 microglobulin, aspirin

INTRODUCTION

Preeclampsia (PE) is a relatively common hypertensive disorder in pregnancy, affecting 4,6% of pregnancies worldwide (Abalos et al., 2013). The specific etiology of PE is, however, still not completely described. According to the most well-accepted model, PE is a two-stage disorder (Redman, 1991; Redman et al., 2014). The abnormal modification of the spiral arteries during

placental development is thought to be the initial stage leading to reduced utero-placental perfusion and increased oxidative stress that in turn causes placental damage. Circulating toxic factors derived from the placenta cross the blood-placenta barrier and leak into the maternal circulation where they in turn trigger an inflammatory response and general endothelial damage. As a consequence of that, general organ damage develops, which leads to the typical manifestations of PE after 20th week of gestation, including hypertension, edema and proteinuria. Circulating syncytiotrophoblast microvesicles (Smarason et al., 1996), free fetal DNA (Zhong et al., 2002), cytokines (Hamai et al., 1997) and antiangiogenic factors (Powe et al., 2011) have been postulated as endothelial toxic factors derived from the fetus and the placenta. However, we still lack a full explanation on how placental damage leads to distinct maternal and fetal manifestations that occur either during early pregnancy or late pregnancy, so called early onset PE and late-onset PE. Early onset PE is linked to poor placentation while the late-onset is more determined by maternal risk factors such as obesity, diabetes mellitus and chronic hypertension, which are associated with a higher pre-pregnancy level of vascular inflammation (Roberts and Redman, 1993; Ness and Roberts, 1996).

Extracellular fetal hemoglobin (HbF) has been introduced in a series of earlier studies and suggested to have a crucial role in the etiology of PE (Hansson et al., 2013). Increased synthesis of HbF in the placenta was indicated by an up-regulation of the HbF genes and there was an accumulation of extracellular HbF in the vascular lumen of PE placenta (Centlow et al., 2008). Extracellular HbF induces oxidative stress by formation of reactive oxygen species resulting in damage to the blood-placenta barrier and leakage of extracellular HbF into the maternal circulation (May et al., 2011). As a consequence, plasma concentration of extracellular HbF has been shown to be increased in maternal plasma as early as the first trimester in women who later develop PE (Anderson et al., 2011). Increased plasma levels in the late third trimester has been shown to correlate to the maternal blood pressure (Anderson et al., 2018).

There are several defense mechanisms which protect against the harmful effects of extracellular hemoglobin (Hb). Haptoglobin (Hp) is the most important protective scavenger protein that binds extracellular Hb in plasma resulting in a complex that in turn is cleared via CD163 receptors on macrophages (Kristiansen et al., 2001; Schaer et al., 2006). Hemopexin (Hpx) has a complementary role to bind extracellular heme that is released as a metabolite when Hb is degraded by the rate-limiting enzyme heme-oxygenase (HO-1) (Nielsen et al., 2010; Tolosano et al., 2010). The resulting complex is cleared from the circulation by liver parenchymal cells via receptor-mediated endocytosis involving CD91/LRP1. Alpha-1-microglobulin (A1M) is another component of the heme scavenger system (Akerstrom and Gram, 2014). It is a lipocalin with heme-binding properties as well as being an antioxidant due to radical-scavenging and reductase properties. In a series of studies (Gram et al., 2015; Anderson et al., 2018), it has been shown that the plasma levels of Hp and Hpx are reduced, suggesting that in cases where the maternal endogenous protection system against extracellular HbF is overwhelmed, PE becomes clinically manifest. Cellular A1M expression of A1M is upregulated by increased oxidative stress and Hb/heme exposure (Olsson et al., 2007) and previous investigations have shown increased circulating plasma levels of A1M in women pregnant with PE (Olsson et al., 2010; Anderson et al., 2011) consistent with high circulating levels of Hb, heme and oxidants in this disease.

In the present study, we analyzed the plasma levels of A1M and Hpx in order to further understand the dynamics of these components of the Hb/heme scavenger system in the second trimester in women with high and low risk of developing PE. The cohort of patients were stratified according to known maternal risk factors, intervention with acetylsalicylic acid (ASA) as well as neonatal outcome.

MATERIALS AND METHODS

Study Population

The present nested case-control study is a part of the multidisciplinary "Prediction and Prevention of Pre-eclampsia and Intrauterine Growth Restriction" (PREDO) project. Women with known risk factors for PE were prospectively recruited between September 2005 and June 2009 at ten participating maternity clinics in Finland. The ethics Committee at the Helsinki and Uusimaa Hospital District approved the study and written informed consent was obtained from all participants.

In total 142 women were included in this study: 42 women diagnosed with PE and 100 controls (49 randomly selected high-risk and 51 low-risk controls). Seven women with PE participated in the ASA trial (part of the PREDO project), as well, and were treated with low dose acetylsalicylic acid (mini-ASA 100mg/d) starting before 14th week of gestation. Three women who were taking mini-ASA and did not develop PE, were included as controls for this sub-group. The inclusion and exclusion criteria are described in **Supplementary Table S1**.

Preeclampsia was defined as a systolic blood pressure $\geq$140 mmHg and/or a diastolic blood pressure $\geq$90 mmHg occurring after 20th weeks of gestation combined with a urinary 24-h protein excretion of $\geq$0.3 g or the dipstick equivalent in two consecutive measurements. Severe PE was defined as systolic blood pressure $\geq$160 mmHg and/or diastolic blood pressure $\geq$110 mmHg and/or proteinuria $\geq$5 g/24 h. Small for gestational age (SGA) was defined as a birthweight $\leq$minus 2 SDs.

All participants had their first visit at 12^{+0}–14^{+0} weeks of gestation. Uterine artery blood flow was measured with Doppler ultrasound examination. Gestational age was confirmed by crown-rump length measurement. The first trimester mean arterial pressure (MAP) was calculated with the equation: MAP = diastolic blood pressure + (systolic blood pressure – diastolic blood pressure)/3.

Fasting blood samples were collected in all three trimesters. Plasma was separated within an hour by centrifugation and stored in -80°C until analysis. In the present study we determined serum A1M and Hpx concentrations from samples drawn at 26^{+0} to 28^{+0} weeks of gestation.

Hemopexin ELISA

The Hpx concentrations were measured with a Human Hemopexin ELISA Kit from Genway Biotech Inc. The analysis was performed according to manufacturer's instructions and the absorbance was read at 450 nm using a Wallac 1420 Multilabel Counter.

A1M ELISA

The A1M concentrations were measured with an in-house A1M ELISA. Flat-bottom ninety six-well microtiter plates were coated with mouse monoclonal anti-A1M antibodies (clone 35.14) by incubation overnight at +4°C under sealing film with 100 μl/well of a 5 μg/ml-solution in PBS. After washing three times with PBS + 0.05% tween-20, 100 μl of human urinary A1M reference standard samples (1.56 – 100 ng/ml in PBS + 0.05% tween-20) or unknown plasma samples (diluted 1000× with PBS + 0.05% tween-20) were added to the wells and incubated under sealing film for 1 h at room temperature, darkness and rotational shaking 250–500 rpm. After washing three times with PBS + 0.05% tween-20, 100 μl/well of the detection antibody solution was added (horse radish peroxidase-coupled mouse monoclonal anti-A1M antibody clone 57.10; 5 ng/ml in PBS + 0.05% tween-20) and incubated under sealing film for 1 h at room temperature, darkness and rotational shaking 250–300 rpm. After washing three times with PBS + 0.05% tween-20, 100 μl/well of TMB substrate (SureBlue^{TM} TMB Microwell Peroxidase Substrate, KPL cat. no. 50-00-04) was added, sealed, and incubated 20 min without shaking, and the reaction was stopped by adding 100 μl/well of 1 M sulfuric acid. Absorbance at 450 nm was read in a Wallac 1420 Multilabel Counter (Perkin Elmer Life Sciences). The monoclonal anti-A1M antibodies were prepared against human urinary A1M by Agrisera AB (Vännäs, Sweden). Human urinary A1M was prepared in our lab as described (Akerstrom et al., 1995).

Statistical Analysis

Statistical analyses were performed using SPSS version 25.0 statistic software package. Normally distributed data were analyzed using one-way ANOVA followed by Tukey's *post hoc* tests. Kruskal-Wallis and Mann-Whitney test were used in case the data were not normally distributed and Bonferroni corrections were used in *post hoc* comparisons. Statistical significance was defined as $p < 0.05$.

RESULTS

Patient demographics and clinical characteristics are shown in **Table 1**, **2**. Women affected by PE had higher body mass index (29,3 kg/m^2) compared to controls (23,7 kg/m^2). There were less primiparas among women affected by PE compared to controls. There were less women with a previous pregnancy complicated by PE among controls compared to women affected by PE.

The distributions of plasma Hpx and A1M across the groups are shown in **Figures 1A,B**. Pairwise comparisons using significance values adjusted by the Bonferroni correction for multiple tests revealed that the concentration of Hpx in women with PE was higher compared to low-risk normotensive women (median concentration 1.21 mg/ml versus 1.04 mg/ml, $p = 0.014$). There was no significant difference in Hpx concentration between women with PE and high-risk normotensive women (median concentration 1.11 mg/ml) or between high-risk and low-risk normotensive women. The concentration of A1M in high-risk controls was higher compared to low-risk controls (median concentration 16.08 μg/ml vs. 13.09 μg/ml, $p = 0.002$). There was no significant difference in A1M concentration between women with PE and high-risk controls or between women with PE and low-risk controls.

The concentrations of plasma Hpx and A1M in women with PE are shown in **Table 3**. We found no difference in Hpx or A1M concentrations between women with PE who gave birth to SGA infant and women with PE who gave birth to non-SGA infant. The concentration of A1M in women with severe PE was higher compared to women with non-severe PE. The distributions of Hpx and A1M across the subgroups of PE, high- and low-risk controls are shown in **Figures 1C,D**.

TABLE 1 | Demographics of patients and controls.

	Women not affected by PE (n = 100)	Women affected by PE (n = 42)	*P*-value	OR[a]	95% CI	
					Lower	Upper
Age, years (SD)[b]	31.3 (4.4)	31.6 (5.2)	0.74	1.01	0.94	1.10
BMI, pre-pregnancy, kg/m^2 (IQR)[c]	23.7 (7.3)	29.3 (10.6)	<0.01	1.09	1.03	1.15
Primiparous, n (%)	42 (42.0)	10 (23.8)	0.04	1.53	1.01	2.30
Infertility treatment, n (%)	11 (11.0)	5 (11.9)	0.77	1.19	0.38	3.70
Chronic disease, n (%)	37 (37.0)	22 (52.4)	0.67	0.18	0.80	3.26
Education, n (%)						
Elementary or less[d]	0 (0.0)	3 (7.9)	0.02			
High school or vocational school	22 (22.0)	8 (19.0)	0.64	0.90	0.36	2.26
Intermediate	32 (32.0)	19 (45.2)	0.83	0.90	0.36	2.26
University	40 (40.0)	7 (17.0)	0.01	0.32	0.13	0.79
Prior preeclampsia	14 (14.0)	21 (50.0)	<0.01	0.16	0.07	0.37
SGA in previous pregnancy	9 (9.0)	4 (9.5)	0.92	0.94	0.27	3.24
Chronic hypertension	14 (14.0)	10 (23.8)	0.16	0.52	0.21	1.29
Prior GDM	4 (4.0)	4 (9.5)	0.32	0.50	0.13	1.96
BMI ≥ 30 kg/m^2	22 (22.0)	16 (38.1)	0.05	0.46	0.21	1.00
Prior fetus mortuus[d]	2 (2.0)	1 (2.4)	1.00			

[a]binary logistic regression, [b]mean, [c]median, [d]fisher's exact test; PE = preeclampsia, OR = odds ratio, CI = confidence interval, SD = standard deviation, BMI = body mass index. There was one type I diabetes mellitus and one Sjögren's syndrome in women who did not develop preeclampsia. There was not systemic lupus erythematosus in either group.

TABLE 2 | Clinical characteristics of patients and controls.

	Women not affected by PE *n* = 100	Women affected by PE *n* = 42	*P*-value	OR	95% CI Upper	95% CI Lower
Weight change during pregnancy, kg/m^2	14.5 (7.8)	12.0 (6.6)	0.18	0.95	0.88	1.02
Gestational diabetes, n (%)	17 (17.0)	12 (28.6)	0.12	0.51	0.22	1.20
I trimester mean arterial pressure, mmHg	89.7 (15.7)	98.8 (13.8)	<0.01	1.07	1.04	1.11
I trimester mean uterine artery PI	0.99 (0.35)	1.25 (0.47)	<0.01	14.64	3.45	62.04
Highest mean arterial pressure, mmHg	100.0 (13.5)	128.5 (15.2)	<0.01	1.16	1.11	1.22
Gestational weeks at birth	40.3 (1.9)	38.4 (3.2)	<0.01	0.53	0.41	0.70
Apgar score at 5 min	9 (1)	9 (2)	0.32	0.80	0.51	1.25
Umbilical artery pH	7.25 (0.13)	7.25 (0.13)	0.24	0.08	<0.01	5.57
Newborn birthweight, g	3590 (651)	3109 (1259)	<0.01	0.999	0.998	0.999
Placental weight, g	605 (175)	540 (173)	<0.01	0.995	0.992	0.998

Continuous variables are presented as median values with interquartile range in parenthesis. n=number of cases, PE=preeclampsia.

Seven PE women received mini-ASA during the pregnancy and one of them gave birth to SGA infant. Thirty-five PE women did not receive mini-ASA and eight of them gave birth to SGA infant. Women who received ASA had higher Hpx concentration compared to women who did not receive ASA (median concentration 1,28 mg/ml versus 1,09 mg/ml, $p = 0{,}025$).

DISCUSSION

To the best of our knowledge, this is the first study evaluating the levels of maternal plasma Hpx and A1M in the late second trimester in PE women as well as in high- and low-risk controls without PE. The strength of the study is a carefully characterized cohort, where both women with predetermined risk factors for PE and a low-risk reference group were prospectively recruited (Girchenko et al., 2017).

Our analysis shows a significantly higher plasma A1M concentration in high-risk controls compared to low-risk controls, while there was no significant difference in concentration of A1M between PE women and controls. In previous studies (Olsson et al., 2010; Anderson et al., 2011, 2016; Gram et al., 2015), the concentration of A1M in PE women was increased compared to controls when it was analyzed during the first trimester and third trimester, 24 h prior to delivery. In the aforementioned studies, normotensive pregnant women had been studied as a single undivided group, irrespective of risk factors for PE. However, it is reasonable to hypothesize that high-risk women are a distinct group characterized by increased oxidative stress compared to low-risk women.

In the present cohort, pre-pregnancy obesity was among the criteria conferring high-risk status to normotensive pregnant women. Obesity is associated with systemic oxidative stress (Furukawa et al., 2004). It can therefore be assumed that in high-risk controls, the endogenous protection system against oxidative stress is activated. The housekeeping protein A1M is an extravascular scavenger and tissue repair protein which has an important role in cleaning oxidative radicals and heme. It is upregulated during oxidative stress in general and by hemolysis specifically (Akerstrom and Gram, 2014). Thus, our results may suggest that increased oxidative stress is present in high-risk normotensive pregnant women compared to low-risk controls in late second trimester. A serious weakness with this argument, however, is that we have not included evaluation of oxidative stress markers. Evaluation of oxidative stress markers and reactive oxidative species measurements need to be undertaken before the association between A1M and oxidative stress in high-risk normotensive women is more clearly understood. Although A1M has not been shown to be an acute-phase protein, increased levels of A1M in high-risk normotensive women as a result of chronic inflammation related to obesity might be another explanation and needs to be clarified in further studies (Akerstrom and Gram, 2014).

Interestingly, the median plasma A1M concentration in high-risk normotensive women was increased compared to PE women, although the difference was not statistically significant. Higher concentration of A1M may confer its protective effect ameliorating the clinical impact of oxidative stress and preventing the development of PE in high-risk controls who remain asymptomatic. In line with this assumption, intravenous administration of a recombinant version of A1M in PE animal models has been successful in eliminating or at least significantly reducing the manifestations of preeclampsia (Sverrisson et al., 2014; Nääv et al., 2015; Gunnarsson et al., 2017). One can therefore speculate that the administration of exogenous A1M might have a similar effect in humans and PE women also.

Recent evidence suggests that extracellular HbF is elevated in the fetal circulation of pregnancies complicated by fetal growth restriction (Brook et al., 2018). We expect that among SGA infants in our cohort there are both constitutionally small infants and infants that have not reached their growth potential because of placental dysfunction. In the latter case, we expect that extracellular HbF in the fetal circulation is elevated causing, as we have previously shown (Centlow et al., 2008; May et al., 2011), damage to blood-placenta barrier and consequently leaking into maternal circulation, depleting Hpx. We hypothesized thus that women with PE and SGA infant would have lower levels of Hpx compared to women with PE and non-SGA infant, because Hpx binds and detoxifies heme and HbF that is released from the feto-placental unit. Although the levels of plasma Hpx was lower in women with PE who gave birth to SGA infant compared to women with PE and non-SGA infant, the difference was not significant. Type II error is possible due to a small sample size. One woman in the group of women with PE who gave birth to SGA infant had exceptionally high plasma Hpx and A1M values and this increased variability substantially. This woman differed from the other in that group by having a medical history of

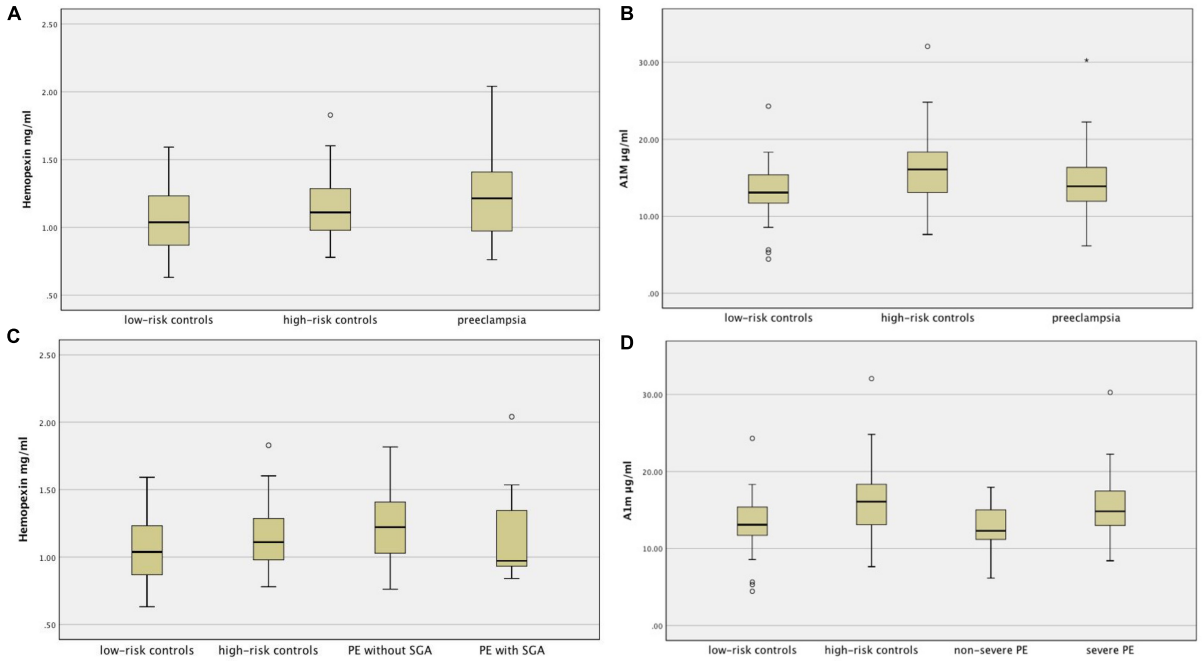

FIGURE 1 | (A): Distribution of hemopexin across groups. **(B)**: Distribution of A1M across groups. **(C)**: Distribution of hemopexin across subgroups of PE, high- and low-risk controls. **(D):** Distribution of A1M across subgroups of PE, high- and low-risk controls.

TABLE 3 | Values of A1M and hemopexin across subgroups of women with PE.

		Hemopexin (mg/ml)	A1M (µg/ml)
PE onset	late onset (n = 31) versus early onset (n = 11)	1.22 (0.32) versus 1.16 (0.64), p = 0.66	13.34 (3.26) versus 16.66 (3.96), p = 0.07
SGA	no (n = 34) versus yes (n = 8)	1.22 (0.41) versus 0.97 (0.52), p = 0.183	13.20 (3.84) versus 16.85 (5.83), p = 0.11
PE severity	mild (n = 21) versus severe (n = 21)	1.22 (0.56) versus 1.21 (0.44), p = 0.84	12.30 (4.46) versus 14.83 (5.23), p = 0.04
ASA	no (n = 35) versus yes (n = 7)	1.20 (0.43) versus 1.31 (0.51), p = 0.16	14.24 (4.42) versus 12.13 (3.10), p = 0.30

Continuous variables are presented as median values with interquartile range in parenthesis. n=number of cases.

chronic hypertension and thus superimposed PE and she was the only woman who received mini-ASA in the group of women with PE who gave birth to SGA infant.

Recent studies (Ferrazzi et al., 2018; Tay et al., 2018) suggest that women with PE and SGA or growth restricted fetus have a distinct cardiovascular phenotype characterized by lower cardiac output and higher peripheral vascular resistance. It is tempting to speculate that there is a connection between the impaired cardiovascular function and the decreased levels of heme scavenger Hpx. A possible explanation may be leakage of extracellular HbF of fetoplacental origin in maternal circulation. As a consequence, heme is released from metabolized extracellular HbF and depleting Hpx as recently shown (Anderson et al., 2018). Imbalance in the scavenging capacity causes vasoconstriction by reducing nitric oxide availability and impaired cardiac function. In fact, heme has been shown to induce contractile dysfunction in human cardiomyocytes *in vitro* (Alvarado et al., 2015). Furthermore, Hpx has been shown to have cardio-protective effect and it preserves systolic function by limiting heme-driven oxidative stress in mice (Ingoglia et al., 2017). Further studies, where cardiac function is evaluated simultaneously with the concentration of heme in maternal circulation, are needed to further clarify whether this suggested mechanism has clinical relevance.

In contrast to previous studies, we found increased plasma concentration of Hpx in PE women compared to low-risk controls. After excluding controls and PE women treated with mini-ASA, there was no statistically significant difference in the concentrations of Hpx between PE women and controls. In previous studies, where women treated with mini-ASA were not included, the concentration of Hpx was decreased, albeit marginally, in early pregnancy in women who later developed PE (Anderson et al., 2016). The concentration of Hpx in PE women has been shown to be even more decreased compared to controls just before delivery (Anderson et al., 2018). Thus, when focusing on women not treated with mini-ASA, our results suggest that Hpx depletion, due to heme-Hpx binding, is a continuous process, which starts slowly in early pregnancy and intensifies in third trimester when PE is manifested clinically. Further studies with a significantly larger patient group are needed if we try to detect the very marginal differences of the early stages of this process in order to clarify the dynamics of this process.

As regards to mini-ASA prophylaxis, 3 high-risk controls and 7 PE women who had participated in ASA trial were included in this study. Overall, these 10 women had significantly higher plasma Hpx concentration compared to controls and PE women who did not receive mini-ASA prophylaxis. Mini-ASA could possibly have an impact on Hpx concentration by improving placental perfusion. We have previously hypothesized that the up-regulation of HbF gene expression in the preeclamptic placenta may be induced by hypoxia caused by insufficient placental perfusion (Gram et al., 2015). Mini-ASA may improve the placental perfusion through inhibition of the potent vasoconstrictor Thromboxane A2 (TXA2) or by preventing placental thrombosis formation improving the blood flow in the PE placenta. Improved placental perfusion could then possibly prevent up-regulation of the HbF gene expression thereby reducing the leakage of HbF and heme into the maternal circulation. As previously shown, small amount of heme administered intravenously in rhesus monkeys increases the Hpx levels by increasing the rate of Hpx synthesis (Foidart et al., 1982). In contrast, large amount of heme has been shown to reduces Hpx levels by increased Hpx catabolism. Further studies are needed to understand the role of mini-ASA on Hpx dynamics.

CONCLUSION

This study shows increased concentration of plasma A1M in high-risk normotensive pregnant women in late second trimester.

AUTHOR CONTRIBUTIONS

SH, BÅ, and HL conceived and designed the analysis. GK and KM performed the analysis and wrote the manuscript. KM, PV, KR, EH, EK, and HL collected the data and contributed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00300/full#supplementary-material

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Conflict of Interest Statement: SH and BÅ holds patent related to diagnosis and treatment of preeclampsia and are co-founders of A1M Pharma and Preelumina Diagnostics (www.a1m.se). The pre-existing intellectual properties involve 4 patents owned by A1M Pharma;

1. HBF and A1M as early stage markers for preeclampsia-1550535
2. Medical use of A1M-2638915
3. Diagnosis and treatment of preeclampsia-201500335
4. Biomarkers for preeclampsia-PA 2015 70146

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