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Heme Oxygenase-1 Modulates Fetal Growth in the Rat

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SUMMARY: Intrauterine growth restriction is associated with increased perinatal morbidity and mortality as well as with lifelong cardiovascular and metabolic complications. Deficiency of heme oxygenase 1 (HO-1) is associated with growth restriction in mice and in humans, suggesting a role for HO-1 in fetal growth and maintenance of pregnancy. We hypothesized that modulation of HO-1 in the pregnant rat would alter fetal growth. In pregnant dams, placental HO activity was significantly inhibited with zinc deuteroporphyrin IX 2,4 bis glycol, and HO-1 protein was increased by transducing adenoviral human HO-1. Inhibition of HO-1 by zinc deuteroporphyrin IX 2,4 bis glycol resulted in a significant decrease in pup size, whereas transfection with hHO-1 resulted in increased pup size. Furthermore, the expression of IGF binding protein-1 and its receptor paralleled the expression of HO-1 in the placenta and were significantly modulated by modification of HO-1 along with the expression of vascular endothelial growth factor. These observations demonstrate that HO-1 modulates fetal growth by its effects on placental growth factors. (*Lab Invest 2002, 82:687–692*).

Intrauterine growth restriction (IUGR) is defined as a birth weight below the 10th percentile for gestational age (Goldenberg et al, 1989). In the United States, 8.6% of live births are growth-restricted (Hediger et al, 1998). Fetal growth in utero is influenced by extrinsic factors such as maternal vascular disease or intrinsic factors resulting from cell or organ dysfunction.

Adverse effects of IUGR are seen immediately at birth, but the long-term morbidity and mortality of IUGR manifest into adulthood. A direct correlation between low birth weight and several adult onset diseases such as Syndrome X (hypertension, non-insulin-dependent diabetes mellitus, high serum triglycerides, and low serum high-density lipoproteins), cardiovascular diseases, and arterial hypertension has been demonstrated (Barker et al, 1993).

The genetic contribution to IUGR is suggested by an increased prevalence of growth restriction in some families. A mutation in the IGF-1 gene can also cause IUGR (Woods et al, 1996). Furthermore, fetal cord serum IGF levels are increased in large-for-gestation-age infants and decreased in IUGR infants (Giudice et al, 1995). However, this correlation is not universal (Wang et al, 1991). A family of binding proteins (BP) mediates the biologic actions of IGFs (Han et al, 1996). The type 1 IGF receptor (IGF-1R) is a transmembrane tyrosine kinase that is widely expressed in fetal tissues. Activation of the receptor after binding of IGF-1 or IGF-2 results in cell proliferation and protection from apoptosis (Granerus and Engstrom, 2001), suggesting an important pathway for intrauterine growth. In fact, targeted mutations of the IGF-1R gene reduce mouse embryonic growth (Accili et al, 1999). In contrast, IGF-BP binds to IGF-1 and inhibits its growth-promoting actions (Price et al, 1992; Woodall et al, 1996).

Vascular endothelial growth factor (VEGF) can alter placental function and thereby affect fetal growth (Ahmed and Perkins, 2000; Cheung, 1997). Targeted disruption of this gene results in embryonic lethality even in the heterozygote state (Ferrara et al, 1996).

Another gene that has been implicated in placental vascular proliferation and cell growth is heme oxygenase-1 (HO-1) (Ahmed et al, 2000; Lyall et al, 2000). A case of HO-1 deficiency demonstrated severe growth restriction (Yachie et al, 1999), as with the HO-1 null mutant mice (Poss and Tonegawa, 1997). HO, which catalyzes the conversion of heme to bilirubin and carbon monoxide (CO) is found in most tissues, including the uterus and the placenta (Odrcich et al, 1998). In human placenta HO-1 is expressed at low levels throughout pregnancy (Lyall et al, 2000). In the rat, HO-1 protein levels peak on Day 16 in the uterus and on Day 19 in the placenta, and decline thereafter (Kreiser et al, 2001). This pattern approximates that of VEGF and of fetal liver erythropoietic activity (Joshima, 1996). HO-1 is also found at higher levels in the fetal liver (Abraham et al, 1988). Other researchers have suggested that HO-1 expression

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decreases placental vasoconstriction via CO (McLaughlin et al, 2000; Yoshiki et al, 2000) and a vasodilator molecule (Zhang et al, 2001), and a direct effect of HO inhibition on placental function has been demonstrated in vitro (Lyall et al, 2000). Furthermore, HO-1 protein is decreased in placentas of mothers with pre-eclampsia and with IUGR fetuses (Ahmed et al 2000), suggesting a role for HO-1 in endothelial cell protection. The increased expression of HO-1 in development, the association of impaired growth with HO-1 disruption, and the effect of HO on placental vasodilatation strongly suggest a role for HO-1 in fetal growth. We hypothesized that modulation of HO-1 in the pregnant rat would alter fetal growth and that this would be associated with changes in placental growth factor expression in vivo.

Results

Ventilatory Excretion of Carbon Monoxide (VeCO)

Transduction with adenoviral human HO-1 construct (hHO-1) or injection of zinc deuteroporphyrin IX 2,4 bis glycol (ZnBG) did not significantly alter VeCO (Fig. 1), suggesting that modulation of placental HO-1 did not alter systemic HO activity.

Tissue HO Activity

Injection of hHO-1 in lower concentrations (10^5 and 10^8 pfu/ml) modestly increased total HO activity (Fig. 2). Injection with a higher dose of hHO-1 (10^{10} pfu/ml) resulted in fetal demise and placental absorption, obviating tissue analysis. In the ZnBG-injected group, HO activity in the placenta decreased 3.5-fold (72%) relative to the controls 24 hours after injection (Fig. 2).

Determination of HO-1 Immunoreactive Protein Levels

Injection of hHO-1 (either 10^5 or 10^8 pfu/ml) increased HO-1 protein levels in the placenta 1.4-fold, whereas

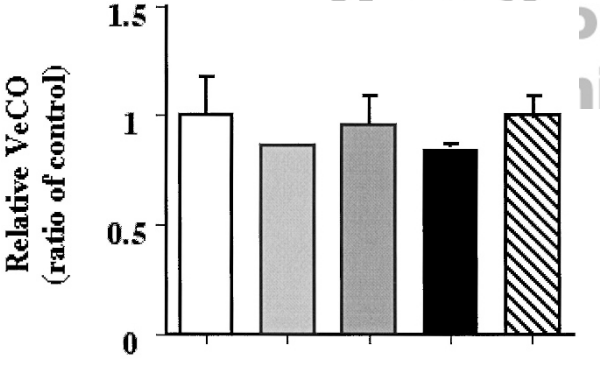

Figure 1.
Ventilatory excretion of carbon monoxide (VeCO) after manipulation of heme oxygenase 1 (HO-1) expression. Animals were evaluated for VeCO as described in "Materials and Methods." Values represent the means ± SE of 5 measurements in each group. *Open bar:* controls injected with vector alone or with saline; *light grey bar:* rats injected with 10^5 pfu/ml of human adenoviral HO-1 construct (hHO-1); *dark grey bar:* rats injected with 10^8 pfu/ml of hHO-1; *black bar:* rats injected with 10^{10} pfu/ml hHO-1; *hatched bar:* rats injected with 10 μmol/kg zinc deuteroporphyrin IX 2,4 bis glycol (ZnBG) to inhibit HO activity. No significant differences were observed between groups.

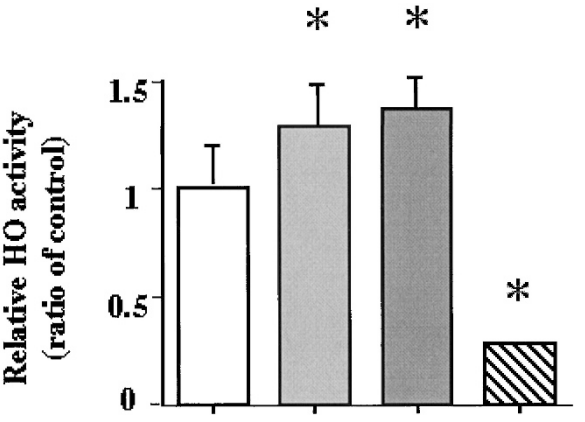

Figure 2.
Placental HO activity after manipulation of HO-1 expression. Placental tissue was evaluated for HO activity as described in "Materials and Methods." Values represent the means ± SE of nine measurements in each group. *Open bar:* controls injected with vector alone or with saline; *light grey bar:* rats injected with 10^5 pfu/ml of hHO-1; *dark grey bar:* rats injected with 10^8 pfu/ml of hHO-1; *hatched bar:* rats injected with 10 μmol/kg ZnBG to inhibit HO activity. * $p < 0.05$ versus saline- or empty vector-injected controls.

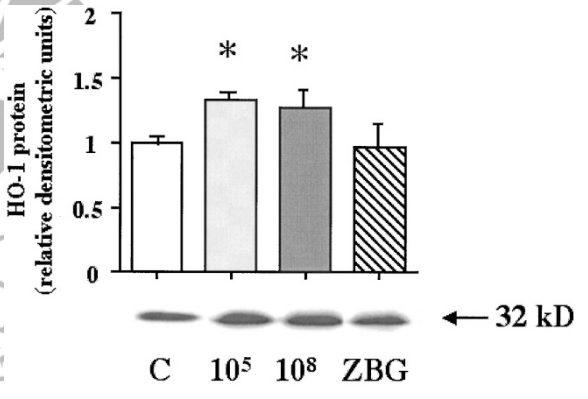

Figure 3.
Placental HO-1 expression. Placental tissue was evaluated for immunoreactive HO-1 protein levels by Western analysis. Lower panel: Representative Western blot of four placentas from rats injected with hHO-1 on Day 15 of gestation or injected with ZnBG to suppress HO activity. Upper panel: Densitometric evaluation of HO-1 protein from the Western blots represented in the lower panel. Values represent the means ± SE of nine measurements in each group. *Open bar:* controls injected with vector alone or with saline; *light grey bar:* rats injected with 10^5 pfu/ml of human adenoviral HO-1 construct (hHO-1); *dark grey bar:* rats injected with 10^8 pfu/ml of hHO-1; *hatched bar:* rats injected with 10 μmol/kg ZnBG (*ZBG*) to inhibit HO activity. *C* = control. * $p < 0.05$ versus saline- or vector-injected controls.

ZnBG injection did not significantly alter placental HO-1 protein levels (Fig. 4A).

Pup Weight

To determine the effect of hHO-1 and ZnBG injections on fetal growth, pups from all litters were weighed in the first 12 hours of life. Injection of hHO-1 resulted in a 9% increase in the average pup weight (6.2 ± 0.3 g versus 5.7 ± 0.6 g). Moreover, ZnBG injection was associated with a 5.5% reduction in the average pup weight (5.4 ± 0.3 g versus 5.7 ± 0.3 g) (Fig. 3).

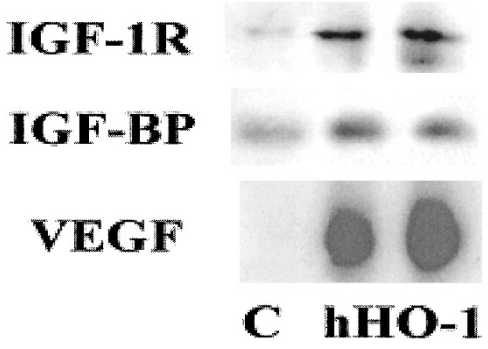

Figure 4.
Effect of hHO-1 transduction on placental growth factors. Representative Western blot of 4 placentas from rats injected with 10^8 pfu/ml of hHO-1 on Day 15 of gestation to enhance HO activity. Lanes are: *C*, control injected with vector alone; and *hHO-1*, rats injected with hHO-1 (2 examples are shown). Equal loading was verified with Coomassie blue staining. *IGF-1R* = insulin-like growth factor-1 receptor; *IGF-BP* = insulin-like growth factor-1 binding protein; *VEGF* = vascular endothelial growth factor.

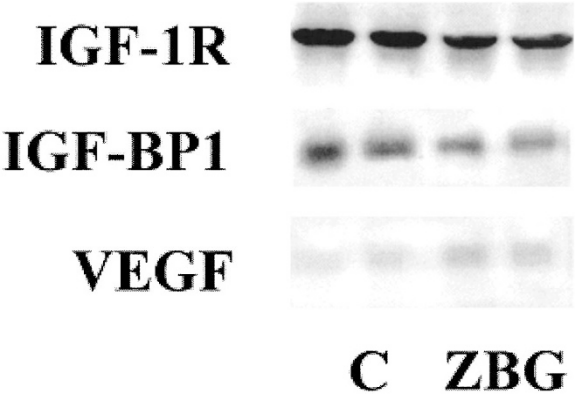

Figure 5.
Effect of HO inhibition on placental growth factors. Representative Western blot of four placentas from rats injected with10 μmol/kg ZnBG on Day 15 of gestation to suppress HO activity. Lanes are: *C*, controls injected with saline; and *ZBG*, rats injected with ZnBG (two examples are shown). Equal loading was verified with Coomassie blue staining. *IGF-1R* = insulin-like growth factor-1 receptor; *IGF-BP1* = insulin-like growth factor-1 binding protein; *VEGF* = vascular endothelial growth factor.

Determination of IGF-1R, IGF-BP-1, and VEGF Immunoreactive Protein Levels

Because some of the byproducts of the HO reaction (in particular CO) serve as signaling molecules (Otterbein et al, 2000), protein levels for various growth factors were evaluated to determine whether HO-1 mediated its effects by modulating IGF-1 or VEGF expression. Because the highest concentration of IGF-1 is in serum (Wang et al, 1991) and we were most interested in placental tissue, we determined the concentration of IGF-1R and IGF-BP-1 in tissues rather than IFG-1 itself. Although injection of 10^8 pfu/ml hHO-1 had no effect on IGF-BP-1 in the placenta, ZnBG injection caused a significant decrease (60%) (Fig. 4B). Additionally, IGF-1R protein levels increased 2.1-fold with hHO-1 adenovirus injection, whereas there was a 57% decline after ZnBG injection (Fig. 4C). As to VEGF, adenoviral transduction of hHO-1 was associated with a dramatic increase in placental expression. Injection of ZnBG did not alter placental VEGF expression compared with controls (Fig. 5).

Discussion

IUGR is associated with many long-term consequences and has many etiologies. Deficiency of HO in a human has been described (Yachie et al, 1999) and, as with the HO-1 null mutant mice (Poss and Tonegawa, 1997), there was growth restriction. An effect of HO-1 on cell proliferation (Clark et al, 1997; Deramaudt et al, 1998), vasodilatation (Thorup et al, 1999; Zhang et al, 2001), and postnatal growth (Sabaawy et al, 2001) has been documented. Gene transfer of hHO-1 into coronary endothelial cells promoted angiogenesis (Deramaudt et al, 1998) and inhibitors of HO increased placental resistance (Lyall et al, 2000). Overall, these examples suggest a role for HO-1 in placental vascularization and fetal growth; however the mechanism by which this is mediated is not well defined.

Placental vascularization and an adequate fetoplacental circulation are essential for promoting fetal growth. In the present work, the fact that inhibition of HO suppressed growth and that increased HO-1 expression promoted growth suggests that increased HO activity improves placental function, perhaps through the vasodilator effects of CO and the vasoproliferative effects of HO-1. Although, HO-1 was modified by ZnBG and hHO-1 locally, there were no changes in the systemic excretion of CO, suggesting tight control of systemic CO excretion.

The increased expression of IGF-1R and VEGF after hHO-1 transduction suggests that HO-1 or its byproducts serve in signaling processes to enhance growth factor expression and activation. Inhibition of IGF-related proteins by ZnBG further attests to the role of HO-1 to modulate growth factor action. This has not been previously demonstrated.

Other researchers have hypothesized that increased CO inhibits VEGF expression via a cGMP-mediated pathway (Ghiso et al, 1999). We clearly demonstrate increased VEGF expression in the placenta after hHO-1 transduction. This implies differential cell-specific effects of CO or HO-1 on VEGF, or that CO regulates other factors that in turn modulate VEGF in the placenta. In a retinal model, IGF-1R activation resulted in increased VEGF activation of a mitogen-activated protein (MAP) kinase but did not modulate VEGF protein expression (Smith et al, 1999). However, in NIH3T3 fibroblasts, incubation with IGF-1 increased VEGF mRNA via MAP kinase activation (Miele et al, 2000). This demonstrates that IGF-1 can both increase VEGF expression and increase its activation. It is therefore plausible to suggest that CO or HO-1 could modulate VEGF through IGF-1, but this does not explain how HO-1 mediates increased IGF-1.

The IGF-1R gene has response elements in the promoter that allow for increased transcription (Werner et al, 1995). Recent evidence suggests that HO-1 can precede activation of other genes, such as

superoxide dismutase (Frankel et al, 2000), and that CO can result in signaling via the p38 MAP kinase (Otterbein et al, 2000). Perhaps IGF-1R gene transcription is enhanced via the effects of CO or another byproduct of the HO reaction. One such byproduct, iron, is important in regulating many genes (Alcantara et al, 2001; Fogg et al, 1999), although no evidence exists as to its regulation of IGF-1 gene expression.

Overall, our results indicate that changes in HO-1 expression influence growth in utero. This is associated with changes in growth factor expression that could affect fetal growth and placental function. We speculate that one of the byproducts of the reaction, such as CO, could result in signaling and increased regulation of these growth factors.

Materials and Methods

Sixty-day-old timed pregnant Wistar rats (Simonson Labs, Gilroy, California) were housed singly in a temperature-controlled room (25° C ± 4° C) on a 12-hour light cycle. The animals were allowed free access to food and water. All animal care was in accordance with National Institutes of Health guidelines and under Institutional Animal Care and Use Committee approval.

Pregnant dams were intraperitoneally injected with either 5 μmol/kg of body weight ZnBG, on Day 16 of pregnancy, or with 300 μl of hHO-1 adenovirus in three distinct dilutions (10^5, 10^8, and 10^{10} pfu/ml) on Day 15 of pregnancy. Control groups were injected with normal saline or an empty vector. VeCO measurements were obtained before and 24 hours after injection. After 24 hours, placental tissue was obtained for HO activity and for HO-1, IGF-1R, and IGF-BP-1 protein levels by Western analysis. On Day 1 of life, pups from all groups were weighed.

ZnBG Preparation

ZnBG (2.87 mg) was dissolved in 240 μl of Na_3PO_4, 0.4 M. An additional 1 ml of H_2O was added and the solution was slowly titrated to pH 7.8 with 1 M HCl (approximately 100 μl). The final volume was adjusted to 8 ml with normal saline.

Construction of Adenoviral hHO-1 cDNA Vector

A 987-bp hHO-1 cDNA fragment was released from the plasmid pRc-CMV-hHO1, and cloned at the *Hin*dIII site of the plasmid pGEM-7zf(+) (Promega, Madison, Wisconsin). The resulting vector pGEM-hHO1 was then linearized with *Eco*RI, and end-blunted with dNTP and Klenow. After digestion with *Bam*HI, a hHO-1 cDNA fragment was released from pGEM7z-hHO1, and cloned at the PmeI/*Bgl*II sites of the adenoviral vector pAdCMV5GFP. The resulting adenoviral vector was designated as pAdCMV5GFP-HO. The linearized pAdCMV5GFP-HO by the digestion with *Eco*RI was cotransfected with $E1^-$, $E3^-$ adenoviral long-arm DNA (QBI-viral DNA, Quantum, Montreal, Québec, Canada) into a human embryonic cell line, 293A cells. The hHO-1 adenovirus construct was replicated and encapsulated into an infectious virus. After a 5-day incubation period, the virus plaque locations were marked on the flasks, and the resultant cytopathic effect on the monolayers was observed microscopically until the plaque reached an adequate size. The plaques were purified and checked for the presence of hHO-1 by PCR with HO-1–specific primer, and amplified by propagation in the 293 cell line. The HO-1 adenovirus was released and collected by rupturing the infected cells through three freeze/thaw cycles 48 hours after infection. After three rounds of plaque purification, recombinant adenovirus was large-scale amplified and purified. Titers of each cesium chloride–purified viral stock were determined from the absorbance at 260 nm (1 absorbance unit = 10^{10} pfu/ml) and were confirmed by plaque assay. The virus was stored at 80° C until use.

Determination of HO Activity

Measurement of HO activity in placental tissue was obtained by pooling placentas from each animal. Tissues were homogenized in four volumes of 0.01 M sodium potassium phosphate buffer at pH 7.4 and then centrifuged for 60 seconds at 12,500 ×*g*. The supernatant was then analyzed for HO activity as previously described (Vreman and Stevenson, 1988). Twenty microliters of tissue supernatant, representing 4 mg fresh weight of tissue, were incubated in 2-ml amber glass vials with 20 μl of 1.5 μM methemalbumin in 150 μl BSA and 20 μl of 4.5 mM NADPH for the total reaction vials. For blank reaction vials, the NADPH was replaced with an equal volume of buffer. The vials were sealed with septum caps and placed in 37° C water bath for 5 minutes of temperature equilibration and then purged with CO-free air. After 15 minutes of further incubation, placing vials on powdered dry ice (−78° C) terminated the reactions. The CO generated in the reaction medium and effused into the vials headspace was quantitated by gas chromatography with a reduction gas analyzer (Trace Analytical, Menlo Park, California). Analyzer response to CO was recorded with an integrating recorder (CR-3A; Shimadzu Scientific Instruments, Columbia, Maryland) through measurement of peak area. The reduction gas analyzer was standardized daily with volumes of 10.8 μl of CO/l air (482 nM). Homogenates were analyzed for protein content by the method of Lowry et al (1951) and read at 595 nm. HO activity was defined as NADPH-dependent CO production and calculated as the difference between the CO in the total and blank reaction vials. HO activity was expressed as mean ± SD pmol CO/μg protein/hour and then normalized to controls.

VeCO Measurements

The animals were weighed and placed in sealed Plexiglas tubes supplied with CO-free air at flow rate of 100 ml/minute. After a 30-minute equilibration period, gas leaving the chamber was analyzed for CO concentration by gas chromatography. For each time point, a minimum of five readings per animal was

taken. VeCO was expressed as mean ± SD microliters per hour per kilogram of body weight (Hamori et al, 1989) and then normalized to control values.

Antibodies

Polyclonal rabbit anti-rat HO-1 antibody was raised against a 30 kd soluble HO-1 protein expressed in *E coli* from rat liver cDNA (Wilks and Ortiz de Montellano, 1993) (gift of Angela Wilks, University of California San Francisco, California) as previously described (Dennery et al, 1997). Rabbit anti-goat IGF-BP-1 and goat anti-rabbit IGF-1 receptor antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, California). HRP-conjugated anti-rabbit and anti-goat polyclonal IgG was obtained from Santa Cruz Biotechnologies.

Determination of HO-1, IGF-1R, and IGF-BP-1 Immunoreactive Protein Levels (Western Analysis)

One hundred and eighty microgram aliquots of uterine or placental homogenates for HO-1, IGF-1R, and IGF-BP-1 were subjected to electrophoresis on a 12% polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (PVDF) (Millipore Corporation, Bedford, Massachusetts). Membranes were incubated for 1 hour with a 1:500 dilution of the primary antibody washed three times for 10 minutes with 0.05% Tween PBS (Dennery et al, 1997). The membranes were then incubated for 1 hour with a 1:800 dilution of anti-goat or anti-rabbit polyclonal IgG at room temperature. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system (Super Signal; Pierce Chemical, Rockford, Illinois) according to the manufacturer's instructions. Equal loading of samples was verified by Coomassie blue stain. Densitometric evaluation was conducted (SGI, Sunnyvale, California) and values from each blot normalized to controls to allow for comparison between blots.

Statistical Analysis

For comparisons between the study groups, the null hypothesis that there was no difference between group means was tested by a single factor ANOVA for continuous variables using Bonferroni multiple comparisons *t* test for multiple groups or unpaired *t* test for two groups or the Fisher Exact tests for dichotomous data (Stat-View 4.02; Abacus Concepts, Berkeley, California). Statistical significance was assumed at $p < 0.05$.

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