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Gestational Pattern of Heme Oxygenase Expression in the Rat

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ABSTRACT

Fetal growth is influenced by many intrinsic and extrinsic factors. Our objective was to determine the pattern of heme oxygenase (HO) expression in the pregnant rat and to study its association with fetal growth and growth factors. Uterine tissues were obtained from nonpregnant and from time-mated rats at 7, 13, 16, 19, and 21 d of pregnancy. Placental tissue was obtained on d 13, 16, 19 and 21 of pregnancy. Tissues were evaluated for HO activity, HO-1, HO-2, leptin and vascular endothelial growth factor protein, and HO-1 and HO-2 mRNA. HO activity in both the uterus and placenta peaked on d 21 of pregnancy. In the uterus, HO-1 and HO-2 protein and total mRNA levels peaked on d 16 of pregnancy, whereas, in the placenta, HO-1 and HO-2 protein levels peaked on d 19. Additionally, placental HO-1 mRNA peaked on d 16, but placental HO-2 mRNA declined toward the end of pregnancy. Placental leptin and vascular endothelial growth factor protein levels followed a similar pattern to placental HO-1 and peaked on d 16. We conclude that there is a clear uterine and placental gestational pattern of HO expression in the rat. This pattern is comparable to that of vascular endothelial growth factor and leptin. (***Pediatr Res* 54: 172–178, 2003**)

Abbreviations

HO, heme oxygenase
NO, nitric oxide
VEGF, vascular endothelial growth factor
CGMP, cyclic GMP
CO, carbon monoxide
NADPH, nicotinamide adenine dinucleotide phosphate, reduced form

HO catalyzes the conversion of heme to bilirubin and carbon monoxide (CO). There are three isoforms of HO, which are regulated under separate mechanisms (1). The proteins of the best-characterized isoforms, HO-1 and HO-2, are immunologically distinct (2). The two genes are located on different chromosomes and there is no hybridization of HO-1 to HO-2 cDNA (1). HO activity is not evenly distributed across tissues, but HO activity is found in most tissues (3),including the uterus and the placenta of rats and other species (4, 5). In human placenta, HO-1 is expressed at low levels throughout pregnancy, whereas HO-2 expression declines throughout pregnancy in the syncytiotrophoblast (6). Recent evidence suggests that HO functions to change vascular tone, reduce oxidative stress, and influence cellular proliferation (7–10).

The diatomic molecule CO is produced through the metabolic reaction of HO. This molecule shares some of the physiochemical properties of NO (11). Like NO, CO has the ability to bind to the iron atom of the heme moiety associated with soluble guanylyl cyclase, thereby activating the enzyme and increasing intracellular cGMP production. Endogenous CO production under physiologic conditions serves to regulate smooth muscle (12) and uterine contraction (13, 14), thrombocyte aggregation (15), and neurotransmission (16). Exogenously added CO has been shown to inhibit platelet aggregation, relax both human and rat uterine smooth muscle (13), and relax isolated blood vessel preparations by elevating intracellular levels of cGMP (3). Therefore, HO could play a role in gestation and fetal growth through CO-mediated effects.

In both humans (17) and mutant mice (18), HO-1 deficiency is associated with growth restriction and fetal loss. The exact mechanism by which HO may mediate growth is not known, although it may be that HO exerts an influence on growth factors such as leptin and VEGF, which are known to be important in the fetal growth (19, 20). Therefore, the purpose of this study was to determine the expression of HO-1 and HO-2 at different stages of gestation and to evaluate the

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relationship of HO to certain growth factors important in gestation in a rat model.

METHODS

Timed pregnant Wistar rats were housed singly in a temperature-controlled room (25 ± 4°C) on a 12-h 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.

Uterine and placental tissues were obtained from timed pregnant Wistar rats at 13, 16, 19 and 21 d of pregnancy, with the day when the sperm plug was observed considered as d 1. Uterine tissue was also removed on the d 7 of pregnancy and from nonpregnant Wistar rat females. The pregnant rats were anesthetized and killed by CO_2 inhalation.

Determination of HO Activity

Measurement of HO activity in placental tissue was obtained by pooling four conceptuses per sample. Tissues were homogenized in four volumes of 0.01M sodium potassium phosphate buffer at pH 7.4 and then centrifuged for 60 s at 12,500 × *g*. The supernatant was then analyzed for HO activity as previously described (21). 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 4.5mM 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 min of temperature equilibration and then purged with CO-free air. After 15 min, the reactions were terminated by placing the vials on powdered dry ice (−78°C). CO generated in the reaction medium and effused into the vials' head space was quantitated by gas chromatography with a reduction gas analyzer (Trace Analytical Inc., Menlo Park, CA, U.S.A.). Analyzer response to CO was recorded with an integrating recorder (CR-3A, Shimadzu Scientific Instruments Inc., Columbia, MD, U.S.A.) through measurement of peak area. The reduction gas analyzer was standardized daily with volumes of 10.8 μL of CO per liter air (482 nM). Homogenates were analyzed for protein content by the method of Lowry *et al.* (22) and read at 595 nm. HO activity is defined as NADPH-dependent CO production and is calculated as the difference between the CO in the total and blank reaction vials. HO activity was expressed as mean ± SD in picomoles CO per microgram protein per hour.

Antibodies

Polyclonal rabbit anti-rat HO-1 antibody was raised against a 30 kD soluble HO-1 protein expressed in *Escherichia coli* from rat liver cDNA (23) (gift of Angela Wilks, University of California, San Francisco, CA, U.S.A.) as previously described (10). Rabbit anti-rat HO-2 antibody was obtained from Stressgen (Vancouver, BC, Canada). Rabbit anti-rat VEGF and leptin antibodies were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). Horse radish peroxidase–conjugated goat anti-rabbit and anti-goat polyclonal IgG was obtained from Santa Cruz Biotechnologies.

Determination of HO-1, HO-2, VEGF, and Leptin Immunoreactive Protein Levels (Western Analysis)

Sixty microgram aliquots of uterine or placental homogenates for HO-1 and HO-2, or 180 μg for VEGF and leptin, were electrophoresed on a 12% polyacrylamide gel and then transferred to polyvinylidene difluoride membrane (PVDF) (Millipore Corporation, Bedford, MA, U.S.A.). Membranes were incubated for 1 h with a 1:500 dilution of the primary antibody washed three times for 10 min with 0.05% Tween PBS (T-PBS) (10). Afterwards, the membranes were incubated for 1 h 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 (ECL) according to the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). Equal loading of samples was verified by Coomassie blue stain. Densitometric evaluation was conducted (SGI, Sunnyvale, CA, U.S.A.) and values from each blot normalized to controls to allow for comparison between blots.

Immunohistochemical Detection of HO-1 and HO-2

Six-micron slices from pregnant rat uterus or placenta were fixed in ice-cold 100% acetone, permeabilized in 0.3% saponin in PBS, and blocked in a PBS solution containing 5% nonfat powdered milk, 1% BSA, and 0.03% saponin. The slides were incubated for 1 h at room temperature with a rabbit anti-rat polyclonal antibody to HO-1 at a 1:20 dilution. The slides were then incubated with a 1:50 dilution of FITC-labeled goat anti-rabbit secondary antibodies (Caltag Laboratories, Burlingame, CA, U.S.A.) for 1 h at room temperature. Slides were then incubated with rabbit anti-rat polyclonal HO-2 antibody at a 1:20 dilution for 1 h at room temperature and finally incubated with Texas red–labeled goat anti-rabbit secondary antibodies at 1:50 for 1 h. The slides were mounted with antifade reagent in glycerol buffer (Slowfade, Molecular Probes, Eugene, OR, U.S.A.) and viewed with a Nikon fluorescence microscope (Tokyo, Japan) using a confocal laser scanning unit (model 2010, Molecular Dynamics, Sunnyvale, CA, U.S.A.). Excitation was set at 488 nm and emission at 515–545 nm. Images obtained here and with FITC staining were processed as anaglyphs. Negative controls for nonspecific binding, incubated with secondary antibody only, were processed and revealed no signal.

Determination of HO-1 and HO-2 Total mRNA

Plasmid and probe preparation. The plasmid pBKRHO1 was constructed in pBluescript II SK− using rat HO-1 and HO-2 cDNA fragments prepared by reverse transcriptast PCR as previously described (10). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PD) was prepared as an *E. coli* digest of the HHCPF19 plasmid by standard methods

(24). Labeled probes were prepared by the random primer method (25) using ^{32}P-dCTP.

Northern hybridization. For HO-1 mRNA determinations, tissue samples were immediately homogenized in 4 M guanidinium thiocyanate, quick frozen in liquid N_2, and stored at −80°C. RNA was isolated by the guanidinium thiocyanate phenol extraction method (26) and quantitated spectrophotometrically at 260 nm. Total RNA was electrophoresed and probed as previously described (10). Briefly, RNA (20 μg) was electrophoresed on a 1.0% agarose gel after formaldehyde denaturation. The RNA was transferred by capillary action onto filter membranes. Filter membranes were prehybridized at 42°C for 4 h. The hybridization solution containing 1×10^6 cpm/mL of probe (^{32}P-labeled HO-1 cDNA or HO-2 cDNA) was then added and the mixture wasallowed to incubate for 72 h at 42°C. The blot was exposed against x-ray film at −80°C with intensifying screens. Autoradiographs were visualized and quantitated by densitometry and normalized to G3PD. For re-probing, membranes were stripped according to the manufacturer's protocol using boiling 0.5% SDS.

Statistical Analysis

For comparison between days of pregnancy, the null hypothesis that there was no difference between group means was tested by a single-factor ANOVA for continuous variables using the Fisher exact test for multiple groups or unpaired *t* test for two groups (StatView 4.02, Abacus Concepts, Berkeley, CA, U.S.A.). Statistical significance was assumed at $p < 0.05$.

RESULTS

Uterine HO activity rose significantly from d 16 of pregnancy compared with that of the nongravid uterus. The HO activity peaked on d 21 of pregnancy (Fig. 1*A*). As with uterine HO activity, placental HO activity was significantly higher beginning on d 16 of pregnancy ($p < 0.05$), when compared with d 13 of pregnancy and peaked on d 21 of pregnancy (Fig. 1*B*).

In contrast to HO activity, uterine HO-1 protein levels were significantly higher ($p < 0.05$) from d 16 of pregnancy compared with the nongravid uterus (Fig. 2*A*). Thereafter, the levels declined until d 21. Similarly, placental HO-1 protein levels were significantly higher ($p < 0.05$) on d 16 compared with d 13 of pregnancy and peaked on d 19 of pregnancy but declined on d 21 (Fig. 2*A*). Uterine HO-2 protein was also highest on d 16 and declined toward the end of pregnancy (Fig. 2*B*). As with the placental HO-1, placental HO-2 protein levels peaked on d 19 of pregnancy but declined on d 21 of pregnancy (Fig. 2*B*).

Immunohistochemical detection of HO protein demonstrated increased HO-1 and HO-2 protein signal in the uterine blood vessels at d 19 and a concentrated expression of HO in the syncytiotrophoblast region of the placenta with maximal expression at d 16 (Fig. 3, *A* and *B*)

As with HO-1 protein, uterine HO-1 m-RNA levels were significantly higher ($p < 0.05$) from d 13 and peaked on d 16 of pregnancy, compared with the nongravid and d 7 uteri. Thereafter, the levels declined until d 21 (Fig. 4*A*). As with the uterus, placental HO-1 m-RNA levels peaked on d 16 and declined through d 21 (Fig. 4*B*). Uterine HO-2 mRNA levels also peaked on d 16 of pregnancy (Fig. 4*C*). In contrast, placental HO-2 mRNA levels declined, starting on d 19 of pregnancy, with the lowest value seen on d 21 (Fig. 4*D*).

We evaluated the abundance of growth factors known to play a role in the fetal growth to see whether these paralleled HO expression. Placental VEGF and leptin protein levels peaked on d 16 of pregnancy (Fig. 2, *C* and *D*). This pattern approximated that of HO-1 and HO-2 protein expression.

DISCUSSION

In the pregnant rat, HO-1 and HO-2 have a gestational pattern of expression that approximates that of key growth factors. Specifically, we showed that uterine and placental HO protein levels were significantly elevated from d 16 to 19. Furthermore, in the placenta there is a localized expression of HO in the syncytiotrophoblast region, where the synthesis of many placental growth factors occurs (27–29). As with HO protein levels, the erythropoietic activity of the fetal liver increases on d 16 with a decline toward the end of pregnancy (30). Additionally, CO, a byproduct of HO, positively influences erythropoiesis in rats (31). Furthermore, erythropoiesis is positively correlated to fetal growth in the rat (32) and the peak of erythropoietic activity and of HO protein levels represent the phase of maximal growth. Based on these observations, we hypothesize that HO plays a role in fetal growth.

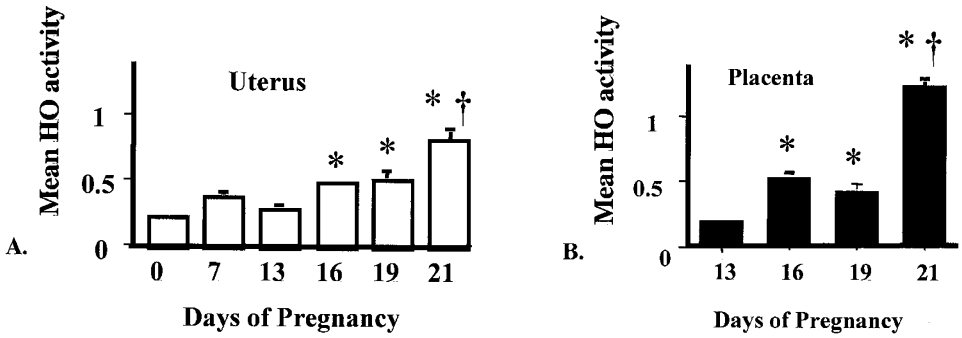

Figure 1. HO activity in rat uterus (*A*) and placenta (*B*) throughout pregnancy. The *open bars* represent the mean (± SE) of four separate uterine samples, and the *solid bars* represent the mean (± SE) of pooled placentas from four animals. The mean HO activity is expressed as pmol CO/μg protein/h. *Lanes 0–21*: days of gestation. $^*p < 0.05$ *vs* d 7 and 13; †$p < 0.05$ *vs* d 16 and 19; ‡$p < 0.05$ *vs* d 0.

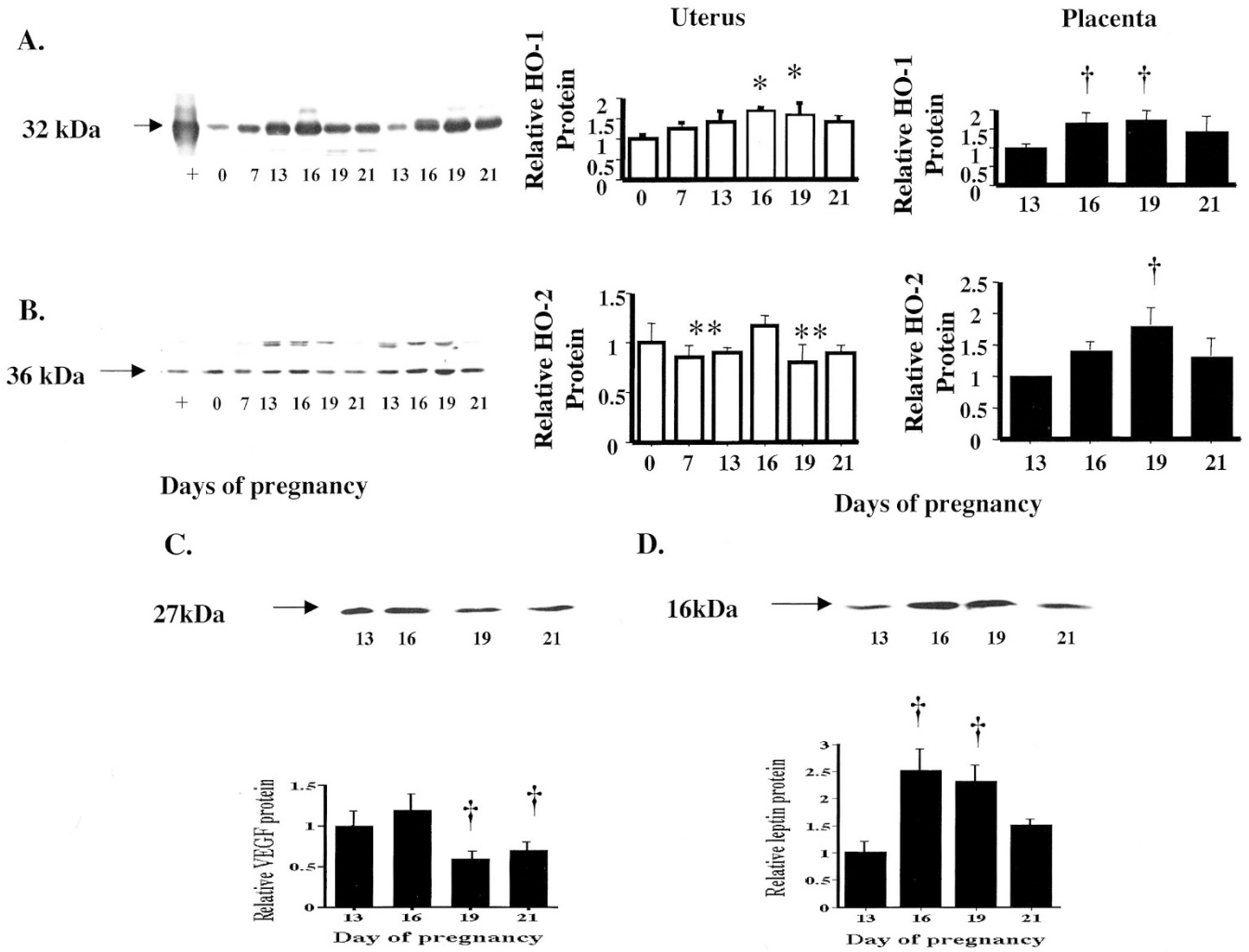

Figure 2. Immunoreactive HO-1 (*A*), HO-2 (*B*), VEGF (*C*), and leptin (*D*) proteins in the pregnant rat. For HO-1 and HO-2 (*A* and *B*), panels on the left are representative of four Western analyses and the densitometric evaluation is shown on the right. +: positive control derived from cobalt chloride injected adult rat liver for HO-1, and noninjected adult rat testis for HO-2. For VEGF amd leptin *(C* and *D*), the *upper panels* are representative of four Western analyses in the placenta and the *lower panels* are the densitometric evaluation from these analyses. In all figures, *lanes 0–21*: days of gestation. Equal loading was verified by Coomassie blue staining. Densitometric values are expressed as a ratio to d 0 values (uterus) or d 13 (placenta) and represent the mean ± SE of four separate experiments. $^{*}p < 0.05$ *vs* d 0; †$p < 0.05$ *vs* d 13.

As with the uterine HO protein expression, HO-1 and HO-2 mRNA levels reached a peak at about d 16 of pregnancy and declined toward the end of pregnancy. In contrast, HO activity showed a peak on d 21 of pregnancy. The lack of correlation between protein levels and HO activity may suggest post-translational regulation of HO-1 and/or HO-2, as both contribute to the total HO activity. This is not unique to this model. Others have demonstrated translational activation of HO-2 in cultured neurons (33). In the neonatal lung, there also appears to be post-transcriptional activation of HO-1 protein in hyperoxia (34).

We demonstrated that HO protein levels are comparable to the expression of VEGF and leptin. These factors have a clear role in fetal growth as they are found at lower levels in pregnancies complicated by intrauterine growth restriction (20, 35). Although this is merely an association, we speculate that HO may play a role in intrauterine growth. This is corroborated by a recent study from our lab demonstrating that overexpression of HO-1 protein or inhibition of HO activity is associated with modulation of VEGF in the placenta (36). Whether CO or another byproduct of HO regulates growth factor expression, or whether HO is regulated by these growth factors is not yet clear. There are several mechanisms by which HO may alter growth factors and mediate fetal growth. First, HO generates CO, which in turn activates guanylyl cyclase *in vitro* (16) and in isolated cells (12). This activation of guanylyl cyclase is similar to that observed with NO, which plays an essential role in fetal perfusion and fetal weight at delivery (37). Chronic reduction of NO production in the last trimester of pregnancy results in significant growth retardation and hemorrhagic disruption of hind limbs in rats (38). CO can also modulate p38 MAP kinase activation, leading to wide-ranging effects, including alteration of cell proliferation and apoptosis (39). Targeted disruption of HO-1 results in abnormal fetal growth as shown in the human and the mouse models (17, 18) and inhibition of HO activity by ZnPP results in decreased fetal growth (36).

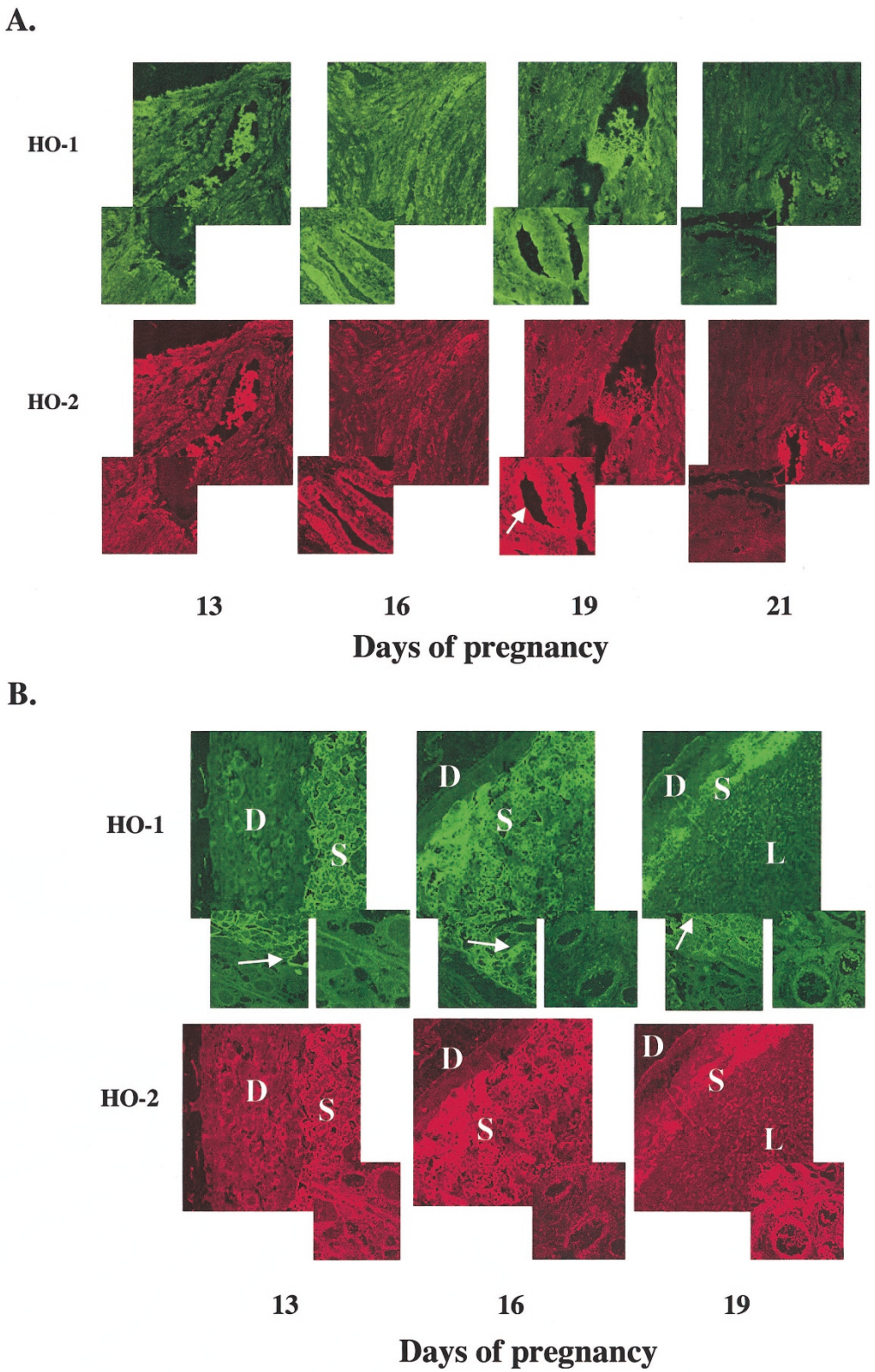

Figure 3. Immunohistochemical detection of HO protein in the uterus (*A*) and the placenta (*B*). Tissue slices were incubated with antibody to HO-1 and HO-2, as described in the methods, and viewed with confocal laser microscopy. d 13–21 represents the gestational age. (*A*) Uterus: all insets show a higher magnification of a blood vessel. No increased HO signal can be observed except in the endothelial layer of the blood vessels on d 19 (*arrow*). (*B*) Placenta: the letters represent the decidua (*D*), the syncytiotrophoblast (*S*), and the labyrinth (*L*). Note the strong HO signal in the syncytiotrophoblast layer compared with the other layers. In all of the panels representing HO-1 signal in the placenta, the leftmost insets demonstrate a higher magnification of the syncytiotrophoblast layer. Note that the increased HO-1 signal is found in the basement membrane (*arrow*). For both HO-1 and HO-2, the right insets show that the blood vessels in the labyrinth had no increased signal.

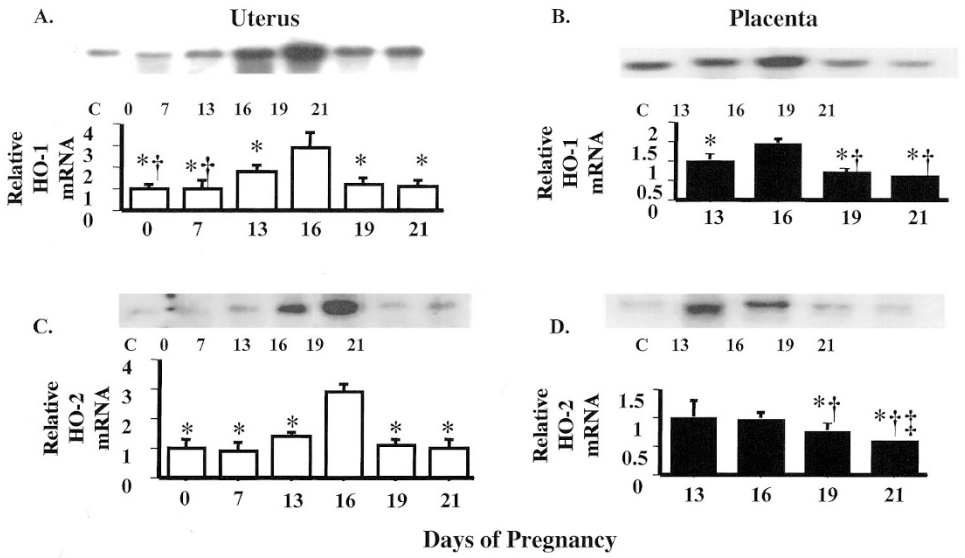

Figure 4. Uterine HO-1 (*A*), placental HO-1 mRNA (*B*), uterine HO-2 (*C*), and placenta HO-2 mRNA (*D*). *Upper panels*: Representative of four Northern analyses. *C*: positive control derived from adult rat liver after CoCl injection for HO-1 and untreated adult rat brain for HO-2. *Lanes 13–21*: days of pregnancy. *Lower panels*: Relative HO-1 mRNA in the uterus (*A*) and placenta (*B*) and HO-2 mRNA in the uterus (*C*) and placenta (*D*). The *open bars* represent the mean (± SE) of four separate uterine samples and the solid bars represent the mean (± SE) of pooled placentas from four animals. Values are normalized to d 0 values (uterus) or d 13 (placenta). *Lanes 0–21*: days of gestation. $^{*}p < 0.05$ *vs* d 0; †$p < 0.05$ *vs* d 13; ‡$p < 0.05$ *vs* d 19.

Perhaps the HO byproduct CO is important in modulating signaling pathways involved in fetal growth

Secondly, because the reaction of HO results in the release of iron, this could also affect growth-related gene regulation through iron response elements (40). In a recent study, iron loading by treatment of cells with ferric salt correlated with loss of endocytic activity, which coincided with the formation of rat hepatocyte leptin-1 (41). Perhaps iron release from HO regulation is important in dictating leptin and VEGF expression. Nonetheless, the changes in HO expression during gestation are not very large in comparison to the substantial maternal iron intake during pregnancy (42), making this possibility less likely. Additionally, with iron release there is often induction of ferritin (43, 44), which sequesters iron. The induction of ferritin in the placenta or uterus throughout gestation has not been examined here or elsewhere.

Another possible way in which HO could affect growth is through the modulation of oxidative tone or inflammation. It is known that up-regulation of HO-1 can ameliorate oxidative stress and decrease inflammation (45, 46). Loss of HO-1 could result in increased susceptibility to oxidative and inflammatory injury. This could subsequently alter growth factors and fetal growth as suggested by others (47). More recently, others have documented that the HO protein devoid of activity can modulate antioxidant gene expression (48, 49), but the mechanism by which this occurs is not yet defined and remains to be investigated systematically.

In summary, in the pregnant rat, HO-1 and HO-2 have a gestational pattern of expression that approximates that of key growth factors important in gestation. The role of gestational HO expression in modifying fetal growth needs to be further investigated.

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