Relationship between tissue damage and heme oxygenase expression in chorionic villi of term human placenta

G. E. LASH, B. E. MCLAUGHLIN, S. K. MACDONALD-GOODFELLOW, G. N. SMITH, J. F. BRIEN, G. S. MARKS, K. NAKATSU, AND C. H. GRAHAM. Relationship between tissue damage and heme oxygenase expression in chorionic villi of term human placenta. Am J Physiol Heart Circ Physiol 284: H160–H167, 2003. First published September 26, 2002; 10.1152/ajpheart.00738.2002.—Heme oxygenase (HO) catalyzes the oxidation of heme to carbon monoxide (CO), biliverdin, and iron and is thought to play a role in protecting tissues from oxidative damage. There are three isoforms of HO: HO-1 (inducible), HO-2 (constitutive), and HO-3 (unknown function). Preeclampsia is characterized by an inadequately perfused placenta and areas of tissue damage. We hypothesized that damaged areas of placentas from women with PE and uncomplicated pregnancies are associated with an alteration in HO expression. Compared with microsomes isolated from morphologically normal and peri-infarct chorionic villi of pathological placentas, microsomes from infarcted chorionic villi from the same placentas had decreased HO activity measured under optimized assay conditions. There was no correlation between microsomal HO levels and activity and tissue damage in uncomplicated pregnancies. Whereas there was no significant difference in HO-1 protein levels across all regions of uncomplicated and mildly preeclamptic pregnancies, HO-2 protein levels were decreased (P < 0.05) in peri-infarct regions and infarcted chorionic villi of mildly preeclamptic pregnancies. Immunohistochemical analysis revealed an apparent decrease in both HO-1 and HO-2 protein expression in damaged tissues. HO-1 and HO-2 were immunolocalized in the syncytiotrophoblast layer of the chorionic villi, the underlying cytotrophoblast, and in the vascular endothelium. This study suggests that the ability of the chorionic villi to oxidize heme to CO, biliverdin, and iron may be compromised in areas of tissue damage in the placenta of women with preeclampsia.

Heme oxygenase (HO) is the rate-limiting enzyme in the metabolism of heme to carbon monoxide (CO), biliverdin, and free iron. There are three isoforms of HO: HO-1, -2, and -3. HO-1 (also known as heat shock protein 32) is inducible by a number of stress stimuli including hypoxia. While HO-2 is constitutively expressed (12) and HO-3 has very little known activity (14), it is not clear how each of these isoforms contributes to the total HO activity of a cell. It was first proposed in 1991 that the CO product of HO enzymatic activity has a biological role in vivo (13). It has since become evident that CO acts as a vasodilator and as a second messenger in some cell types. In addition, biliverdin is further metabolized into bilirubin in a reaction catalyzed by biliverdin reductase; both metabolites are potent antioxidants and have been suggested to have cytoprotective properties (18).

Preeclampsia is a multisystemic disorder of pregnancy characterized by maternal hypertension and proteinuria. This condition affects 5–10% of all pregnancies and is a major contributor to perinatal mortality and morbidity. While the precise etiology of this disease is unclear, associated with preeclampsia is a lack of trophoblast-mediated remodeling of the uterine spiral arterioles, such that they are unable to accommodate the increase in blood flow demanded during pregnancy and retain their ability to respond to vasoactive molecules. Whereas the maladapted spiral arterioles associated with preeclampsia may be inconsequential in the first trimester, an increase in blood flow demanded by the rapidly growing fetus of the second and third trimesters can lead to an inadequately perfused placenta. In fact, blood flow to the placenta of preeclamptic women has been proposed to be intermittent or pulsatile (1, 8). This may lead to regional hypoxia followed by reperfusion, which in turn may result in oxidative damage and an adaptive response in the placenta involving the transcription of genes, such as oxidative stress response genes. Indeed, the profile of oxidative stress markers observed in the placenta of women with preeclampsia has been reproduced in an in vitro ischemia-reperfusion injury model (10). It is believed that placental infarcts (or areas of tissue damage) are caused by localized regions of hypoxia followed by reperfusion. Whereas there are areas of tissue damage in the placenta of uncomplicated pregnancies as well as in pregnancies complicated by preeclampsia, the extent of placental damage in a pregnancy compli-
The presence of HO has been demonstrated in the human placenta using CO formation as an index of HO enzymatic activity (15) and by immunohistochemistry (11, 16). It has recently been reported that there were no differences in the protein content of HO-2 in human placental homogenate from pregnancies complicated by preeclampsia and fetal growth restriction compared with controls (5). However, immunohistochemical analysis showed decreased HO-2 expression in endothelial cells in preeclampsia and fetal growth restriction compared with normotensive controls (5). It was also reported that the human placenta contains relatively little HO-1 protein, based on Western blot and immunohistochemical analysis (5, 11). However, these previous studies concentrated on either morphologically healthy tissue or did not make a distinction between healthy and injured tissue. Furthermore, previous studies have not correlated HO enzymatic activity with HO protein levels.

On the basis of the concept that HO plays an important role in protecting tissues from oxidative damage, we hypothesized that microsomal HO activity is altered in areas of tissue damage compared with morphologically normal areas of the chorionic villi of placenta from uncomplicated and pathological pregnancies. To test this hypothesis, we examined HO activity and HO-1 and HO-2 protein levels in microsomes isolated from normal, peri-infarct, and infarct regions of chorionic villi of the placenta of women with mild preeclampsia and uncomplicated pregnancies.

MATERIALS AND METHODS

Chemicals. Hemin, ethanalamine, BSA, and NADPH were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were obtained from VWR CanLab (Toronto, Ontario, Canada) and were at least reagent grade. Working solutions of methemalbumin were prepared as described previously (17).

Tissue samples. Placentas (n = 4 for each group) were collected after lower segment cesarean section (LSCS) from the following groups of women. 1) Women whose pregnancies were complicated by mild preeclampsia. Preeclampsia was defined by the development of blood pressure of at least 140 mmHg systolic and 90 mmHg diastolic on two or more separate occasions after the 20th wk of pregnancy, having previously been normotensive and without a history of renal disease. The definition also included proteinuria (either >300 mg per 24-h collection or 2+ on a voided random urine sample) in the absence of urinary tract infection (7). Gestational ranges ranged from 38 to 42 wk. 2) Women whose pregnancies were uncomplicated, with normal fetal growth and no incidence of hypertension, and who were having a LSCS for nonobstetric reasons such as previous LSCS or breach presentation. Gestational ages ranged from 38 to 42 wk.

Tissue samples from three regions (morphologically normal chorionic villi, peri-infarct chorionic villi, and infarct chorionic villi) of each placenta were taken and either frozen immediately in liquid nitrogen and stored at -80°C until required or fixed in paraformaldehyde for preparation of paraffin sections.

Preparation of the microsomal fraction. Each frozen placental sample was homogenized, the microsomal fraction was retained, and the protein content was determined as described previously (17) by spectrophotometric analysis using a microplate reader (EL 800, BioTek Instruments; Winooski, VT). The microsomal samples were stored at -80°C until required for analysis.

Microsomal HO enzymatic activity. For each microsomal sample, HO enzymatic activity was determined in vitro by measuring the rate of CO formation during the NADPH-dependent oxidative biotransformation of heme (19) using optimized experimental conditions (15). Briefly, for each microsomal fraction duplicate, 1-ml reaction mixtures consisting of phosphate buffer (100 mM KH₂PO₄), microsomal protein (0.2 mg/ml), methemalbumin (25 μM heme and 2.5 μM BSA), and NADPH (500 μM) were mixed in 3.5-ml amber vials. Each vial was sealed with a Teflon-lined silicon septum and a screw cap (Chromatographic Specialties), and the headspace was flushed with CO-free air introduced by means of a needle system used to pierce the septum and incubated for 15 min at 37°C. CO in the headspace gas was measured by gas-solid chromatography as previously described (15, 19). For each microsomal sample, a third vial was prepared in which NADPH was not added to the reaction mixture (sample blank). The amount of CO in the headspace gas of each sample was determined by interpolating the peak area value of the CO chromatographic signal on the linear CO standard curve (10–156 pmol CO). NADPH-dependent formation of CO was calculated by subtracting the value for CO produced in the microsomal sample not containing NADPH (blank) from the value for CO formed in the tissue sample containing NADPH. HO enzymatic activity in the microsomal fraction of the homogenate of each placental region was expressed as nanomoles of CO formed per gram tissue wet weight per hour.

HO-1 and HO-2 Western blot analysis. To determine the specificity of the HO-1 and HO-2 antibodies, Western blot analysis was performed on protein samples extracted from a first trimester extravillous trophoblast cell line (HTR-8/SVneo) (9). Cells were incubated for 24 h with or without methemalbumin (30 μM), a known inducer of HO-1 expression in trophoblast cells (S. D. Appleton, personal communication), before the extraction of total cellular protein.

For HO-1 and HO-2 analysis, an extract of total cellular protein containing 50 or 30 μg, respectively, was loaded onto a 12.5% (wt/vol) polyacrylamide gel, separated by electrophoresis under reducing conditions, and then transferred onto Immobilon-P membranes (Millipore; Bedford, MA). Membranes were blocked overnight at 4°C in PBS containing 0.1% (vol/vol) Tween 20 (PBS-T) and 10% (wt/vol) skim milk powder. The blots were then incubated (1 h) with a 1:2,000 dilution of either the polyclonal anti-HO-1 (SPA-896, StressGen; Victoria, British Columbia, Canada) or anti-HO-2 (SPA-897, StressGen) antibodies. Separate blots were incubated with the anti-HO-1 that had been preincubated (1 h) with an equal dilution of the peptide immunogen used to generate either the anti-HO-1 antibody (SPT-896, StressGen) or the anti-HO-2 antibody (SPT-897, StressGen). Separately, separate blots were incubated with the anti-HO-2 antibody that had been preincubated (1 h) with an equal dilution of the peptide immunogen used to generate either the anti-HO-1 antibody (SPT-896, StressGen) or the anti-HO-2 antibody (SPT-897, StressGen). After being washed in PBS-T, the blots were incubated with a peroxidase-labeled goat anti-rabbit IgG secondary antibody (Vector Laboratories; Burlingame, CA). Antigen was detected by enhanced chemiluminescence (Amersham; Oakville, Ontario, Canada) and
exposure of the membrane onto Kodak X-Omat Blue film (Kodak; Rochester, NY). Western blot analysis was also performed for HO-1 and HO-2 on the microsomal fractions prepared from the different regions of placentas of uncomplicated and preeclamptic pregnancies. The intensity of the bands was determined using the Un-Scan-It software package (Silk Scientific; Orem, UT). Relative amounts of HO protein were obtained after normalization against the intensity of the HO bands from extracts of morphologically normal areas of uncomplicated pregnancies.

**RESULTS**

**Microsomal HO activity.** There was no difference in microsomal HO activity of chorionic villi from women with mild preeclampsia compared with uncomplicated pregnancies for each of the three chorionic villi regions that were studied (Fig. 1, A and B). For chorionic villi from the placenta of women with preeclampsia, there was a decrease in microsomal HO activity in the infarct regions (P < 0.05) compared with the morphologically normal chorionic villi and peri-infarct regions (Fig. 1B). With the use of a P value of <0.05 for statistical significance, there was no significant decrease in HO activity across the morphologically normal, peri-infarct, and infarct chorionic villi regions of placentas from uncomplicated pregnancies. However, there was a trend (P < 0.07) toward decreased HO activity in the infarcted regions compared with the morphologically normal and peri-infarcted regions (Fig. 1A).

**Western blot analysis of HO-1 and HO-2 expression.** Specificity of the anti-HO-1 and anti-HO-2 antibodies was determined by Western blot analysis using extracts from HTR-8/SVneo first trimester trophoblast cells treated with methemalbumin. The antibody against HO-1 protein detected a band at 32 kDa, which corresponds in mass to the HO-1 protein (Fig. 2A). Moreover, additional bands at ~90, 70, 50, and 20 kDa were also detected. Preincubation of the anti-HO-1 antibody with the immunogen peptide used to generate the antibody resulted in loss of detection of only the 32-kDa band (Fig. 2B). There was no loss of the HO-1 band (or any other bands) after preincubation of the HO-1 antibody with the HO-2 blocking peptide (Fig. 2C). Similarly, the antibody against HO-2 protein predominantly detected a band at 36 kDa, which is the predicted mass of HO-2, as well as nonspecific bands at 70, 50, and 20 kDa (Fig. 2D). Preincubation of the HO-2 antibody with the HO-1 peptide did not affect the detection of any bands (Fig. 2E). However, the 36-kDa band corresponding to HO-2 was not detected after preincubation of the anti-HO-2 antibody with the HO-2 peptide (Fig. 2F). Again, the nonspecific additional bands were not removed by the blocking peptide.

With the use of the above antibodies, relative HO-1 and HO-2 protein levels in the microsomal fractions of placentas from women with uncomplicated or preeclamptic pregnancies were determined by Western blot analysis. HO-1 was detected as a band with a molecular weight of 32 kDa (Fig. 3A). Several nonspecific bands were also detected in the microsomal fractions with the anti-HO-1 antibody corresponding to

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**Fig. 1.** Heme oxygenase (HO) activity in microsomes of morphologically normal (MN), peri-infarct (PI), and infarct (I) chorionic villi. A: uncomplicated pregnancies; B: pregnancies complicated with preeclampsia. *Statistical significance in the I group from the MN and PI groups in placentas of women with preeclampsia (P < 0.03, ANOVA, MN vs. I; P < 0.02, PI vs. I; P < 0.05, Fisher’s post hoc test). There were no differences between the preeclampsia and uncomplicated pregnancy groups for any of the regions studied (MN: P > 0.6; PI: P > 0.2; I: P > 0.1, Student’s t-test). Bars represent means ± SD of microsomal preparations from four separate placentas.
~120 and 70–50 kDa (data not shown). There was a high degree of variability in HO-1 protein levels in microsomal fractions obtained from placentas from both uncomplicated and pathological pregnancies. However, there was no significant difference in HO-1 protein levels between the two groups or across the regions of the placenta sampled from each group. A 36-kDa band corresponding to HO-2 was detected in all samples (Fig. 3B). There was also a nonspecific band at ~120 kDa detected using the anti-HO-2 antibody (data not shown). Similar to the HO-1 protein levels, there was a high degree of variability in the levels of HO-2 protein detected in the microsomal fractions obtained from placentas of both uncomplicated and pathological pregnancies. There was no statistical difference in the levels of HO-2 protein detected for each of the placental regions studied between the two groups. When comparing HO-2 protein levels according to the degree of tissue damage, in the uncomplicated pregnancies there was a trend toward a decrease in HO-2 protein levels in the microsomal fraction prepared from infarct chori- onic villi compared with microsomes prepared from morphologically normal chorionic villi (P = 0.1; Fig. 3B). In the microsomal fractions of placentas prepared from pregnancies complicated by preeclampsia, there were lower HO-2 protein levels in both the peri-infarct and infarct chorionic villi compared with the morphologically normal chorionic villi (peri-infarct, P < 0.01; infarct, P < 0.005; Fig. 3B).

**Immunohistochemical localization of HO-1 and HO-2.** With the use of the same antibodies, HO-1 and HO-2 proteins were immunolocalized to the syncytiotrophoblast, the underlying cytotrophoblast cells, and the endothelial cells of the vasculature (Figs. 4 and 5). There was no staining with either antibody when the antibody was preincubated with the appropriate blocking peptide (Figs. 4E and 5E). There was only faint diffuse staining of the sections when the primary
antibody was omitted from the protocol and replaced with normal rabbit serum (Figs. 4F and 5F).

Staining for HO-1 was apparently highest in the syncytiotrophoblast of morphologically normal chori-
tonic villi from placentas of both the uncomplicated (Fig. 4A) and pathological (Fig. 4B) groups. There was also staining of the endothelial cells of the placental vasculature of the morphologically normal chorionic villi. There was apparently less intense staining of the noninfarcted chorionic villi of women with mild pre-
eclampsia compared with the normotensive controls. Decreased staining for HO-1 was observed in the syn-
cytiotrophoblast and endothelial cells in the areas of chorionic villi that were damaged (peri-infarct) in the placenta of both normotensive (Fig. 4C) and pre-
eclamptic (Fig. 4D) pregnancies; however, there was no difference in staining intensity between these two groups.

There was no apparent difference in the staining intensity for HO-2 in the syncytiotrophoblast and endo-
thelial cells of the placental vasculature in the morphologically normal regions of the chorionic villi from the placenta of women with mild preeclampsia (Fig. 5B) compared with the normotensive controls (Fig. 5A). There was an apparent decrease in staining intensity for HO-2 with increasing tissue damage in the chori-
onic villi of placentas from women with preeclampsia (Fig. 5D) or uncomplicated pregnancies (Fig. 5C) in both the syncytiotrophoblast and endothelial cells of the vasculature.

DISCUSSION

The main finding of the present study is that HO activity is lower in microsomal preparations from se-
verely injured regions of placental chorionic villi from women with mild preeclampsia than in noninjured (or mildly injured) chorionic villi from the same placenta. This decrease in microsomal HO activity was associ-
ated with a decrease in HO-2 protein levels as indicated by the immunoblotting findings. In contrast, no significant correlation was observed between tissue damage and microsomal HO activity in placental chori-
onic villi of women with uncomplicated pregnancies.

Western blot analysis demonstrated that both HO-1 and HO-2 were detected in the microsomal fraction of placental samples. This is in contrast to Barber et al. (5), who could not detect HO-1 in placental samples. Consistent with the HO activity data, there was no difference in HO-1 or HO-2 protein levels between samples collected from the uncomplicated and pre-
eclamptic pregnancies. However, there was also no significant difference in HO-1 protein levels between the three regions of the placenta sampled from either of the two groups. In contrast, there was a statistically significant decrease in the levels of HO-2 with increasing tissue damage in samples from women with pre-
eclampsia and a trend toward decreased levels of this enzyme in damaged tissue from women with uncom-
plicated pregnancies. These results parallel the micro-
somal HO enzymatic activity findings and support data suggesting that HO-2 but not HO-1 protein levels cor-
relate with HO activity in microsomal samples from the placenta (B. E. McLaughlin, unpublished results).

Immunohistochemical analysis of sections from mor-
phologically normal or peri-infarct chorionic villi of placentas from women with mild preeclampsia and uncomplicated pregnancies was consistent with the microsomal HO activity results. Although the HO-1 and HO-2 staining intensity was apparently similar in tissues from the placenta of women with preeclampsia versus normotensive controls, there was a general de-
crease in staining intensity in the peri-infarct regions.
compared with the morphologically normal chorionic villi of both normal and pathological groups. Consistent with previous studies, we demonstrated immunolocalization of HO-2 in endothelial cells of the chorionic villi vasculature, syncytiotrophoblast, and in the smooth muscle cells of anchoring villi (2, 5, 11). In contrast to our findings, Barber et al. (5) demonstrated a decrease in endothelial HO-2 staining in chorionic villi samples from women with preeclampsia compared with normotensive controls. These differences may be attributable to differences in the patient populations selected in both studies. In our study, tissues from four late-gestation mildly preeclamptic women were analyzed, whereas the study of Barber et al. (5) employed tissues from a higher number of patients with an earlier onset, more severe form of the disease.

The immunohistochemical analysis of the present study also demonstrated the presence of HO-1 in chorionic villi. This is in contrast to the studies of Lyall et al. (11) and Barber et al. (5), who provided evidence of HO-2 expression but little or no HO-1 expression. It is possible that these discrepancies are due to differences in the antibodies used. Whereas a new anti-human HO-1 antibody from StressGen was used in our study, other studies have used anti-rat HO-1 antibodies as well as other anti-human HO-1 antibodies. The specificity of the antibodies used in the present study was confirmed by the fact that incubation with the blocking peptides completely abolished the immunostaining with both the anti-HO-1 and anti-HO-2 antibodies. In addition, the peptides also abolished the detection of the 32-kDa HO-1 and 36-kDa HO-2 bands in the im-

Fig. 4. Immunohistochemical analysis for localization of HO-1 protein. A: uncomplicated pregnancy MN villi; B: preeclamptic pregnancy MN villi; C: uncomplicated pregnancy PI villi; D: preeclamptic pregnancy PI villi; E: HO-1 blocking peptide; F: normal goat serum. S, syncytium; E, endothelial cells.
munoblots of HTR-8/SVneo cell extracts. Although other bands were also detected in the immunoblots, it is unlikely that the immunostaining is due to the binding of the antibodies to these proteins because preincubation with the blocking peptides completely abolished the staining of the sections.

Physiologically, preeclampsia is characterized by deficient trophoblastic remodeling of the uterine spiral arterioles, such that these vessels retain their ability to respond to vasoactive molecules. It is likely that when an increase in utero-placental blood flow is demanded by the rapidly growing fetus during the second and third trimesters the lack of vascular remodeling leads to an inadequately perfused placenta. This may lead to repeated cycles of hypoxia/reperfusion, which could lead to tissue damage and/or an adaptive response of the placenta involving the transcription of genes, such as those encoding oxidative stress response proteins. Although the functional HO levels in the microsomal fractions of chorionic villi from women with preeclampsia were not different from those of uncomplicated pregnancies, the in vitro conditions in which enzymatic activity was measured were optimized by providing excess substrate and cofactors. A recent study (3) from our laboratory demonstrated a decrease in HO enzymatic activity (as measured by the rate of CO formation) as the oxygen content of the enzymatic reaction mixture was decreased from 20% to 1%. Thus it is likely that the in vivo HO enzymatic activity in certain regions of the placenta of women with preeclampsia is compromised due to inadequate perfusion, which leads to reduced oxygen availability.

HO has been proposed to play a role in vasodilation and cytoprotection of the placenta. Lyall et al. (11) and

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Fig. 5. Immunohistochemical analysis for localization of HO-2 protein. A: uncomplicated pregnancy MN villi; B: preeclamptic pregnancy MN villi; C: uncomplicated pregnancy PI villi; D: preeclamptic pregnancy PI villi; E: HO-2 blocking peptide; F: normal goat serum.
Bainbridge et al. (4) demonstrated that CO (from heme metabolism) is responsible in part for a decrease in the perfusion pressure of placental vessels using an in vitro placental perfusion system. The study by Bainbridge et al. (4) also suggested that the CO-mediated decrease in placental perfusion pressure occurs via the activation of the enzyme soluble guanylyl cyclase and the production of the second messenger cGMP. One of the other products of the HO-catalyzed degradation of heme is biliverdin, which is further reduced to bilirubin, both of which have antioxidant properties. Ahmed et al. (2) demonstrated in vitro that increased HO-1 protein levels in term chorionic villi explants leads to increased cytoprotection from proapoptotic factors.

The precise reasons for the decreased microsomal HO-2 levels and HO enzymatic activity in damaged areas of placentas from preeclamptic pregnancies remain unclear. However, it is possible that this decrease in HO expression is a consequence of the extent of tissue damage. Whereas infarcts are present in placentas from uncomplicated pregnancies, infarcts in placentas from preeclamptic pregnancies tend to be larger and more numerous (6). HO-2 is the constitutive form, and the decrease in the expression of this enzyme in areas of tissue damage may be part of a general inability of that tissue to synthesize proteins de novo. Therefore, in the placentas of women with preeclampsia, it is likely that the overall magnitude of HO activity and, hence, the capacity for heme metabolism is decreased, resulting in lower CO and biliverdin concentrations. In turn, the decrease in these bioactive substances may contribute to more tissue damage.

The authors thank Drs. Hendrik J. Vreman and David K. Stevenson, Department of Pediatrics, Stanford University School of Medicine, for the use of the gas chromatograph to measure CO formation.

This research was supported by Heart and Stroke Foundation of Ontario Grants NA-4438 and T-3361. G. Lash was the recipient of a Post-Doctoral Fellowship from the Canadian Hypertension Society.

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