Carbon Monoxide Promotes Proliferation of Uterine Natural Killer Cells and Remodeling of Spiral Arteries in Pregnant Hypertensive Heme Oxygenase-1 Mutant Mice

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Abstract—Heme Oxygenase-1 (HO-1) and its metabolite carbon monoxide (CO) promote implantation and placentation. Pregnancy disorders such as preeclampsia and intrauterine growth restriction are linked to both HO-1 diminution and impaired remodeling of maternal spiral arteries (SAs). Here, we investigated whether CO is able to prevent preeclampsia and intrauterine growth restriction through the modulation of uterine natural killer (uNK) cells that are necessary for initiation of SA remodeling. Hmox1−/− or Hmox1+/− implantations presented fewer uNK cell numbers and lower expression of uNK-related angiogenic factors compared with Hmox1+/+ sites. Quantitative histology revealed that Hmox1−/− and Hmox1+/− implantations had shallow SA development that was accompanied by intrauterine growth restriction and gestational hypertension. Application of CO at low dose during early to midgestation prevented intrauterine growth restriction in Hmox1−/− mothers, this being associated with enhanced in situ proliferation of uNK cells and normalization of angiogenic parameters. Most importantly, CO improved SA remodeling and normalized blood pressure, ensuring a proper fetal growth. Thus, CO emerges as a key molecular player in pregnancy success by modulating uNK cells, which results in promotion of SA remodeling, adequate fetal support/growth, and prevention of hypertension. (Hypertension. 2014;63:00-00.) • Online Data Supplement

Key Words: blood pressure ■ hypertension ■ pregnancy

Adequate vascular remodeling is fundamental for placental and fetal development. Defects in human spiral artery (SA) development are associated with placental underperfusion, leading to gestational pathologies such as intrauterine growth restriction (IUGR) and preeclampsia.12 In both, reduced levels of Heme Oxygenase-1 (HO-1), the enzyme that catalyzes heme degradation, were reported.4 Hmox1-deficient female animals conceive but do not carry offsprings to term because of defective placentation followed by IUGR and fetal death.5 We recently reported that exogenous application of carbon monoxide (CO), HO-1’s main metabolite, restores placentation and fetal growth while enhancing live birth in Hmox1-deficient and Hmox1-sufficient mice.5,6

Uterine natural killer (uNK) cells are the major immune cell population at the early fetomaternal interface where they regulate maternal uterine vasculature remodeling.2 Differing from peripheral NK cells, uNK cells have unique tissue-related phenotypes and functions. They are mainly localized close to vessels in the decidua basalis (DB) and the mesometrial lymphoid aggregate of pregnancy (MLAp).6,9 CD3+CD122+ uNK cells do not express the common NK markers NK1.1 and DX5, but bind selectively to the Dolichus biflorus agglutin (DBA) lectin. DBA lectin is a specific uNK marker that binds uniquely to both cytoplasmic and granule membranes of uNK cells. This dual compartment labeling is not seen in blood NK cells or NK cells from other organs.10 Recent studies revealed the existence of 3 distinct uNK cell subpopulations, characterized as either CD3−CD122−DBA- or CD3+CD122−DBA− and their vascular endothelial growth factor (VEGF) production. The DBA+ cell population becomes progressively dominant as pregnancy proceeds and can be subdivided by their ability to produce VEGF.11 Besides production of proangiogenic factors including VEGF,12 placental growth factor (PGF),13 and Delta-like ligand (DLL1),14 mouse uNK cells provide local interferon-γ (IFN-γ) necessary and sufficient for the initiation of SA remodeling.15 Mice lacking uNK cells, for example, interleukin-15 (IL-15) KO mice, have impaired SA modification and placentation development, leading to IUGR and smaller progeny.16

Recent studies from our group revealed the importance of HO-1 in sustaining pregnancy by promoting optimal placentation.13 We postulate that HO-1 and its metabolite CO activate uNK cells to achieve their salutory effect. Here, we address whether genetic ablation of HO-1 impacts on uNK numbers and SA modification that may lead to IUGR or preeclampsia. We further analyzed whether the application of CO can prevent preeclampsia and IUGR in Hmox1−/− mothers by modulating uNK cells.
Materials and Methods

Animals
For analysis of implantation sites, Hmox1−competent or Hmox1−deficient BALB/c mice were used. These mice were initially provided by Dr Saw Feng-Yet and bred and maintained in our facility. The progeny obtained from breeding Hmox1−/− female animals and Hmox1+/− male animals were genotyped and recorded. C57BL/6 male animals were obtained from Charles River (Sulzfeld, Germany). The mice were maintained in our husbandry with a 12-hour light/dark cycle and with food and water ad libitum. All experiments were approved by German authorities (Saxon-Anhalt’s Ministry 2 – 868).

Hmox1−/−, Hmox1−/−, or Hmox1+/− BALB/c female animals were paired with Hmox1+/− or Hmox1+/− BALB/c male animals. For CO treatment, Hmox1−/− female animals were paired allogeneically with C57BL/6 male animals and treated either with CO or with mixed air as described below. For investigating whether CO can prevent the development of hypertension during pregnancy, Hmox1+/− and Hmox1−/− BALB/c female animals were paired with either Hmox1−/− or Hmox1−/− BALB/c male animals. Blood pressure was measured every other day beginning at day 0 of pregnancy.

For all experiments, the detection of a vaginal plug indicated day 0 of pregnancy. Animals were euthanized on days 8, 10, 12, or 18 of pregnancy. Whole implantation sites for paraffin embedding were kept in 4% paraformaldehyde with 0.1% saccharose for 6 hours. For RNA isolation, tissue of MLAp and DBA lectin+ uNK cell numbers were assessed in pregnant mice treated with mixed air or CO as indicated in the online-only Data Supplement.

Protein Isolation and Western Blot
Protein isolation and Western blot were performed following our standard protocol, and more details are available in the online-only Data Supplement.

Ki67 or IFN-γ/DBA Lectin Double Immunofluorescence
The expression of Ki67 and DBA or IFN-γ and DNA was analyzed at the fetomaternal interface of Hmox1−/− animals treated with mixed air or CO as indicated in the online-only Data Supplement.

Total RNA Isolation, cDNA Synthesis, and Real-Time RT-PCR
MLAp and DB tissue were homogenized in 1 ml of TRIZOL (Invitrogen). RNA was extracted with chloroform (Sigma), precipitated in isopropanol (Otto Fischer, München, Germany), and redisolved in RNase-free water (Berlin-Chemie, Berlin, Germany). After determination of RNA quantity and quality, cDNA synthesis was performed as previously described.

Amplification reactions for IL-15, VEGF, PGE2, and IFN-γ were performed on the iQ5 Real-time PCR System (Biorad). All samples were normalized regarding their β-actin content. Primer sequences are included in the online-only Data Supplement.

Blood Pressure Measurements
First, we conditioned nonpregnant female animals in the tail-cuff blood pressure machine every 2nd day for 20 minutes daily for 2 weeks. Pregnant mice were then evaluated for blood pressure at days 0, 2, 4, 6, 8, 10, 12, 14, 16, and 18 of pregnancy. After placing animals onto a 37°C warm plate, a 17-mm tail-cuff and a pulse trans-mitter were applied to the tail. The apparatus (TSE BP-Systems, Bad Homburg, Germany) was calibrated to inflate from 90 to 200 mm Hg.

Morphometric Analyses of Whole Implantation Sites, SA, and Kidneys
For morphometric analyses, implants were screened for their genotype. Hmox1−/−, Hmox1−/−, or Hmox1+/− mid sagitally cut implantations from gestation days 8, 10, and 12 and implantations from Hmox1+/− female animals treated either with mixed air or CO were analyzed blindly for uNK cell counting, size measurements, and SA modification. UNK cells were identified as DBA lectin reactive cells. Size measurements of H&E-stained midsagittally cut whole fetoplacental units, MLAp, DB, placenta, and embryo cavity, and identification of wall-to-lumen diameter ratio of SA were done with AxioVision (Carl Zeiss, Germany). Kidney sections were stained with H&E and blindly analyzed.

Flow Cytometry
For flow cytometry analysis, lymphocytes were isolated from MLAp and DB from Hmox1+/− female animals either treated with air or CO from day 3 to 8 of pregnancy. Briefly, the tissue was minced and mashed through a 100-μm mesh. The collected decidual cells were washed followed by a Ficolyte-M (Cedarlane Laboratories, Burlington, Canada) gradient.

Thereafter, the lymphocytes were washed once with buffer containing 1% BSA (Sigma, Steinheim, Germany) and 0.1% sodium azide (Merck, Darmstadt, Germany) in PBS and incubated with biotinylated DBA lectin (Sigma) diluted 1/200 for 5 minutes at 4°C. After washing, the lymphocytes were incubated for 30 minutes at 4°C with following extracellular antibodies: CD122 (FITC conjugated) or CD3 (preeclampsia conjugated) diluted 1/100 and a Streptavidin complex (PerCP conjugated) diluted 1/200. The samples were measured with the BD FACSCalibur and later analyzed with CellQuest Pro (both BD Biosciences).

Results
HO-1 Deficiency Is Associated With Reduced Number of uNK Cells and Diminished IL-15 Expression at the Fetomaternal Interface

DBA lectin+ uNK cell numbers were assessed in Hmox1−/−, Hmox1−/−, or Hmox1+/− midsagittally cut whole implantation sites on gestation days 8, 10, and 12. At all 3 time points, the number of uNK cells was significantly lower in MLAp and DB of Hmox1−/− or Hmox1−/− when compared with Hmox1+/− implantations (Figure 1A and 1B). IL-15 is the major cytokine...
supporting uNK cell differentiation and maintenance at the fetomaternal interface.\textsuperscript{16} qRT-PCR studies at gestation days 8, 10, and 12 showed that IL-15 was lower in MLAp and DB of Hmox1\textsuperscript{+/−} or Hmox1\textsuperscript{+−} implantations compared with Hmox1\textsuperscript{++} implantations. A significant reduction of IL-15 mRNA expression was observed at day 10 in MLAp and at day 12 in DB of Hmox1\textsuperscript{+−} implantations when compared with Hmox1\textsuperscript{++} implantations (Figure 1C).

Low uNK Number in HO-1–Deficient Mice Is Associated With Aberrant Expression of Angiogenic Factors and Defective SA Modification

Expression of uNK–related angiogenic molecules in Hmox1\textsuperscript{+/−}, Hmox1\textsuperscript{+−}, or Hmox1\textsuperscript{−−} implantations sites at gestation days 8, 10, and 12 was analyzed by qRT-PCR. The mRNA levels of VEGF and PGF were diminished in MLAp and comparable in DB of Hmox1\textsuperscript{+/−} and Hmox1\textsuperscript{+−} implantations when compared with Hmox1\textsuperscript{++} implantations at gestation day 8 (Figure S1A and S1B in the online-only Data Supplement). At days 10 and 12 of pregnancy, VEGF and PGF mRNA levels were comparable in MLAp and slightly decreased in DB of Hmox1\textsuperscript{+/−} and Hmox1\textsuperscript{+−} implantations compared with Hmox1\textsuperscript{++} implantations (Figure S1D, S1E, S1G, and S1H). IFN-\gamma mRNA levels were comparable in MLAp of Hmox1\textsuperscript{+/−}, Hmox1\textsuperscript{+−}, and Hmox1\textsuperscript{−−} whole placental units at days 8 and 10 of pregnancy (Figure S1C and S1F). By contrast, differences could be observed in specimens from day 12 of pregnancy, with Hmox1\textsuperscript{+−} and Hmox1\textsuperscript{−−} samples presenting decreased IFN-\gamma levels.

Figure 1. Heme Oxygenase-1–deficient implantations show reduced uterine natural killer (uNK) cell numbers and diminished interleukin-15 (IL-15) mRNA expression. A, Representative pictures from Dolichus biflorus agglutin lectin–stained Hmox1\textsuperscript{+/+}, Hmox1\textsuperscript{+−}, or Hmox1\textsuperscript{−−} mid-sagitally cut whole fetoplacental units at gestation day 8, 10, and 12 (×10; scale bar, 1000 μm). B, Number of uNK cells per mm\textsuperscript{2} in mesometrial lymphoid aggregate of pregnancy (MLAp) and DB of Hmox1\textsuperscript{+−} (n=12), Hmox1\textsuperscript{+−} (n=12), or Hmox1\textsuperscript{−−} (n=5/2) whole placental units at days 8, 10, and 12 of pregnancy. At all 3 gestation days, counts of uNK cells were significantly decreased in Hmox1\textsuperscript{+−} or Hmox1\textsuperscript{−−} implantations compared with Hmox1\textsuperscript{++} implantations. Data are presented as means. For statistical analysis, unpaired t test was performed. C, In comparison to Hmox1\textsuperscript{++} (n=6/5) whole fetoplacental units, the IL-15 mRNA expression was decreased in MLAp and DB of Hmox1\textsuperscript{+−} (n=6) or Hmox1\textsuperscript{−−} (n=3/2) implantations from gestation day 8, 10, or 12 of pregnancy. Data are presented in medians, and Mann–Whitney U test was used for statistical analyses. *P<0.05; **P<0.01; ***P<0.001. DB indicates decidua basalis; E, embryo cavity; M, MLAp; and P, placenta.
mRNA levels when compared with wild-type fetoplacental units (Figure S11). We further confirmed IFN-γ expression in DBA+ uNK cells by immunofluorescence (Figure S2). We next studied whether low uNK number and diminished levels of angiogenic factors affected SA remodeling. We, therefore, analyzed SA morphology at days 10 and 12, because at day 8 they are not completely differentiated.17 At both time points, SA remodeling was dramatically impaired in DBA+ Hmox1+/− and Hmox1−/− implantation sites when compared with Hmox1+/+ controls (Figure 2A). The ratio of wall-to-lumen diameter of SA was significantly increased in Hmox1+/− and Hmox1−/− implantations when compared with Hmox1+/+ implantations at gestation days 10 and 12 (Figure 2B).

**Hmox1+/− Pregnant Mothers Develop Gestational Hypertension, and Their Fetuses IUGR**

Because SA remodeling was impaired in Hmox1+/− and Hmox1−/− implantations in contrast to Hmox1+/+ implantations, we wondered whether this had an impact on fetal growth. The areas of midsagittally cut whole fetoplacental unit, MLAp, DB, placenta, and embryo cavity were defined in growth. The areas of midsagittally cut whole fetoplacental units, at gestation days 8, 10, and 12 (Figure S3; Table 1). Furthermore, areas at which uNK cells are located at the fetomaternal interface, the so-called MLAp and DB of Hmox1+/− implantations, were significantly smaller when compared with Hmox1+/+ controls at days 8, 10, and 12 of pregnancy. The MLAp of Hmox1+/− implantations was significantly smaller on days 10 and 12 of pregnancy, whereas the DB of Hmox1+/− implantations was significantly smaller on day 10 and, by trend, on day 12 of pregnancy when compared with Hmox1+/+ controls (Figure 3A, Table 1). An underdeveloped placenta resulted in significantly smaller embryo cavities in Hmox1+/− and Hmox1−/− implantations in comparison to Hmox1+/+ controls (Table 1). Because no differences in the neuronal development of the fetuses at day 10 of pregnancy were observed (data not shown), fetal growth retardation rather than developmental delay characterized this IUGR phenotype (Figure 3A; Table 1). Furthermore, disturbed remodeling and smaller fetoplacental units, seen as early as day 8 of pregnancy in Hmox1+/− female animals, were associated with a dramatic rise in systolic blood pressure beginning at day 14 of pregnancy (Figure 3B) that reached 151.5 mm Hg at day 18 (Hmox1+/+ controls, 116.3 mm Hg; *P<0.001*).

**Exogenous CO Application Elevates uNK Number, Normalizes the Expression of Angiogenic Factors, Avoids the Development of Hypertension, and Promotes Normal Fetal Growth**

We next treated Hmox1+/− female animals with low-dose CO (50 ppm) following our protocol.5 CO was able to enlarge embryo cavities in fetuses from Hmox1+/− female animals at day 10 (Figure 4A; Table 2). We found a statistically significant higher number of CD3+CD122+DBA lectin+ uNK cells in animals treated with CO as compared with Hmox1+/− controls treated with air (Figure 4B). The elevation in uNK numbers at the fetomaternal interface of CO-treated Hmox1+/− animals was independent of the IL-15 mRNA expression (Figure S4A and S4B). Augmentation of double-positive Ki67+/DBA uNK cells after CO treatment confirmed their in situ proliferation (Figure 4C). The normalization of uNK cell numbers at the fetomaternal interface after CO treatment had a positive effect on angiogenesis; it augmented the levels of VEGF, PGF, and IFN-γ mRNA in MLAp and the levels of VEGF and PGF mRNA in DB by trend (Figure S5A–S5C). At protein level, CO treatment significantly augmented both VEGF and IFN-γ expression in DB (Figure S6A and S6B). Because CO augmented the number of DBA+ uNK cells but not DBA− uNK cells (Figure S7), we suggest that the main source of induced

**Figure 2.** Low numbers of uterine natural killer cells in Heme Oxygenase-1-deficient mice cause poor spiral artery (SA) modification. A, Representative pictures of SA in decidua basalis (DB) of Hmox1+/+, Hmox1+/−, or Hmox1−/− midsagittally cut whole fetoplacental units from day 10 or 12 of pregnancy (×400; scale bar, 50 μm). B, The wall-to-lumen diameter ratio of the SA was significantly increased in Hmox1+/− or Hmox1−/− DB in contrast to Hmox1+/+ DB. The mean of 5 to 10 SA from individual Hmox1+/+ (n=12), Hmox1+/− (n=12), or Hmox1−/− (n=5/2) implantations is displayed as single dot, square, or triangle. Data are presented as means, and for statistical analyses, the unpaired t test was performed. ***P<0.001.
areas of not only promoted normotension but also normalized the size of pregnant animals (Figure 4D). CO application during days 3 to 8 of pregnancy impressedely prevented development of late gestational hypertension (Figure 4E) that began at day 14 of pregnancy in air-treated mice (Figure 3B). CO application not only promoted normotension but also normalized the size areas of Hmox1+/− implantation sites at day 18 (Figure S8). Intriguingly, CO inhalation diminished the Hmox1+/− implantation sizes (Figure S8). CO-positive effects were independent of systemic levels of sFlt-1 or sEndoglin, which remained comparable among all groups (Figure S9A and S9B). We observed that kidneys from air-treated Hmox1+/− pregnant mothers at day 18 presented enlargement of the capsule glomeruli connective tissue, abnormal mesangial cell proliferation, and severe hemorrhage in the tissue surrounding the glomeruli (Figure 4G). All these signs were not observed in kidneys from Hmox1++ mothers that were previously treated with CO, or in Hmox1+/− mothers treated with air or CO (Figure 4G). Additionally, CO treatment was able to diminish the rate of fetal death (measured at day 18) in Hmox1++ and Hmox1+/− combinations (Figure S10).

Discussion

A successful pregnancy depends to a great extent on adequate placentation that ensures oxygen and nutrient supply to the developing fetus. Uteroplacental blood flow increases dramatically during gestation, facilitated by the growth and remodeling of the maternal uterine SA system. Insufficient uterine vascular remodeling leads to pregnancy-associated pathologies such as IUGR and preeclampsia. The partial or total absence of HO-1 in Hmox1−/− or Hmox1+/− mothers negatively affects implantation and placentation, and breathing low doses of CO, HO-1’s most prominent metabolite, can rescue fetuses from intrauterine death. Thus, HO-1 and its gaseous metabolite, CO, emerge as pivotal regulators of pregnancy and fetal wellbeing.

In this work, we investigated whether the positive effects of HO-1 and CO on pregnancy rely on the fact that they interfere with the remodeling of SA either directly or indirectly by modifying uNK cells and the metabolites they secrete. In addition, we pursued the goal of dissecting whether HO-1 deficiency was related to the development of hypertension as claimed for the human system and whether the CO delivery can ameliorate preeclampsia signs in mice. This was already suggested because of the fact that smokers have a reduced risk of developing preeclampsia.

Here, we show that HO-1 deficiency is associated with diminished uNK cell numbers at the fetomaternal interface. A significant reduction in the absolute number of uNK cells could be observed as early as gestation day 8 in Hmox1−/− as well as Hmox1+/− implantations. These findings are supported by earlier results from Zhao et al showing alteration of uNK cell differentiation and maturation, but not proliferation, at the fetomaternal interface of Hmox1−/− mothers at day 10. However, in Zhao et al’s study, fetal phenotype was not considered in data analysis. Because IL-15 is a key regulatory molecule of uNK cell development in the decidua, we next embarked on studying its expression in embryos partially or totally deficient in HO-1 to understand whether their altered lower uNK number responds to lower IL-15. IL-15 mRNA expression in MLAp and DB of partially and completely HO-1−/− deficient implantations was reduced at midgestation. At the fetomaternal interface, IL-15 mRNA and protein are mainly expressed by uterine macrophages and perivascular cells surrounding decidual SA. HO-1 is known to modulate macrophages to the anti-inflammatory phenotype (M2) that represents the majority of uterine macrophages during pregnancy. Whether HO-1 diminution or absence directly influences IL-15 expression was not previously studied. From our results, a possible scenario is that the absence of HO-1 interferes with the phenotype of macrophages (M1 rather than M2) that in turn provokes less IL-15 secretion in MLAp and DB, thereby reducing uNK cell numbers.

In accordance with the known dependency of SA remodeling in uNK cells, we found that HO-1 deficiency resulted not
only in fewer uNK cells but also in shallow SA remodeling and IUGR. The administration of CO from gestation day 3 until day 8 provoked a significant increase in the number of uNK cells in MLAp and DB. The increase in uNK cell numbers on CO treatment was, however, independent of IL-15 at the implantation sites. Several studies could demonstrate a direct immune regulatory effect of exogenous application of CO on different cell types, for example, macrophages, endothelial, and vascular smooth muscle cells. Seeing no differences in IL-15 mRNA expression after CO treatment, it is clear that CO acts directly on uNK cells, provoking their in situ proliferation as we confirmed by Ki67 staining, perhaps via modulation of IFN-γ.

HO-1 positively influences angiogenesis via CO in several models by stimulating the production of VEGF and PGF, causing remodeling of the maternal uterine vascular system. VEGF and PGF mediate this process by inducing vasodilation, stimulating endothelial mitosis, and enlarging pre-existing vessels. An attenuation of VEGF and PGF signaling in the placenta is aligned with IUGR and preeclampsia in humans. uNK cells are reportedly the primary mediators of SA enlargement, stimulating endothelial mitosis, and enlarging pre-existing vessels. The midsagitally cut whole fetoplacental units, mesometrial lymphoid aggregate of pregnancy (MLAp), decidua basalis (DB), and placenta of Hmox1−/− or Hmox1−/− implantations were significantly smaller compared with Hmox1+/+ implantations at days 10 and 12 of pregnancy. Furthermore, the embryo cavities of Heme Oxygenase-1-deficient implantations were significantly smaller compared with wild-type ones, resembling intrauterine growth restriction. Data are presented in mm² and as medians.

**Table 1. Size of Midsagitally Cut Whole Fetoplacental Units, MLAp, DB, Placenta, and Embryo Cavity of Hmox1+/+, Hmox1−/−, or Hmox1−/− Implantations at Gestation Day 10 or 12**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Day of Pregnancy</th>
<th>Hmox1+/+(n=12)</th>
<th>Hmox1−/−(n=12)</th>
<th>Hmox1−/−(n=5)</th>
<th>Hmox1+/+(n=12)</th>
<th>Hmox1−/−(n=12)</th>
<th>Hmox1−/−(n=2)</th>
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<tr>
<td>Whole fetoplacental unit</td>
<td>10</td>
<td>25.2</td>
<td>18.7</td>
<td>12.9†</td>
<td>38.20</td>
<td>29.40‡</td>
<td>22.60‡</td>
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<tr>
<td></td>
<td>12</td>
<td>1.93</td>
<td>1.52</td>
<td>0.97‡</td>
<td>1.85</td>
<td>1.50‡</td>
<td>0.95</td>
</tr>
<tr>
<td>MLAp</td>
<td>10</td>
<td>6.35</td>
<td>4.44‡</td>
<td>4.31*</td>
<td>3.81</td>
<td>3.27</td>
<td>2.80</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.50</td>
<td>1.80</td>
<td>0.80‡</td>
<td>5.20</td>
<td>4.40*</td>
<td>3.50</td>
</tr>
<tr>
<td>Placenta</td>
<td>10</td>
<td>8.40</td>
<td>5.70†</td>
<td>2.10†</td>
<td>21.90</td>
<td>15.50§</td>
<td>10.30</td>
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<td>12</td>
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<tr>
<td>Embryo cavity</td>
<td>10</td>
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*P<0.01 significant to Hmox1+/+.
†P<0.01 significant to Hmox1−/−.
‡P<0.05 significant to Hmox1+/+.
§P<0.001 significant to Hmox1−/−.

The administration of CO during early pregnancy can actually prevent hypertension and IUGR, supporting former assumptions and studies about the beneficial effect of smoking and CO on preeclampsia development. CO therapy should be explored in more detail, especially after the recent report on decreased risk of preeclampsia after maternal exposure to moderate ambient CO. Most importantly, in animals that spontaneously develop preeclampsia, the rise in blood pressure began at day 14 of pregnancy. Systolic blood pressure in Hmox1−/− animals reached 151 mm Hg at day 18. Thus, these animals resemble a model of naturally occurring preeclampsia that is helpful to establish therapies to avoid the development of harmful signs and protect the fetus from hypertension-related damage. These findings are supported by previous studies from Zhao et al that showed an increase in diastolic blood pressure in pregnant HO-1 heterozygous mice. Here, we demonstrate that CO application during days 3 to 8 could compensate HO-1 deficiency and avoid the development of hypertension and IUGR. Cigarette smoking during pregnancy is associated with a wide range of adverse maternal, placental, and fetal effects, for example, preterm delivery, abnormal placentation, and too-small-for-age fetuses. However, women who smoke during pregnancy have a reduced risk of developing preeclampsia. One mechanism through which cigarette smoking may decrease the risk of developing preeclampsia in pregnancy is the essential element CO, but other cigarette components may account for the negative effects. We show here that CO application during early pregnancy can actually prevent hyper-tension and IUGR, supporting former assumptions and studies about the beneficial effect of smoking and CO on preeclampsia development. CO therapy should be explored in more detail, especially after the recent report on decreased risk of preeclampsia after maternal exposure to moderate ambient CO. Most importantly, in animals that spontaneously develop preeclampsia, the rise in blood pressure began at day 14, whereas CO treatment that could prevent this was performed beginning on day 3 until day 8 of pregnancy. It is clear that the events leading to preeclampsia development are triggered and can be corrected beginning as early as day 3. It is known that in humans a shallow invasion of the trophoblast at week 14, long before
Figure 4. Exogenous CO treatment of allogeneically paired Hmox1−/− female animals from gestation day 3 until day 8 augmented the number of uterine natural killer cells, enhanced spiral artery modification, and reduced intrauterine growth restriction and high blood pressure. A, Representative Dolichus biflorus agglutinin (DBA) lectin staining of midsagittally cut whole implantation sites from Hmox1−/− pregnant female animals at gestation day 10, treated with either air for control or with 50 ppm CO from pregnancy day 3 until day 8 (×10; scale bar, 1000 μm). B, Count of CD3−/CD122+DBA lectin+ cells was significantly increased in mesometrial lymphoid aggregate of pregnancy (MLAp) and DB of CO (50 ppm)-treated animals (n=5) in contrast to air-treated control animals (n=5). Data are presented as median and analyzed with Mann–Whitney U-test. C, Representative double immunofluorescence pictures of DBA lectin– and Ki67-stained MLAp of either mixed air– or CO-treated Hmox1−/− female animals at gestation day 10 of pregnancy. The number DBA lectin+Ki67+ cells per mm² was significantly elevated in MLAp of CO (50 ppm)-treated animals (n=5) when compared with air-treated control animals (n=5). Data are presented as median and analyzed with Mann–Whitney U test. D, Representative pictures of spiral artery (SA) at the fetomaternal interface of Hmox1−/− female animals exposed to air or CO from day 3 until day 8 (×400; scale bar, 50 μm). E, Exposure of Hmox1−/− female animals from gestation day 3 until day 8 with CO (n=6) causes a significant reduction in the wall-to-lumen diameter ratio of SA when compared with air-treated animals (n=5). The mean of 5 to 10 SA from individual implantations of Hmox1−/− female animals treated with air or CO are displayed as dot or square. Data are presented as mean and statistically analyzed by unpaired t test. F, After treatment of Hmox1−/− female animals (n=5) with CO (50 ppm), no significant differences could be observed when compared with CO-treated Hmox1−/− female animals (n=5). The mean of 10 individual measurements per female animal and day is displayed as dot or square. Variation within 1 group over time was analyzed with Friedman test, and differences between groups was analyzed by unpaired t test. G, Representative H&E staining of capsula glomeruli in air- or CO-treated Hmox1−/− or Hmox1+/+ mothers. The air-treated Hmox1−/− mothers display severely damaged glomeruli (connective tissue enlargement, distinct mesangial cell proliferation, and severe hemorrhage) compared with control animals. Glomeruli damage was reduced by CO treatment (×400; scale bar, 50 μm). *P<0.05; **P<0.01; ***P<0.001. DB indicates decidua basalis; E, embryo cavity; M, MLAp; and P, placenta.
preeclampsia symptoms arise in patients, is responsible for the pathophysiology of this complex disease.\textsuperscript{36} Whether clinically approved HO-1 inducers such as hemin will induce CO and, therefore, be a therapeutic option for preeclampsia is not known. We are rather skeptical and would like to state that this might depend on the dose used. We showed that the application of 20 mg/kg body weight hemin to pregnant animals provoked 100% fetal death. However, it is of great translational importance to test in animal models, similar to the one described here, whether clinically used drugs that enhance endogenous CO have a positive effect in preeclampsia if administered at the same or a lower dose used against porphyria. In this line of thought, we propose that excess of free heme in Hmox1\textsuperscript{−/+} animals contributes in part to gestational hypertension. These animals are not hypertensive if not pregnant or at the beginning of pregnancy. However, as pregnancy advances and circulating blood volume increases, the insufficient levels of HO-1 are not enough to remove the excess of free heme, known to be highly cytotoxic.\textsuperscript{7} This may explain the direct renal effect of Hmox1\textsuperscript{−/+} pregnant animals present impaired SA remodeling, IUGR, and hypertension. All these abnormalities can be corrected by the application of low-dose CO that emerges as a promising therapy against preeclampsia. CO therapy should be explored in more detail, especially after the recent report on decreased risk of preeclampsia after maternal exposure to ambient CO.

Sources of Funding

The present work was supported by a grant from The GEMI Fund (Gas Enabled Medical Investigation) and a grant from the DFG (Deutsche Forschungsgemeinschaft; ZE 526-5/1) to A.C.Z.

Disclosures

None.

References


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Table 2. Size of Midsagitally Cut Whole Fetoplacental Units, MLAp, DB, Placenta, and Embryo Cavity of Hmox1\textsuperscript{−/+} Female Animals Treated With Either Air or CO (50 ppm) From Day 3 Until Day 8 of Pregnancy

<table>
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<th>Treatment</th>
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<td>Whole fetoplacental unit</td>
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<tr>
<td>MLAp</td>
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<td>1.47†</td>
</tr>
<tr>
<td>DB</td>
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<tr>
<td>Placenta</td>
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<td>Embryo cavity</td>
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The treatment of Hmox1\textsuperscript{−/+} animals with 50 ppm CO from gestation day 3 to day 8 led to a significant enlargement of midsagitally cut whole fetoplacental unit, mesometrial lymphoid aggregate of pregnancy (MLAp), placenta, and embryo cavity in contrast to air-treated control Hmox1\textsuperscript{−/+} animals. No differences in the size of decidua basalis (DB) could be observed. Data are presented in mm\textsuperscript{2} and as medians.

*P<0.05.
†P<0.01.
15. Ashkar AA, Croy BA. Functions of uterine natural killer cells are medi-


17. Charalambous F, Elia A, Georgiades P. Decidual spiral artery remodeling during early post-implantation period in mice: investigation of associa-


Carbon Monoxide Promotes Proliferation of Uterine Natural Killer Cells and Remodeling of Spiral Arteries in Pregnant Hypertensive Heme Oxygenase-1 Mutant Mice
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CARBON MONOXIDE PROMOTES PROLIFERATION OF UTERINE NATURAL KILLER CELLS AND REMODELING OF SPIRAL ARTERIES IN PREGNANT HYPERTENSIVE HEME OXYGENASE-1 MUTANT MICE

Running title: Targeting uNKs to suppress hypertension

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Total word count: 7214, Figures: 1848
Abstract word count: 177
Total number of figures: 4, 10 Supplementary Figures
**Supplementary Material and Methods**

**DBA-lectin histochemistry**

For DBA-lectin histochemistry paraffin samples were rehydrated with xylene and an alcohol series. The sections were washed with TBS and blocked with 3% hydrogen peroxide in methanol for 30 min, Avidin/Biotin Blocking Kit (Vectorlabs, Peterborough, UK), according to the manufacturer instructions, and 5% BSA in TBS for 20 min at room temperature (RT). The slides were then incubated overnight (ON) at 4°C with biotinylated DBA-lectin (Sigma, Steinheim, Germany) diluted 1:250 in 100mM BSA in TBS. After washing, the samples were incubated for 30 min with an AB-Complex/HRP solution (Dako, Germany) at RT, developed with AEC+ Substrate Chromogen (Dako), counterstained with Hematoxylin and mounted.

**Protein Isolation and Western Blot**

Proteins were extracted by homogenizing frozen *decidua basalis* tissue in 30 µl lysis buffer (10% NP4, 0.1 mg/ml LM, 500 mM sodium fluoride, 10 mM sodium metavanadate, 100mM PMSF, 1M Tris (pH 7.5), 0.5 M EDTA and 5M NaCl) and quantified using the BioRad Proteinassay (Bio-Rad Laboratories, Munich, Germany), according the manufacturer instructions. The proteins (50 µg) were separated with a 15% SDS–polyacrylamide electrophoresis gel (4h, RT and 90 V) and transferred to a 0.2 µm nitrocellulose membrane (O.N., 4°C and 30 V). The membrane was afterwards blocked for 2h at RT in 5% Milk/TBS and than incubated ON at 4°C with the first antibody (VEGF: 1:100, Santa Cruz, Dallas, USA; IFN-γ: 1:100, Mabtech, Nacka Starnd, Sweden). After washing with 0.1% Tween20/TBS, the membranes were incubated with a horseradish peroxidase conjugated secondary antibody (for VEGF: 1:5000 donkey anti-goat, Jackson ImmunoResearch, Suffolk, UK; for IFN-γ: 1:5000 goat anti-rat, Santa Cruz) for 2 h at RT. As loading control, GAPDH (Sigma) was used. Bands were revealed by chemiluminescence and detected with the GeneGnome Imaging System (Syngene, Cambridge, UK). Intensity of the bands was quantified with the help of GeneTools.

**Ki-67 or IFN-γ and DBA-lectin double immunofluorescence**

Paraffin samples were rehydrated with xylene and alcohol series. Antigen retrieval of the tissue was performed by cooking in citrate buffer (pH 6.0) for 10 min. The sections were washed with TBS/Tween20 and incubated ON at 4°C with the first antibody (Ki-67: 1:500, eBioscience, San Diego, USA; IFN-γ: 1:50, Mabtech). After washing, the samples were incubated for 2h with the second antibody (AF555 conjugated goat anti-rat: 1:250, life technologies, Darmstadt, Germany) at RT. Afterwards the slides were washed with TBS/Tween20, incubated with FITC-conjugated DBA-lectin (1:50, US Biological, Hamburg, Germany) for 2h at RT, mounted and counterstained with DAPI (Vectashield, Vectorlabs).

**Primer Sequences**

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**Figure S1.** Low number of uNK cells in HO-1 deficient implantations correlates with diminished mRNA expression of angiogenic factors when compared to HO-1 sufficient implantations (A) The VEGF mRNA expression is significantly lower in the MLAp and lower in the decidua basalis of Hmox1<sup>−/−</sup> (n=3) whole placental units compared to Hmox1<sup>+/+</sup> (n=6), Hmox1<sup>+/−</sup> (n=6) implantations at day 8 of pregnancy. (B) The PGF mRNA expression in Hmox1<sup>+/−</sup> MLAp (n=6) was significantly reduced and
reduced in Hmox1−/− (n=6) MLAp when compared to Hmox1+/+ implantations (n=3) from day 8 of pregnancy. In Hmox1+/+ (n=5), Hmox1+/− (n=6) and Hmox1−/− (n=2) decidua basalis no differences in the PGF mRNA expression was found. (C) In Hmox1+/+ (n=6), Hmox1+/− (n=5) and Hmox1−/− (n=3) MLAp, no differences in the IFN-γ mRNA production was found at day 8 of pregnancy. (D-F) At day 10 of pregnancy no differences were observed in the VEGF, PGF and IFN-γ mRNA expression in Hmox1+/+, Hmox1+/− or Hmox1−/− MLAp. Whereas in the decidua basalis of Hmox1+/+ (n=6) or Hmox1−/− (n=2) whole implantations sites from gestation day 10 the VEGF and PGF mRNA expression was diminished compared to Hmox1+/+ implantations (n=6). (G-I) The VEGF, PGF and IFN-γ mRNA expression in the MLAp and decidua basalis of Hmox1+/− (n=6) or Hmox1−/− (n=2) whole placental units from gestation day 12 was reduced in contrast to Hmox1+/+ implantations (n=5). A detection of IFN-γ in the decidua basalis was not possible on day 8, 10 or 12 of pregnancy. Every implantation of the relevant phenotype is represented by a single dot, square or triangle in the graphs and displayed with medians. Statistical analyses were performed with the help of Mann – Whitney U-test. *P < 0.05. N.D., not detectable.
**Fig. S2**

**Figure S2. DBA-lectin⁺ uNK cells do express IFN-γ.** (X1000, scale bar: 10 µm) Immunofluorescent staining of the decidua basalis of an Hmox1⁺⁻ female treated with co (50 ppm) from gestation day 3 until day 8. (A) Immunofluorescent staining with a rat anti-mouse IFN-γ Ab visualized by an Alexa flour 555 coupled mouse anti-rat Ab. (B) The DNA was visualized with a DAPI staining. (C) The uNK cells were labeled with DBA-lectin and visualized with biotinylated FITC. (D) Merge of the immunofluorescent staining of IFN-γ, DAPI and DBA-lectin, demonstrating the expression of IFN-γ in DBA⁺ uNK cells.
Figure S3. HO-1 deficient implantations from gestation day 8 with low uNK cell counts show impaired growth. (A) HE staining of Hmox1+/+, Hmox1+/- and Hmox1-/- whole implantations at day 8 of pregnancy. (X10, scale bar: 1000 µm) (B) Hmox1-/- whole placental units and the decidua basalis (n=9) from day 8 of pregnancy are significant smaller compared to Hmox1+/+ (n=12) and Hmox1+/- (n=12) whole placental units and decidua basalis. Individual implantations are displayed as dots, squares or triangle with median. Statistical analyses were performed with the Mann – Whitney U-test. *P < 0.05 and ** P < 0.01. DB, decidua basalis; E, embryo cavity.
Figure S4. uNK cell augmentation in CO-treated females is independent of the IL-15 mRNA expression at the feto-maternal interface. (A) Regarding the IL-15 mRNA expression in the MLAp of air (n=5) or CO (50 ppm) (n=6) treated Hmox1<sup>+/−</sup> animals no difference was found. (B) No differences in the decidua basalis of air (n=5) or CO (50 ppm) (n=6) treated Hmox1<sup>+/−</sup> animals could be observed. Every dot or square represents an implantation from a different animal. Data is presented with a median and statistical analyzed with Mann – Whitney U-test.
Figure S5. Elevation of uNK cell numbers in CO-treated females correlates with increased expression of angiogenic factors at the feto-maternal interface. (A) The VEGF mRNA expression was augmented in the MLAp and decidua basalis of Hmox1<sup>+/−</sup> animals after treatment with 50 ppm CO (n=6) from gestation day 3 until day 8 when compared to air treated control females (n=5). (B) CO treatment in Hmox1<sup>+/−</sup> animals lead in the MLAp to a significant higher and in the decidua basalis to a higher PGF mRNA expression by trend. (C) The most important angiogenic factor produced by uNK cells, IFN-γ, was significant elevated on the mRNA level in the MLAp of implantations from Hmox1<sup>+/−</sup> females treated with CO from gestation day 3 until 8 when compared to air treated control animals. The IFN-γ mRNA expression was not detectable in the decidua basalis. Dots and squares represent a single implantation from a different animal, treated either with air as a control or CO (50 ppm). Statistical analyses were done with Mann – Whitney U-test. * P < 0.05. N.D. not detectable.
**Fig. S6**

![Graph A](image1)

![Graph B](image2)

Figure S6. CO-treated females presented increased protein expression of VEGF and IFN-γ in the **decidua basalis**. (A) The VEGF protein expression was significant augmented in the *decidua basalis* of *Hmox1<sup>+/−</sup>* animals after treatment with 50 ppm CO (n=5) from gestation day 3 until day 8 in comparison to air-treated control females (n=5). (B) CO treatment of *Hmox1<sup>+/−</sup>* animals induced a significant higher IFN-γ protein expression in the *decidua basalis*. The samples correspond to animals sacrificed at day 10 of pregnancy. Individual implantations are displayed as dots, squares or triangle with median. Statistical analyses were performed with the Mann – Whitney *U*-test. *P* < 0.05 and **P* < 0.01.
**Fig. S7**

**Figure S7.** The CO treatment of HO-1 deficient females during pregnancy had no effect in the DBA-lectin⁺ uNK cell population. The percentage of CD3⁺ CD122⁺ DBA-lectin⁺ CD49b⁺ is comparable in Hmox1⁻/⁻ females treated either with air (n=5) or CO (50 ppm) (n=5) from gestation day 3 until day 8. Statistical analyses were done with Mann – Whitney U-test.
**Fig. S8**

Figure S8. CO treatment had positive effects on the implantation size of fetuses. Implantation size was analyzed in HE-stained mid-sagitally cut implantation sites from mixed air or CO treated $Hmox1^{+/+}$ and $Hmox1^{+/−}$. Data is presented in mm$^2$ and medians and was statistically analyzed by Mann–Whitney $U$–test. * $P < 0.05$ and ** $P < 0.01$. 
Figure S9. CO treatment did not modify the levels of s-Flt-1 or s-endoglin. The levels of s-Flt-1 (pg/ml) and s-Endoglin (pg/ml) were analyzed by ELISA using serum samples from 18-days pregnant Hmox1+/+ or Hmox1+/- mothers treated either with mixed air (Hmox1+/+; n=5 and Hmox1+/-; n=6) or 50 ppm CO (Hmox1+/+; n=5 and Hmox1+/-; n=5) during days 3-8 of pregnancy. No differences were found among the groups as analyzed by Kruskall-Wallis test.
**Fig. S10**

**Figure S10.** CO treatment prevented fetal death in *Hmox1*<sup>+/+</sup> and *Hmox1*<sup>+/−</sup> mothers. After the treatment with 50 ppm CO from days 3-8 of pregnancy the number of dead fetuses was diminished in *Hmox1*<sup>+/+</sup> and *Hmox1*<sup>+/−</sup> mothers treated with CO when compared to *Hmox1*<sup>+/−</sup> females treated with air. Individual implantations are displayed as dots, squares or triangle with median. Statistical analyses were performed with the Mann – Whitney *U*-test. *P* < 0.05 and ** *P* < 0.01.