S-nitrosoglutathione reductase deficiency causes aberrant placental S-nitrosylation and preeclampsia.

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Abstract:

Preeclampsia (PE), a leading cause of maternal and fetal mortality and morbidity, is characterized by an increase in S-nitrosylated (SNO) proteins and reactive oxygen species (ROS), suggesting a pathophysiologic role for dysregulation in nitrosylation and nitrosative stress. Here we show that mice lacking S-nitrosoglutathione reductase (*GSNOR*$^{-/-}$), a denitrosylase regulating protein S-nitrosylation, exhibit a PE phenotype, including hypertension, proteinuria, renal pathology, cardiac concentric hypertrophy, decreased placental vascularization, and fetal growth retardation. ROS, nitric oxide (NO) and peroxynitrite levels are elevated. Importantly, mass spectrometry reveals elevated placental SNO-amino acid residues in *GSNOR*$^{-/-}$ mice. Ascorbate reverses the phenotype except for fetal weight, reduces the difference in the S-nitrosoproteome, and identifies a unique set of SNO-proteins in *GSNOR*$^{-/-}$ mice. Importantly, the human preeclamptic placenta exhibits decreased GSNOR activity and increased nitrosative stress. Therefore, deficiency of GSNOR creates dysregulation of placental S-nitrosylation and preeclampsia in mice, which can be rescued by ascorbate. Coupled with similar findings in human placentas, these findings offer valuable insights and therapeutic implications for PE.

Introduction

Preeclampsia (PE), is a life-threatening disorder of pregnancy, characterized by new onset hypertension, proteinuria, abnormal maternal cardiovascular and renal adaptations, poor placental vascularization and fetal growth restriction. PE affects up to 10% of pregnancies, is a leading cause of maternal and fetal/neonatal mortality and morbidity worldwide (1), and maternal mortality rates have been steadily rising over the past 30 years in large part due to cardiovascular complications (2). The pathogenesis of PE is incompletely understood, but emerging data support a role for impaired protein S-nitrosylation, which coupled with increased ROS, can produce nitroso-redox imbalance (3-5). A paradoxical finding in preeclampsia is the elevation in circulating S-nitrosylated (SNO)-albumin (4, 5) because elevated SNO-albumin would traditionally be considered a vasorelaxant and an anti-oxidant (6). Furthermore, cellular SNO level is elevated despite factors that are assumed to abrogate the formation of nitroso-thiols, including oxidative stress and defects in nitric oxide (NO) bioavailability (5, 7). These findings raise two alternative pathogenic possibilities: Either SNO increases to compensate for the increased ROS levels or elevated SNO directly reflects abnormal regulation of SNO in preeclampsia. To differentiate between these two possible roles, we studied the impact of the loss of an important denitrosylase, S-nitrosogluathione reductase (*GSNOR*$^{-/-}$) in pregnant mice.

Protein S-nitrosylation participates in numerous pregnancy-related processes including placental trophoblast cell migration, apoptosis, angiogenesis, immunomodulation and oxygen delivery (8, 9). Protein SNO is enhanced in a transnitrosylation reaction using S-nitrosogluathione (GSNO), which thus acts as a

second messenger to transduce NO bioactivity (5). This process is tightly regulated by GSNOR, which selectively metabolizes GSNO thereby depleting the levels of S-nitrosylated proteins in equilibrium with GSNO. Although homozygous deletion *GSNOR*$^{-/-}$ mice have increased numbers of S-nitrosylated proteins (10, 11), which can in some circumstances lead to favorable outcomes (e.g. recovery from myocardial infarction) (12), deficiency of this enzyme can also disrupt physiological nitrosylation-denitrosylation dynamic cycles (11, 13). Furthermore, GSNO infusion into 10 women with severe PE resulted in a dose-dependent reduction in blood pressure and uterine artery resistance (14). Accordingly, we anticipated that *GSNOR*$^{-/-}$ mice would exhibit favorable maternal and fetal adaptations to pregnancy, and were surprised to discover that pregnant *GSNOR*$^{-/-}$ mice recapitulate all of the features of preeclampsia.

Results and Discussion

Development of hypertension and proteinuria are hallmark features of PE, and *GSNOR*$^{-/-}$ mice are hypotensive compared to wild type mice prior to pregnancy (10), consistent with GSNOR regulation of endothelium-dependent vasodilation. We studied the cardiovascular, renal, and maternal-fetal physiology of these mice and identified that by day 17.5 of pregnancy, *GSNOR*$^{-/-}$ mice develop hypertension (Figures 1, Table S1) and proteinuria (Figure 1), characterized by elevated urine macroglobulin levels, enlarged glomeruli (Figures 1, S1); changes that recapitulate human preeclampsia (15, 16).

We next studied cardiac responses to pregnancy in the *GSNOR*$^{-/-}$ mouse. The heart responds to sustained hypertension by an increase in wall thickness leading to concentric hypertrophy. Concentric hypertrophy is an independent predictor of adverse outcome in preeclamptic pregnancies (17). At late gestation, left ventricular end-diastolic dimension was lower, whereas the anterior wall at diastole (Table S1) was thicker, contributing to higher relative wall thicknesses in pregnant *GSNOR*$^{-/-}$ mice as compared to controls (Figure 1, Table S1). We next isolated cardiomyocytes (CMs) and observed that CM width at late gestation was greater in *GSNOR*$^{-/-}$ mice, whereas CM length was not different between the two strains (Table S1). These factors likely contributed to the enlarged *GSNOR*$^{-/-}$ hearts at late gestation even when normalized to tibia length (Figure 1, Table S1). Furthermore, the normal physiologic increases in maternal cardiac output and stroke volume were completely abrogated at late gestation in *GSNOR*$^{-/-}$ mice (Figure 1, Table S1), consistent with the phenotype of lower cardiac output and stroke volume in preeclamptic patients with concentric heart geometry (17). This observation, combined

with our data suggests that SNO homeostasis plays an important role in limiting pathological hypertrophy (18).

Placental insufficiency is believed to contribute directly to the development of preeclampsia. The placentas of preeclamptic pregnancies often exhibit fetoplacental hypovascularity and decreased fetoplacental perfusion (19), both of which decrease transfer of oxygen and nutrients to the placenta, thereby limiting fetal growth. Fetal litter size was significantly lower in *GSNOR*$^{-/-}$ mice at 17.5 d of gestation (Figure 1). Fetal body weights were significantly lower in *GSNOR*$^{-/-}$ mice at late gestation (Figure 1). To evaluate the role of GSNOR on placental vascularization and fetoplacental perfusion, we examined the placentas at late gestation. GSNOR activity was present in the control placentas, and as anticipated was absent in the knockout mice (Figure S2). Placental weight (Figure S3) and umbilical arterial blood flow (Figure S4) were not significantly different between the two strains. However, placental efficiency (fetal weight to placental weight ratio), and umbilical venous blood flow were significantly lower in *GSNOR*$^{-/-}$ placentas as compared to controls at late gestation (Figures S3, S4). In addition, placental vascularization was decreased in *GSNOR*$^{-/-}$ placentas (Figure 1) suggesting the involvement of the vascular endothelial growth factor (VEGF) pathway.

Next we examined the VEGF pathway in pregnant *GSNOR*$^{-/-}$ mice, as VEGFR2 levels are decreased in human preeclamptic pregnancies (20). Whereas, VEGF protein abundance in the placentas was not different between the two strains at late gestation (Figure S5), nitrosylation of VEGF protein was significantly lower in *GSNOR*$^{-/-}$ placentas (Figure S5). VEGF binds to its receptor VEGFR2 and signals through Akt and eNOS pathways. Like human PE, we found that VEGFR2 ($P=0.058$) and eNOS ($P<0.05$) total

protein quantities were lower in the *GSNOR*$^{-/-}$ placentas as compared to controls (Figure S6), whereas the quantity of AKT remained unchanged (data not shown). Thus, alterations in the VEGF signaling may represent a contributory mechanism for impaired placental development in GSNOR$^{-/-}$ mice.

Aberrant ROS and nitric oxide signaling has been implicated in the pathogenesis of PE (21, 22). To address this issue, we measured the cellular ROS levels in *GSNOR*$^{-/-}$ mice. Prior to pregnancy, cellular ROS generation was higher in CMs isolated from *GSNOR*$^{-/-}$ mice as compared to control mice (Figure 2), yet ROS levels were not significantly different between the two groups (Figure 2), suggesting increased involvement of antioxidant scavengers. As in the case with humans, pregnancy (23) itself increases ROS generation as seen in hearts obtained from control pregnant animals as compared to those from non-pregnant animals (Figure 2). Physiologic oxidative stress is proposed to be necessary for a wide array of physiological functions, including antioxidant defense and continuous placental remodeling (23). In pregnancy, ROS generation and levels were significantly higher in *GSNOR*$^{-/-}$ CM as compared to controls (Figure 2) confirming the presence of oxidative stress. Increased oxidative stress can lead to alterations in NO/SNO signaling, so we next measured NO levels. As expected, there was a significant increase in NO levels in isolated CMs of control mice during pregnancy (Figure 2). This increase likely plays a critical role in vasodilation leading to the normal adaptations to pregnancy. Furthermore, NO levels were significantly higher in CMs isolated from pregnant as compared to non-pregnant *GSNOR*$^{-/-}$ mice. With the presence of elevated ROS and NO/SNO levels in *GSNOR*$^{-/-}$ mice, we predicted an increase production of the potent pro-oxidant, peroxynitrite. Prior to pregnancy, peroxynitrite levels

were significantly higher in *GSNOR*$^{-/-}$ CMs as compared to controls. This preexisting elevation in peroxynitrite levels suggests that the *GSNOR*$^{-/-}$ mice were less able to respond to the stress of pregnancy. At late gestation, peroxynitrite levels were, indeed, significantly higher in isolated CMs of *GSNOR*$^{-/-}$ mice (Figure 2), suggesting the presence of nitrosative stress. NO signals via SNO-proteins exerts a protective role against an oxidative environment by competing with other post-translational modifications and shielding critical cysteine residues from the damaging effects of irreversible oxidation as shown in ischemic preconditioning (24, 25). Together these findings support the concept that SNO-based mechanisms that protect physiologic signaling could be impaired in pregnant *GSNOR*$^{-/-}$ mice contributing to the PE phenotype.

To test this hypothesis, we treated the animals or isolated CMs with the antioxidant, ascorbate. In addition to being an antioxidant, ascorbate also functions as a reductant, promoting the release of biologically active NO from nitrosylated thiols, and plasma ascorbate levels are commonly diminished in preeclamptic patients (26). Importantly, ascorbate treatment completely prevented the onset of hypertension, and proteinuria (Figures 3) and prevented the enlargement of glomeruli seen in untreated *GSNOR*$^{-/-}$ mice (Figure S1). The enlarged anterior wall thickness, relative wall thickness and CM width, all indicative of concentric hypertrophy, returned to normal levels in ascorbate treated pregnant *GSNOR*$^{-/-}$ mice (Table S1). Ascorbate also significantly improved cardiac output and stroke volume in *GSNOR*$^{-/-}$ mothers at late gestation, whereas it lowered both of these parameters in pregnant B6 controls (Figure 3, Table S1). Ascorbate treatment significantly improved placental vascularization along with placental VEGFR2 and eNOS protein levels (Figure 3, S6). In addition, ascorbate

improved umbilical venous blood flow and litter size, but did not improve placental efficiency, which may account for the failure of fetal weights to improve at late gestation in the *GSNOR*$^{-/-}$ mice (Figures 3, S3). Acute and chronic treatment of ascorbate similarly reduced ROS, NO and peroxynitrite levels in isolated CMs from pregnant *GSNOR*$^{-/-}$ mice (Figure 2). Thus, ascorbate may work as a potent scavenger of free radicals as seen in experimental models of hypertension (27, 28) and in rat models of nephrotoxicity (29) to balance the nitroso-redox system and in turn rescue the PE-like phenotype in pregnant *GSNOR*$^{-/-}$ mice.

Next in order to directly test the prediction that SNOylation is dysregulated in *GSNOR*$^{-/-}$ placentas, we performed mass spectrometry using both thiol reactive biotin-HPDP and cysTMT labeling to maximize coverage (30). Consistent with our prediction, this analysis revealed a marked increased number of SNOylated residues in *GSNOR*$^{-/-}$ placentas (459 corresponding to 351 proteins) compared to controls (264 SNOylated residues corresponding to 198 proteins) (Figures 2, S7). Importantly, placentas from ascorbate treated mice exhibited an increased net number of SNOylated proteins in both control and *GSNOR*$^{-/-}$ placentas (consistent with the transnitrosylation properties of chronic ascorbate (31)), but this increase was less in the *GSNOR*$^{-/-}$ mice (Figure S7) decreasing the difference in number of SNO-proteins between the two groups. To gain insights into the most important signaling pathways affected by excess S-nitrosylation we examined the subset of SNO-proteins found exclusively in GSNOR versus WT, and which exhibited denitrosylation with ascorbate. Of all of the detected peptides, there were 50 SNO-residues unique to the *GSNOR*$^{-/-}$ placentas, but only 16 residues unique to the B6 placentas (Figures 2, S7). All 50 SNO-residues were reversed by ascorbate. From these

50 proteins, 14 have been linked to important roles in processes essential in pregnancy, including angiogenesis, inflammation, cell migration and apoptosis (Figure 3G), supporting the pathophysiological relevance of these proteins.

Collectively, these results demonstrate that absence of a single gene, GSNOR, alters large numbers of downstream signaling pathways. Ascorbate, which creates a net increase in the number of SNO-proteins, decreases the differences between the GSNOR$^{-/-}$ and WT mice. As such these results suggest that the regulation of the SNO-integrated post-translational modification system accounts in large part for the phenotype of PE in the *GSNOR*$^{-/-}$ mice. Together, these results suggest that this system-wide alteration in SNO-proteins has a detrimental effect on the function of these pathways and as such, could be a key mechanism involved in the pathological phenotype seen in multiple organ systems, including the heart, kidney, placenta and the offspring of the KO animals with PE.

To examine the potential relevance of this pathway in human preeclampsia, we measured GSNOR mRNA, protein and activity levels in the placenta of patients with pregnancies complicated with PE (Figure 4, Table S2). Whereas GSNOR mRNA and protein abundance were similar (data not shown), GSNOR activity was significantly lower in placentas of human pregnancies complicated with preeclampsia compared to controls (Figure 4). Furthermore, affected placentas also had increased nitrosative stress as indicated by increased protein expression of nitrotyrosine and decreased antioxidant capacity as shown by decreased SOD levels (Figure 4). These findings support the physiologic importance of GSNOR in regulating human placental homeostasis and are consistent with the murine model.

Several clinical trials have examined the effectiveness of antioxidants (ascorbate) in preventing PE. Small, randomized placebo-controlled trials showed reduced PE with ascorbate treatment, whereas all large multicenter randomized trials have yielded disappointing results. These large trials showed that ascorbate did not decrease the risk of PE in either high risk (women with type-1 diabetes, nutritionally deficient, poor social economic status) or low risk populations (32-34). In turn, increased incidence of low birthweight, gestational hypertension, fetal loss, stillbirth or premature rupture of membranes have been reported in some trials, but these findings were not confirmed across all studies; therefore their significance remains uncertain (32-34). Dosage used and/or timing of the antioxidant treatment (8-22 weeks) may have played a role in the unsuccessful outcomes in these large clinical trials. Alternatively, oxidative stress may be relevant to the pathogenesis in only a subgroup of women, with no appreciable benefit of antioxidant therapy for the overall population. Furthermore, preeclampsia is a multi-systemic/multi-factorial disease and based on the heterogeneity of the clinical presentation, there may be different “subtypes” of preeclampsia (35). This observation may explain, in part, why many of the clinical trials using ascorbate have yet to show favorable outcomes. Therefore, identifying women showing dysregulation in nitrosylation and/or altered GSNOR activity levels may be the ideal target sub-population for treatment with ascorbate, permitting a precision medicine approach for future clinical trials.

Development of effective therapies for PE is hampered by a failure to understand the causative mechanisms involved and the absence of robust animal models that exhibit all the clinical features of PE. Therefore, the identification of the GSNOR$^{-/-}$ mice as a model that exhibits essentially all the clinical features of preeclampsia, with the causative

role of dysregulation in nitrosylation contributing to nitrosative stress and dysregulation of physiologic post-translational modification in a large number of signaling pathways as one of the primary mechanisms contributing to this disorder, has important implications for developing novel therapies and identifying clinically useful biomarkers for this difficult to treat maternal-fetal syndrome.

Data and material availability

All data is available in the main text or the supplementary materials.

Methods

Detailed methodology is presented in Supplemental Materials

Human subjects

Tissue collection was approved by the University of Miami Institutional Review Board, and patient and family privacy were assured under conditions of the Health Insurance Portability and Accountability Act. Written informed consent was received from participants prior to inclusion in the study.

Animals

All animal care was carried out in accordance with approval by the Institutional Animal Care and Use Committee.

Author contributions

S.K. conceived, designed and executed the experiments and wrote the manuscript. R.A.D., M.A.B., J.F., R.K.T., E.P., H.A. performed experiments. C.I.M, D.S., J.E.V. performed mass spectrometry experiment and analysis. W.B. assisted in manuscript writing. J.M.H. conceived of and designed experiments, co-wrote the manuscript, and provided funding.

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Conflict of interest. JMH reported having a patent for cardiac cell-based therapy. He holds equity in Vestion Inc. and maintains a professional relationship with Vestion Inc. as a consultant and member of the Board of Directors and Scientific Advisory Board. JMH is the Chief Scientific Officer, a compensated consultant and advisory board member for Longeveron, and holds equity in Longeveron. JMH is also the co-inventor of intellectual property licensed to Longeveron. Longeveron LLC and Vestion Inc. did not participate in funding this work. None of the other authors have anything to report.

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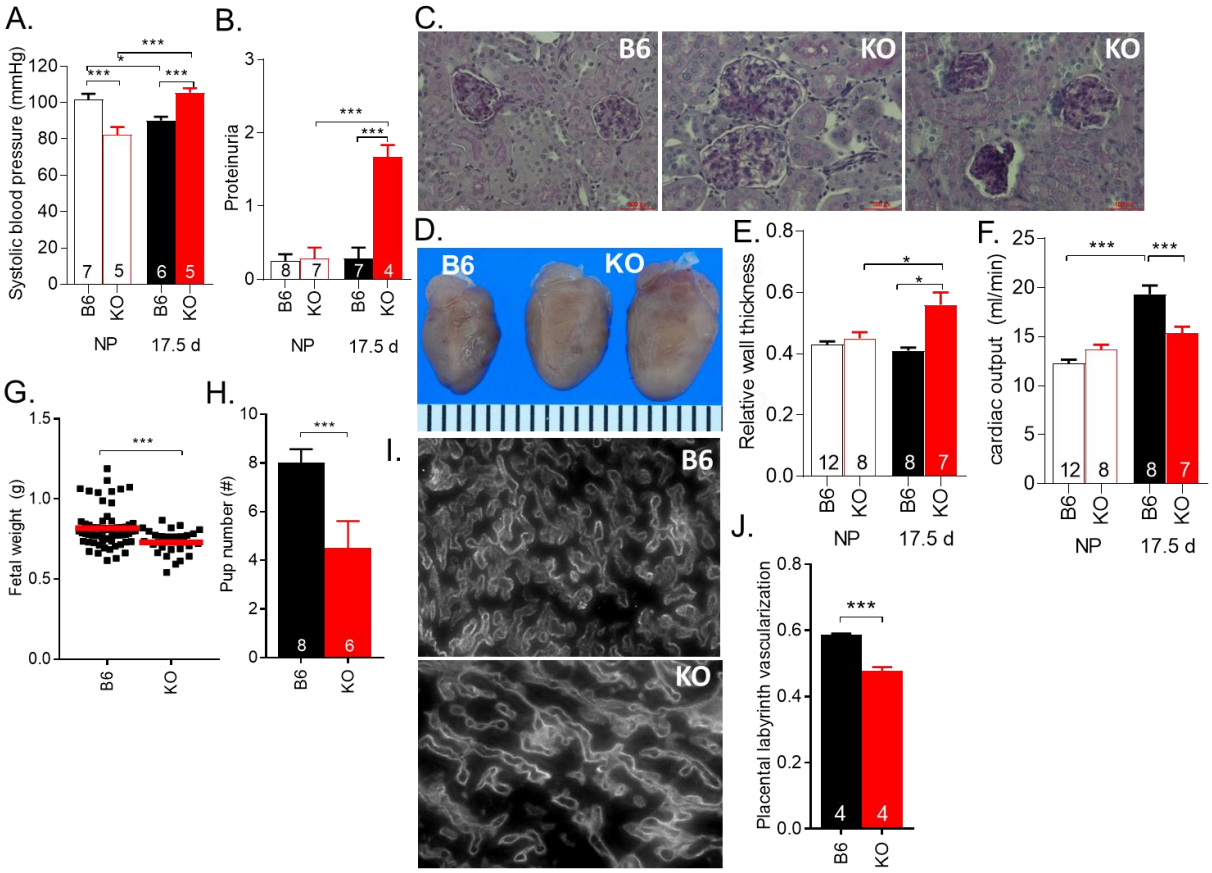

Fig 1: **GSNOR$^{-/-}$ mice exhibit a phenotype of PE**. Non-pregnant and pregnant (17.5 d) (N=4-12 mothers per group) C57Bl/6J (B6) and GSNOR$^{-/-}$ (KO) mice were examined. At late gestation, KO mothers exhibited (**A**) hypertension and (**B**) proteinuria. (**C**) Kidney sections at late gestation stained with periodic acid-Schiff showed enlarged glomeruli and focal and sclerosis with collapsed glomerular capillaries. (**D**) Heart weight and (**E**) relative wall thickness were significantly bigger in KO mice as compared to controls, indicating the presence of concentric hypertrophy. (**F**) The normal increase in cardiac output was absent in KO mice at late gestation. (**G**) Fetal weight and (**H**) number were significantly lower in KO mice. (**I,J**) Placental vascularization determined using isolectin immunostaining was significantly lower in KO as compared to B6 at late gestation. Results are shown as mean ± SEM. *** P<0.001, *P<0.05. NP, non-pregnant; B6, C57Bl/6J mice; KO, GSNOR$^{-/-}$ mice.

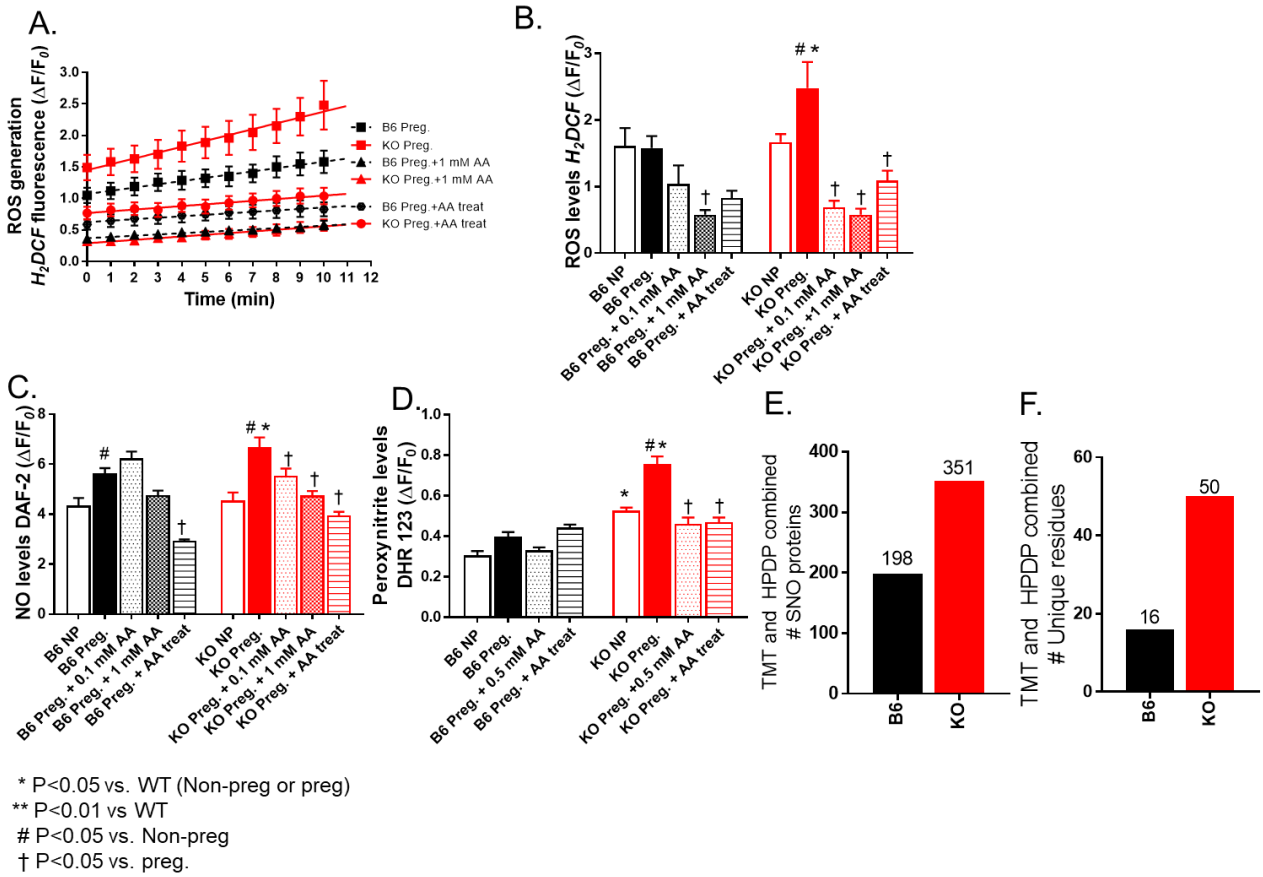

Figure 2: ***GSNOR*$^{-/-}$ mice exhibit nitroso-redox imbalance and nitrosative stress that is rescued with ascorbate treatment. GSNOR$^{-/-}$ placentas showed increase SNO residues**. In non-pregnant (NP) and pregnant (17.5 d) C57Bl/6J (B6) and *GSNOR*$^{-/-}$ (KO) mice, reactive oxygen species (ROS), nitric oxide (NO) and peroxynitrite levels were determined in isolated cardiomyocyte using epifluorescence by 2',7'dichlorodihydro-fluoresceine (H2DCF–DA), 4,5-diaminofluorescein (DAF-2DA) and dihydrorhodamine 123 (DHR 123), respectively. Ascorbate (AA) studies included both acute (0.1 mM, 0.5 mM, 1 mM) and chronic (AA provided in drinking water from day 0.5) treatments. (**A-B**) ROS generation and levels, and (**C**) NO and (**D**) peroxynitrite levels were significantly higher in CM isolated from pregnant *GSNOR*$^{-/-}$ mice. This increase was prevented with acute and chronic AA treatment. Duel-labeling mass spectrometry revealed more SNOylated proteins in the placentas from the knockout animals (**E**) of these there were more unique SNOylated residues (**F**) in *GSNOR*$^{-/-}$ placentas as compared to controls. Results are shown as mean ± SEM. N=3-5 animals per experiment. NP, non-pregnant; B6, C57Bl/6J mice; KO, *GSNOR*$^{-/-}$ mice; AA, Ascorbate.1-way ANOVA with Newman Keuls post-hoc test.

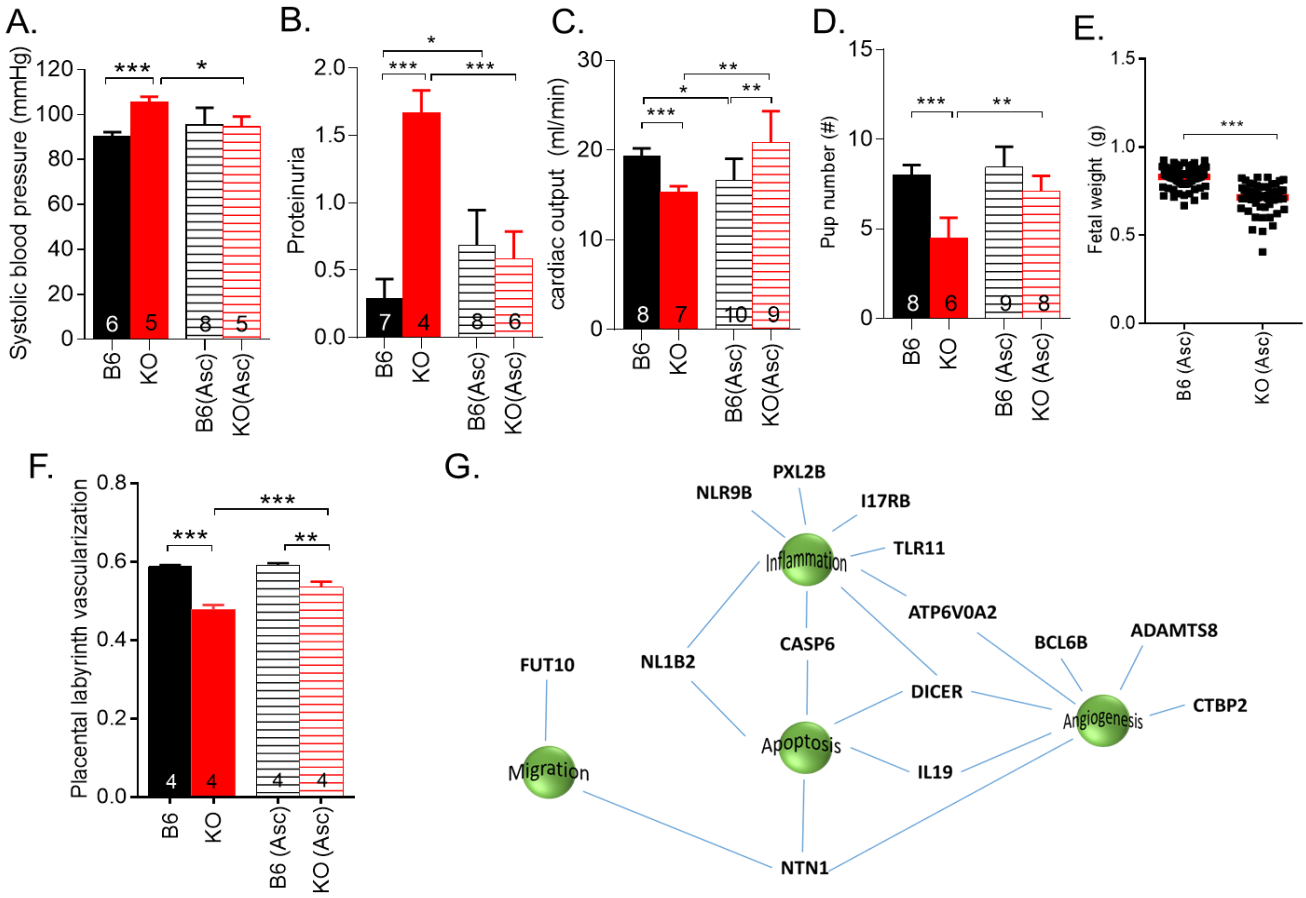

Fig 3: **PE phenotype in the mother and litter size is rescued with ascorbate**. Late pregnant (17.5 d) C57Bl/6J (B6) and GSNOR$^{-/-}$ (KO) mice were examined. (**A**) Hypertension and (**B**) proteinuria were completely rescued with ascorbate treatment. Ascorbate treatment increased (**C**) cardiac output in KO mice at late gestation. (**D**) Pup number was improved, whereas (**E**) fetal weight remained significantly lower in KO mice treated with ascorbate. (**F**) Impaired placental vascularization was rescued with ascorbate treatment in KO placentas. (**G**) Schematic showing proteins unique in GSNOR$^{-/-}$ placentas but absent in the WT placentas, and WT- and GSNOR$^{-/-}$ placentas treated with ascorbate. CASP6 = Caspase-6 (CASP-6); NLR9B = NACHT, LRR and PYD domains-containing protein 9B; TLR11 = Toll-like receptor 11; DICER = Endoribonuclease Dicer; PXL2B = Prostamide/prostaglandin F; IL17RB = IL-17 receptor B; FUT10 = Alpha-(1,3)-fucosyltransferase 10; NTN1 = Netrin-1; CASP6 = Caspase-6; NL1B2 = NACHT, LRR and PYD domains-containing protein 1b allele 2; IL-19 = Interleukin-19; BCL6B = B-cell CLL/lymphoma 6 member B protein; CTBP2 = C-terminal-binding protein 2; ADAMTS8 = A disintegrin and metalloproteinase with thrombospondin motifs 8; ATP6V0A2 = V-type proton ATPase 116 kDa subunit a isoform 2. Results are shown as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. Asc, ascorbate; B6, C57Bl/6J mice; KO, GSNOR$^{-/-}$ mice.

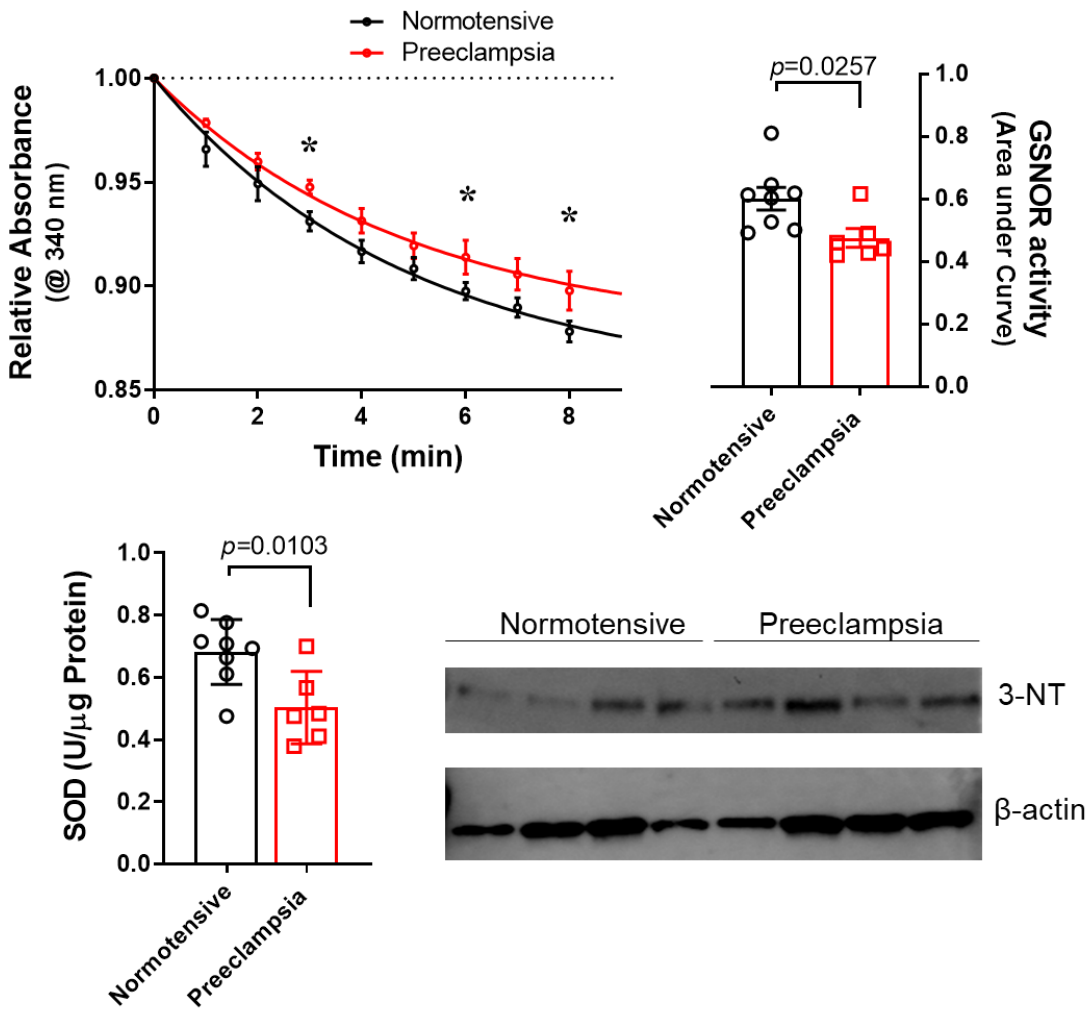

Figure 4: **GSNOR activity is reduced and contributes to nitroso-redox balance in the human preeclamptic placenta**. Human preeclamptic placentas exhibited decreased GSNOR activity, decreased antioxidant capacity (determined by superoxide dismutase levels), and increased nitrosative stress (determined by increased protein expression of nitrotyrosine). Significance for relative absorbance of GSNOR activity was determined by 2-way Anova with Newman Keuls post hoc test. Rest were determined by Student's T-test. Results are shown as mean ± SEM. *P<0.05, N=6-8 mothers.