Heme Oxygenase Inhibition Increases Blood Pressure in Pregnant Rats

Eric M. George, Peter A. Hosick, David E. Stec, and Joey P. Granger

BACKGROUND

During normal gestation, the placenta is a relatively hypoxic organ and, as such, is subject to significant oxidative stress. In the preeclamptic patient, inadequate remodeling of the maternal vasculature severely exacerbates placental oxidative stress, which has been shown to be an important component of maternal hypertension. There is emerging evidence that Heme Oxygenase-1 (HO-1) acts as an important regulator of placental and cardiovascular function during normal pregnancy. Here, we have examined the effect of Heme Oxygenase (HO) inhibition in late gestation on maternal blood pressure, angiogenic balance, and placental oxidative stress in pregnant rats.

METHODS

HO activity was inhibited with tin mesoporphyrin (SnMP), which was administered on gestational day 14, and blood pressure was measured on gestational day 19. Placental angiogenic balance and plasma Vascular Endothelial Growth Factor (VEGF) were determined by sandwich enzyme-linked immunosorbent assay. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity was measured by lucigenin chemiluminescence.

RESULTS

In response to SnMP treatment, maternal mean arterial pressure (MAP) was significantly increased (99 ± 1 vs. 113 ± 2 mm Hg; P < 0.05; n = 15 per group). Placental soluble fms-like tyrosine kinase-1 (sFlt-1) (631 ± 47 vs. 648 ± 26 pg/mg; P = 0.76) levels in the placenta were not affected by HO inhibition. Additionally, there was no significant difference in free VEGF in the maternal circulation (287 ± 22 vs. 329 ± 14 pg/ml; P = 0.11). There was, however, a significant decrease in placental VEGF (23 ± 2 vs. 16 ± 1 pg/mg; P < 0.05) and a significant increase in placental NADPH oxidase activity in SnMP-treated rats (2021 ± 238 vs. 3005 ± 301 RLU/min/mg; P < 0.05).

CONCLUSIONS

Our results demonstrate that HO is an important regulator of blood pressure and an important antioxidant in the developing placenta.

Keywords: blood pressure; heme oxygenase; hypertension; NADPH oxidase; preeclampsia; pregnancy; sFlt-1.

doi:10.1093/ajh/hpt045

Maintenance of blood pressure and vascular function during pregnancy is dependent on a carefully orchestrated program of placental development that adequately supplies the developing uteroplacental unit with blood. Failure to adequately remodel the uterine vasculature can lead to chronic placental ischemia, now recognized as a central causative agent in several pregnancy pathologies, most notably preeclampsia. In recent years, research from numerous labs has identified a number of pathogenic agents produced by the placenta in response to placental ischemia—the VEGF antagonist soluble fms-like tyrosine kinase-1 (sFlt-1) and oxidative stress being major activated targets. However, even under non-pathological conditions, the placenta is a remarkably hypoxic organ, and it is not entirely clear what intrinsic pathways are responsible for keeping these potentially pathogenic agents in check. There is emerging evidence, however, that these 2 factors can be negatively regulated by the heme oxygenase (HO) system.

HO is commonly considered a housekeeping protein responsible for the degradation of heme released from the breakdown of heme-containing proteins. Heme, a powerful pro-oxidant, is converted by HO into biliverdin, releasing the bioactive molecule carbon monoxide (CO) in the process. Biliverdin is then rapidly converted to bilirubin by biliverdin reductase, and both biliverdin and bilirubin have been shown to have potent antioxidant properties. HO-1, and particularly the inducible isozyme HO-1, has recently become a target of interest in several pathological states, perhaps most notably hypertension. HO-1 or its metabolites have demonstrated beneficial effects in the treatment of many forms of experimental hypertension, including AngII-dependent, pulmonary, and renovascular hypertension. Manipulation of the HO-1 system as a therapeutic remains an active area of research. sFlt-1 is perhaps the best-characterized factor released by the ischemic placenta and is a soluble form of the VEGF receptor Flt-1. In the soluble form, sFlt-1 binds free VEGF and makes it unavailable for normal signaling, thereby acting as a potent VEGF antagonist. Interestingly, sFlt-1 is also produced at significantly elevated levels by the placenta.
during even healthy pregnancies, but this is balanced out by a concomitant increase in the VEGF family member placental growth factor (PIGF). Preeclampsia then is the imbalanced increase in sFlt-1 in proportion to VEGF/PIGF, but what keeps these factors in balance during normal gestation remains obscure. Intriguingly, several recent reports have demonstrated that HO-1, CO, and bilirubin could negatively affect sFlt-1 production in response to various stimuli. Oxidative stress has also been intensively studied as a cause of hypertension in response to placental ischemia. Preeclamptic placentas exhibit significant elevations in oxidative stress, and in experimental placental ischemia–induced and sFlt-1–induced hypertension, inhibition of reactive oxygen leads to significant attenuation of the associated hypertension. Given the hypoxic nature of the healthy placenta, it is not clear how the organ keeps oxidative stress under control.

Several recent reports have demonstrated that HO-1 is important for placental development. Crossing of heterozygous HO-1/animals leads to significant defects in placental vascular development and structure and placental morphology. This is associated with prevalent fetal demise of the homozygous offspring, lowered fetal weight, decreased litter size, and altered vascular structure and placental morphology regardless of fetal genotype. However the effects of HO-1 disruption on blood pressure regulation, angiogenic balance, and oxidative stress in late gestation have not been examined. In this study, we have investigated the effect of pharmacological HO inhibition on blood pressure, angiogenic balance, and oxidative stress in late gestation. We demonstrate that HO inhibition leads to an antiangiogenic shift in the placenta, increased placental oxidative stress, and subsequently increased maternal blood pressure.

METHODS

Animals

Timed pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) were received on gestational day 11. All protocols were approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Rats were maintained on a 12:12 hour light:dark cycle at 23 ºC constant temperature and a 30% humidity at 464 nm to 530 nm with an extinction coefficient of 40 mM/cm. Activity was expressed as picomoles of bilirubin per milligram of protein per hour. Six samples from each group were measured.

Measurement of sFlt-1 and VEGF

Placental protein was extracted from a random placenta from individual rats. Briefly, the frozen tissue was mechanically ground by mortar and pestle in liquid nitrogen. Tissue fragments were resuspended in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail, phenylmethanesulfonyl-fluoride (PMSF), and sodium orthovanadate (Sigma, St. Louis, MO). Homogenization was performed in glass tissue dounces, and the solution was cleared by centrifugation at 12,000 g for 20 minutes. Resulting protein concentration was measured by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL). VEGF and sFlt-1 were both measured by sandwich enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) in duplicate according to manufacturer's protocols. Measurements of free plasma VEGF were performed with the same kit. Each group had placentas from 6 rats.

Determination of placental superoxide

Placental extracts were prepared and protein concentration determined as above. Lysates were incubated with lucigenin at 5 µM final concentration. Samples were allowed to dark equilibrate 3 minutes before measurement. Luminescence was recorded continuously for 15 minutes by luminometer (Berthold, Oak Ridge, TN). Values from lucigenin-only

Tissue harvest

Rats were anesthetized as above. The uterus was externalized, thoroughly a ventral midline incision, and blood was collected by cannulation of the abdominal aorta. Records were made of the viable and reabsorbed pups present in each rat, and individual pups and placentas were weighed and recorded. Representative placental samples from each horn, the thoracic aorta, and the liver were flash frozen in liquid nitrogen and stored at −80 ºC for later analysis.

Measurement of HO activity

Measurement of HO activity was carried out as previously described. Briefly, liver and placental samples were homogenized in Radio-immunoprecipitation assay (RIPA) buffer, and 0.5 mg of protein was combined with 2 mM glucose-6-phosphate, 0.2 U of glucose-6-phosphatedehydrogenase, 0.8 mM of nicotinamide-adenine dinucleotide phosphate, and 20.0 µM hemin in a total reaction volume of 1200 µl. The samples were incubated at 37 ºC for 1 hour. Bilirubin produced in that time was chloroform extracted, and its concentration was measured by the bicinchoninic acid method. Six samples from each group were measured.

Measurements of placental superoxide

Placental extracts were prepared and protein concentration determined as above. Lysates were incubated with lucigenin at 5 µM final concentration. Samples were allowed to dark equilibrate 3 minutes before measurement. Luminescence was recorded continuously for 15 minutes by luminometer (Berthold, Oak Ridge, TN). Values from lucigenin-only
blanks were subtracted from final numbers. Levels are expressed as relative light units per minute per milligram of total protein in the lysate, and each group had 7 or 8 samples.

**Statistical analysis**

All figures display mean data ± standard error. Comparisons between groups were performed by unpaired Student *t* test with a significance threshold value of *P* < 0.05. All statistical comparisons and graphs were generated with OriginPro 8 (Microcal, Northampton, MA).

**RESULTS**

**HO inhibition increases mean arterial pressure**

On gestational day 14, rats were injected with either vehicle or the HO inhibitor tin mesoporphyrin (SnMP), a competitive inhibitor that has been shown to chronically inhibit HO activity with weekly administration in rodents. On gestational day 18, all rats were implanted with fluid-filled arterial catheters for direct blood pressure measurement on gestational day 19. As seen in Figure 1a, control rats exhibited a mean arterial pressure of 99 ± 1 mm Hg. In response to SnMP administration, however, mean pressure was significantly increased to 113 ± 2 mm Hg (*P* < 0.05). HO inhibition did not result in changes in pup weight (Figure 1b), placental mass (Figure 1c), or litter size (data not shown).

**SnMP administration significantly attenuates HO activity in both the liver and placenta**

To determine the inhibitory effect of SnMP on HO activity in the treated groups, HO activity in both the liver and placenta were determined *ex vivo* by monitoring the conversion of bilirubin by tissue lysate exposure. As seen in Figure 2a, liver HO activity was significantly reduced in the SnMP-treated rats (391 ± 56 vs. 249 ± 67 pg/mg/h; *P* < 0.05). An even more dramatic decrease was seen in the placentas of the treated group (Figure 2b), demonstrating approximately 75% reduction in HO activity (195 ± 52 vs. 40 ± 13 pg/mg/h; *P* < 0.05). Together, these data demonstrate that even 5 days after administration, SnMP has a significant inhibitory effect of HO activity *in vivo*.

**HO inhibition has differential effects on sFlt-1 and VEGF**

To determine the effects of HO inhibition on angiogenic balance during pregnancy, we measured placental levels of both VEGF and sFlt-1. There was no significant difference in placental sFlt-1 between control and SnMP-treated rats (631 ± 37 pg/mg in controls vs. 648 ± 26 pg/mg in SnMP-treated group) as seen in Figure 3a. Interestingly, HO antagonism produced a significant reduction in placental VEGF when compared with control rats (16 ± 1 pg/mg in SnMP rats vs. 23 ± 2 pg/mg in control rats; *P* < 0.05) as demonstrated in Figure 3b. To determine whether this decrease in VEGF would have an effect on maternal circulating free VEGF, we measured plasma levels of free VEGF in the dams (Figure 3c). Interestingly, there was no significant effect of SnMP administration on free VEGF levels in these rats (287 ± 22 pg/ml in control rats vs. 329 ± 14 pg/ml in SnMP-treated rats).

**NADPH oxidase activity is increased by HO inhibition**

Because of its known antioxidant effects, we examined the effect of HO inhibition on placental NADPH oxidase (NOX) activity. To that end, placental lysates were assayed for NADPH-dependent chemiluminescence (Figure 4). In response to SnMP administration, there was an approximately 50% increase in NOX activity (2021 ± 238 RLU/min/mg vs. 3005 ± 301 RLU/min/mg; *P* < 0.05) as
shown in Figure 3, suggesting that decreased HO activity was having a direct effect to decrease NOX activity. Basal superoxide levels, as indicated by NADPH-independent chemiluminescence, were not significantly different between the 2 groups (data not shown).

**DISCUSSION**

Although our overall understanding of the gestational process is well established, we are still discovering new molecular pathways that are important for maintenance of a healthy pregnancy. In particular, recent research has demonstrated the importance of stress-inducible factors in the placenta, especially in response to the low oxygen tension found in the tissue. Much of the recent attention has been focused on these factors in a pathophysiological manner, as the importance of placental insufficiency and ischemia have become more recognized as an important factor in the development of obstetrical disorders, preeclampsia in particular. However, the normal physiological role for many of these pathways remains unclear.

The last decade has seen an emerging appreciation of the HO system in the maintenance of gestation. Several studies have examined the expression and localization of the 2 HO isoforms in the placenta and have found distinct temporal and spatial patterns of expression for both. Multiple studies have suggested that HO could be an important regulator of trophoblasts migration and invasion—the pivotal early step in remodeling the maternal vasculature to adequately supply the placenta. Several groups have examined the effect of...
genetic deletion of the HO-1 isoform and have convincingly demonstrated that HO-1 is necessary for proper placental vascular development in mice. In all these reports, very low (approximately 2%–7%) viability of HO1−/− offspring are reported when heterozygous knockouts are crossed, well below the expected 25%. Morphologically, the placentas of even the heterozygous fetuses have junctional zone thinning and decreased spiral artery remodeling, possibly through changes in natural killer cell activity. Intriguingly, these effects can be significantly improved by administration of low-dose CO, suggesting the mechanism by which HO deletion is causing these detrimental effects. However these studies largely reveal the effects of HO deficiency in early pregnancy, when maternal arterial remodeling and placental vascular development are taking place. The role of HO in later pregnancy has not been thoroughly addressed. Here we have demonstrated that SnMP causes significant inhibition of HO in both the liver and placenta of pregnant rats in late gestation (Figure 2). We also show that this is associated with increased mean arterial pressure (Figure 1a) but does not result in intrauterine growth restriction or overall changes in placental size (Figure 1c,d) in contrast with the changes seen in the abovementioned mouse HO-1 knockouts. We next wished to look at the possible mechanisms that could lead to changes in arterial blood pressure in response to HO inhibition.

Several lines of evidence suggest that HO could be an important negative regulator of sFlt-1, a major pathogenic factor in the gestational hypertensive disorder of preeclampsia. Cudmore et al. initially demonstrated that HO-1 induction or a CO donor could significantly reduce VEGF or interferon γ–induced sFlt-1 production in vitro. Recently, we have demonstrated that HO-1 induction could also block hypoxia-induced sFlt-1 production from placental tissue, as could exogenous CO and bilirubin, suggesting a possible crosstalk between oxidative stress and sFlt-1 production. Surprisingly, in this study, with administration of the HO inhibitor SnMP, placental sFlt-1 levels were not affected, but intriguingly, production of VEGF in the placenta was significantly decreased with HO antagonism (Figure 2a,b). This is in line with a number of previous reports that suggest that HO activity could positively regulate VEGF expression, specifically through the production of CO. It is interesting, however, that this did not translate to a decrease in free VEGF in the maternal circulation (Figure 2c). The full effects of a localized shift in the sFlt-1/VEGF ratio in the placenta and its effects on the offspring remain to be elucidated.

In addition to CO, increased production of bilirubin can also have an important physiological effect through its activity as an antioxidant. Oxidative stress is a well-known contributor to several forms of experimental hypertension, including placental ischemia–induced hypertension. We have also demonstrated that induction of HO-1 can attenuate the placental oxidative stress associated with chronic placental ischemia, suggesting an inverse relationship between HO and oxidative stress in the placenta. Indeed, when placental NOX activity was examined in this study, rats receiving the HO inhibitor exhibited a significant increase in NADPH-dependent lucigenin chemiluminescence (Figure 3). Again, this is consistent with reports in the literature that bilirubin can inhibit NOX activity in vitro and in vivo and with it the associated oxidative stress. It is possible then that the increased expression of HO in the placenta is at least in part a necessary response to reduce hypoxia-induced oxidative stress through inhibition of NOX. Although this study focused on the role of HO in placental function, the link between oxidative stress and hypertension is well established, and it is possible that oxidative stress in the maternal vasculature could be a contributing factor to the observed hypertension.

There has been a great deal of speculation about the role of HO in preeclampsia both as a cause and as a therapy. Although the majority of attention in the literature goes to HO-1, there are some tantalizing suggestions that HO-2 might be significantly decreased in the preeclamptic placenta. Studies have suggested decreased HO-2 expression in endothelial cells, syncytiotrophoblasts, and invasive trophoblasts of preeclamptic placentas, although the functional effect of this remains unclear. It is possible that some of the changes in VEGF and reactive oxygen species (ROS) that are demonstrated here are due to inhibition of HO-2 in these pivotal cell types. Whether this has functional significance in the development of preeclampsia is an interesting area of future study. Although the data here do not suggest a direct role for decreased HO as a primary cause of preeclampsia, the molecular changes observed do suggest that HO can have an important effect on several of the pathways that are dysregulated during the symptomatic phase of the disorder. It is possible that decreased HO activity in humans could exacerbate the symptoms of preeclampsia and that HO could be acting as an important buffer for the oxidative stress and ischemia that is seen in severe preeclampsia.

The data presented here are part of a larger story that suggests that HO is an important player in placental development and maintenance throughout gestation. The roles of the individual isoforms at different stages of gestation and their
distinct localized effects are still an area of active investigation. Here we demonstrate that general HO inhibition in late gestation leads to mild hypertension, likely through localized effects of decreased placental VEGF and increased oxidative stress. However, there are well-documented roles for HO in the regulation of renal hemodynamics and blood pressure, so some direct effects of HO inhibition in these studies remains an open avenue of investigation. Future studies looking at the effects of HO modulation on individual placental cell types should help fully elucidate the mechanistic basis for the in vivo effects demonstrated here.

ACKNOWLEDGMENTS

This work was supported by National Heart, Lung, and Blood Institute grants HL-108618, HL-51971, HL-088421, and HL-088421-S1, and a postdoctoral fellowship from the American Heart Association.

DISCLOSURE

The authors declared no conflict of interest.

REFERENCES

5. Gilbert JS, Babcock SA, Granger JP. Hypertension produced by reduced uterine perfusion in pregnant rats is associated with increased soluble fms-like tyrosine kinase-1 expression. Hypertension 2007; 50:1142–1147.