Effects of hypoxia on heme oxygenase expression in human chorionic villi explants and immortalized trophoblast cells

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Am J Physiol Heart Circ Physiol 284: H853–H858, 2003; 10.1152/ajpheart.00655.2002.—Although hypoxia induces heme oxygenase (HO)-1 protein and mRNA expression in many cell types, hypoxia has also been shown to decrease HO-1 mRNA and protein expression. We tested the hypothesis that 24-h preexposure to hypoxia in human placental preparations suppresses HO protein expression and enzymatic function. Immortalized HTR-8/SVneo first-trimester trophoblast cells and explants of normal human chorionic villi (CV) from term placentas were cultured for 24 h in 1%, 5%, or 20% O2. HO-2 protein content was decreased by 17% and 5% in human trophoblast cells after 24-h exposure to 1% and 5% O2, respectively, versus 20% O2. In contrast, HO-2 protein content in CV explants was unaffected by changes in oxygenation. Similarly, HO enzymatic activity was unchanged in both preparations after 24-h exposure to 1%, 5%, or 20% O2. The above data do not support the hypothesis that hypoxia in the human placenta suppresses both HO protein content and HO protein function. The present observations reinforce the necessity to determine both HO protein expression and function.

Hemoglobin (Hb) oxygenase (HO) catalyzes the breakdown of heme in the presence of oxygen (O2) and NADPH to form carbon monoxide (CO), biliverdin, and iron. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Both biliverdin and bilirubin have been shown to be potent antioxidants in several tissues (30), and CO has been proposed to play a role in cell-cell communication and in the relaxation of blood vessels (19, 32–34). Earlier studies on the stoichiometry of the HO reaction demonstrated that HO consumed 3 molecules of O2 and 2 molecules of NADPH for every molecule of heme biotransformed (23, 31). Recently, we (2) demonstrated that the magnitude of placental chorionic villi HO activity is dependent on O2 availability.

There are two predominant isoforms of HO. HO-1 is the inducible isoform, whereas the expression of HO-2 is constitutive; hence, HO-2 is responsible for basal HO activity in cells. It has been proposed that HO-1 is responsible for the increase in cellular HO activity observed in various pathological conditions, and it is therefore categorized as a stress enzyme (16). Several studies have revealed that hypoxic exposure of cells from various origins increases their expression of HO-1 at both the protein and mRNA levels (7, 8, 13, 26, 28, 29).

In contrast, Shibahara and colleagues (22) reported inhibition of HO-1 expression by hypoxia. These investigators exposed cultured human umbilical vein endothelial cells, human coronary artery endothelial cells, and astrocytes to 1% O2 (vs. 20% O2) and found that HO-1 mRNA and protein expression was reduced after 24-h incubation. Similar reductions in HO-2 expression have been observed in endothelial cells of placentas obtained from pregnancies characterized by impaired uteroplacental blood flow, such as preeclampsia and those associated with intrauterine growth restriction (IUGR) (3). On the basis of these observations, the objective of the current study was to test the hypothesis that a 24-h preexposure to hypoxia in human placental preparations suppresses HO protein expression and enzymatic function.

MATERIALS AND METHODS

Reagents and solutions. Hemin, ethanolamine, BSA, and NADPH were obtained from Sigma (St. Louis, MO). All other chemicals were at least of reagent grade and were obtained from BDH (Toronto, Ontario, Canada). The stock solution of methemalbumin (1.5 mM hemin and 0.15 mM BSA) was prepared as previously described (35). Briefly, hemin was dissolved in 0.5 ml of aqueous 10% (wt/vol) ethanolamine. BSA dissolved in 2 ml of deionized water was added to the hemin solution. The volume was made up to 7 ml and slowly

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adjusted to pH 7.4 with 1 M HCl and vigorous stirring. The final volume for the stock solution was adjusted to 10 ml with deionized water. The methemalbumin stock solution was prepared with the laboratory lights turned off and was stored at −20°C for up to 1 mo.

Preparation of cultured choriocilllli explants from human placenta. Human placentas of gestational age 37−42 wk were obtained from elective caesarean deliveries of uncomplicated pregnancies at Kingston General Hospital. Within 1 h of delivery, samples of choriocilllli from placental cotyledons were dissected. This region was selected because it is highly vascularized and has been previously shown to possess high levels of functional HO (21). Noninfarcted areas of choriocilllli were identified based on gross morphology and the absence of calcium and fibrin deposits, which characterize areas of infarction.

Choriocilllli explants (CVE) were washed in PBS and then cultured in 1%, 5%, or 20% O2 in RPMI 1640 medium containing 5% FBS and 300 U/ml penicillin-20 μg/ml streptomycin (GIBCO/Invitrogen; Burlington, Ontario, Canada). Aerating with 5% O2 results in a PO2 of ~35−40 mmHg, or the rate that found in vivo in placentas. On the contrast, 1% O2 (<10 mmHg) would be considered hypoxic for many tissues, including choriocilllli at term. After 24 h, the explants were removed from the culture medium and stored at −80°C until required for analysis. To isolate microsomal fractions, 10% (wt/vol) homogenates of thawed explants were prepared in ice-cold, homogenizing buffer (20 mM KH2PO4, 135 mM KCl, and 0.10 mM EDTA adjusted to pH 7.4 at 4°C) using an ultrasonic probe (Sonic Dismembrator, Fisher Scientific; Toronto, Ontario, Canada). Microsomal fractions of the homogenates were obtained by centrifugation at 10,000 g for 20 min at 4°C, followed by centrifugation of the supernatant at 100,000 g for 60 min at 4°C. The 100,000-g pellet (microsomal fraction) was resuspended in 100 mM KH2PO4 buffer (adjusted to pH 7.4 with 1 M KOH) using a Potter-Elvehjem homogenizing system. The microsomal fraction was divided into equal aliquots, placed into microcentrifuge tubes, and stored at −80°C for up to 1 mo. HO enzymatic activity remains stable under these storage conditions. Total protein concentrations of the microsomal fractions were determined by the Biuret method (9), which was modified as described previously (20).

Preparation of HTR-8/SVneo first-trimester human trophoblast cells. HTR-8/SVneo trophoblast cells were obtained from explant cultures of human first-trimester placentas (8−10 wk of gestation) and immortalized by transfection with a cDNA construct encoding the simian virus 40 (SV40) large T antigen (11). These cells, although nontumorigenic and metastatic, are highly invasive in vitro and exhibit phenotypic properties of extravillous placent al cytotrophoblasts in situ. Cells were cultured in RPMI 1640 containing 5% FBS in 1%, 5%, or 20% O2 for 24 h. As a positive control for HO-1 induction, cells were also incubated in 20% O2 in the presence of 30 μg/ml methemalbumin or ethanolamine (vehicle control) for 24 h. After the incubation, the culture medium was decanted, and the cells were rinsed with PBS. Upon addition of 2 ml of 100 mM KH2PO4 buffer, the cells were scraped with a rubber policeman and transferred into 3-ml glass vials. The contents of the vials were then sonicated using a Sonic Dismembrator (Fisher Scientific). The homogenate was aliquoted into microcentrifuge tubes and stored at −80°C until required for analysis. Total protein concentrations were determined using the Biuret method (9).

Measurement of HO-1 and HO-2 protein expression. Thirty to fifty micrograms of total protein were loaded onto 12.5% (wt/vol) SDS-polyacrylamide gels, separated by electrophoresis under reducing conditions, and then transferred onto Immobilon-P membranes (Millipore; Bedford, MA). Membranes were blocked overnight at 4°C in a PBS-buffered solution containing 0.1% Tween 20 (PBS-T) and 10% skim milk powder. The blots were then incubated with a 1:2,000 dilution of polyclonal anti-HO-1 (SPA-896, StressGen; Victoria, British Columbia, Canada), anti-HO-2 (SPA-897, StressGen), or anti-PROXY-1 antiserum. The latter reacts with a 43-kDa protein shown previously by us to be induced in cells and tissues exposed to hypoxia (1−2% O2) (27). To determine and confirm specificity, the anti-HO antibodies were preincubated for 1 h at room temperature with the specific peptides (1,200 dilution) used by the manufacturer (StressGen) as immunogens for the generation of the antisera. This preincubation completely abolished HO immunostaining of placental and umbilical tissues and eliminated the bands in the Western immunoblots corresponding in molecular weight to HO-1 and HO-2, respectively (16a). Blots were subsequently incubated with a peroxidase-labeled goat anti-rabbit IgG secondary antibody (Vector Laboratories; Burlingame, CA). Antigen was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Oxnard, CA and Toronto, Ontario, Canada). Each blot was quantitated by optical densitometry (Un-Scan-It, Silk Scientific; Orem, UT).

Measurement of HO enzymatic activity. HO activity in the sonicate of HTR-8/SVneo cells or microsomal fractions of cultured CVE homogenate was determined by measuring the rate of CO formation during the NADPH-dependent oxidation of heme, as originally described by Vreman and Stevenson (36) and modified by Cook et al. (4). For cultured CVE of each placenta or each set of HTR-8/SVneo cells, HO activity in all homogenates was determined in the presence of three different O2 concentrations (1%, 5%, or 21%). To each of four 3.5-ml amber glass vials (Chromatographic Specialties; Brockville, Ontario, Canada) was added 100 mM KH2PO4 (pH 7.4), 0.2 mg microsomal protein, or 0.6 mg sonicate protein and methemalbumin (final concentration of 25 μM hemin and 2.5 μM BSA) in a final volume of 1 ml. While kept on ice, each vial was sealed with a Teflon-lined silicon septum and a screw cap (Chromatographic Specialties), and the headspace gas was purified with a gas mixture containing 1% O2 (balance N2; certified free of CO contamination) introduced with the use of a needle system used to pierce the septum while the contents were stirred constantly. The samples were then preincubated for 5 min in the dark at 37°C in a shaking water bath. NADPH (0.5 mM) was added to three of four vials, the headspace gas was replaced for 10 s with 1% O2, and the incubation was continued for another 15 min. The fourth vial, to which no NADPH was added, was used as a blank. The reaction was stopped by placing all vials on pulverized dry ice (−78°C), where they remained for 30 min until the headspace gas was analyzed for CO content. The above protocol was repeated using 5% and 21% O2 concentrations. For each O2 concentration, CO production was corrected for the CO produced in the reaction vial that contained no NADPH (blank). To determine total HO activity in the microsomal fractions of homogenates of choriocilllli of human placenta, a sample was prepared that was not purged with gas before the HO enzymatic reaction but was equilibrated with ambient air. This was done to verify that our experimental treatment, which included purging, did not exhibit altered HO activity.

CO levels in the headspace gas of each sample were quantitated using a RGA3 gas chromatograph (Trace Analytical; Menlo Park, CA) equipped with a ×13 molecular sieve and a
chemical spectrophotometric detector that quantitates, at 254 nm, elemental Hg formed from the reaction of CO with HgO, as described by Odrich et al. (24). The amount of CO in the headspace gas was determined by interpolating the peak area of the chromatographic signal on the linear CO standard curve (10–170 pmol CO), which had a correlation coefficient of 0.999 (n = 4 determinations). The rate of formation of CO in the microsomal fractions of chorionic villi homogenates was expressed as nanomoles of CO formed per milligram of protein per hour.

Data analysis. The HO enzymatic activity in the microsomal fractions of chorionic villi homogenates incubated at each O2 concentration was expressed as nanomoles of CO formed per milligram of protein per hour. The data are presented as group means ± SD. Parametric statistical analysis of the HO activity data of microsomal fractions of CVE homogenates for the different O2 concentrations was conducted by repeated-measures one-way ANOVA. For a statistically significant F-statistic (P < 0.05), a post hoc Newman-Keuls test was conducted to determine which experimental groups were statistically different (P < 0.05). For a statistically significant F-statistic for O2 concentration (P < 0.05), a repeated-measures one-way ANOVA was conducted, followed by a Newman-Keuls post hoc test to determine which experimental groups were statistically different (P < 0.05).

RESULTS

HO protein expression in human trophoblast cells cultured under hypoxic conditions. The results of Western blot analysis of HTR-8/SVneo first-trimester extravillous trophoblast cells preincubated in various O2 concentrations for 24 h indicated a significant decrease in HO-2 protein expression in cells incubated in 5% and 1% O2, respectively, relative to incubation in 20% O2 (P < 0.05; Fig. 1A). Also, the results indicated that HO-1 expression was barely detectable and not significantly different among treatment groups in these cells (Fig. 1A).

To assess whether HO-1 protein expression could be increased in response to a known inducer of HO-1, cells were incubated in 30 μM methemalbumin for 24 h. Figure 1B shows a significant increase in the expression of HO-1 protein but not HO-2.

To verify the degree of hypoxia within this system, Western immunoblot analysis was performed for the expression of PROXY-1. The expression of PROXY-1 was increased by 20% in HTR-8/SVneo cells incubated in 30 μM methemalbumin for 24 h relative to cells incubated in 20% O2 (P < 0.05). However, incubation of HTR-8/SVneo cells in 30 μM methemalbumin did not elicit a change in PROXY-1 protein expression (data not shown).

Functional HO levels in human trophoblast cells cultured under hypoxic conditions. HTR-8/SVneo first-trimester extravillous trophoblast cells were cultured in 1%, 5%, or 20% O2 for 24 h. Total HO activity was measured by assessment of CO formation in crude homogenates of the cells under optimized assay conditions (20% O2, 500 μM NADPH and 25 μM methemalbumin). As shown in Fig. 2A, there was no significant difference in functional HO levels among cells cultured in 1%, 5%, or 20% O2.

Heme is an exogenous inducer of HO protein levels and activity (1). To verify that functional HO levels could be increased in response to another known inducer of HO-1, HTR-8/SVneo cells were incubated in 30 μM methemalbumin (MHA; B) for 24 h. The results are representative of an experiment repeated 3 times.

DISCUSSION

Our results using HTR-8/SVneo trophoblast cells and third-trimester chorionic villi revealed very low HO-1 protein content, which was not significantly affected by 24-h incubation with low O2 concentration. A time course of HO-1 expression revealed no upregulation within the 24-h exposure to hypoxia. The 24-h time point was chosen based on the fact that the
viability of the CVE were compromised beyond 24 h. These novel findings are in contrast to those of Nakayama et al. (22), who reported inhibition of HO-1 expression by hypoxia, as well as to those of several groups, who reported hypoxia-induced increase in HO-1 expression in a variety of cell types (7, 8, 13, 28, 29, 38). Because of their location within the placenta, trophoblast cells experience pronounced variations in O2 levels, and, as a consequence, there is an increased demand for antioxidant molecules. Therefore, one might have anticipated an increase in HO-1 expression in trophoblast cells.

HO-1 expression in certain cell types is regulated by the transcription factor known as hypoxia-inducible factor-1 (HIF-1). Preliminary observations in HTR-8/SVneo cells showed an increase in HIF-1 protein expression and activity in response to hypoxia (unpublished data). Moreover, HTR-8/SVneo cells have been shown to respond to hypoxia with increased expression of various genes (6, 10, 25). While HIF-1 is involved in the hypoxic upregulation of many genes, increased HO-1 expression in response to hypoxia may also occur in a HIF-1-independent manner (37).

The present study also revealed that 24-h exposure of HTR-8/SVneo cells to 1% and 5% O2, versus 20% O2, resulted in 17% and 5% respective decreases in HO-2 protein content. However, these changes in HO-2 protein expression were not reflected in concomitant decreases in HO enzymatic function as determined by the rate of CO formation under optimized enzyme assay conditions. This apparent discrepancy may be explained by the fact that phosphorylation of HO-2 can increase HO activity in some cell types (5). Thus it is possible that phosphorylation of HO-2 in trophoblast cells maintains enzymatic activity despite a decrease in protein levels. A second possibility is that, due to their strategic location at the fetal-maternal interface, trophoblast cells are resistant to physiological changes in the microenvironment that otherwise would impair HO function and that a greater decrease in the magnitude of HO-2 protein levels must occur before HO enzymatic function is decreased. Such a decrease in HO-2 protein content may be brought about by pathological conditions that involve impaired uteroplacental blood flow, such as preeclampsia. Indeed, with the use of intact chorionic villi obtained from third-trimester placentas of pregnancies complicated by preeclampsia

![Fig. 2. Total HO enzymatic activity under optimized condition for HTR-8/SVneo first-trimester human extravillous trophoblast cells cultured in 1%, 5%, or 20% O2 (A) or 30 μM MHA or vehicle [1% (vol/vol) ethanolamine] (B) for 24 h. Bars represent mean HO activities ± SD of 3 determinations.](image)

![Fig. 3. Western immunoblot analysis of HO-1 and HO-2 protein expression in chorionic villi explants cultured in 1%, 5%, or 20% O2 for 24 h. The results are representative of an experiment repeated 3 times.](image)

![Fig. 4. Total HO enzymatic activity for chorionic villi explants cultured in 1%, 5%, or 20% O2 for 24 h measured under optimized conditions (20% O2) (A) and when O2 in the headspace of the reaction was decreased to 1% and 5% (B). *a–c* Group means with different letters are significantly different (P < 0.05). Bars represent mean HO activities ± SD of 4 determinations.](image)
and IUGR, Barber et al. (3) demonstrated 33–50% decrease in HO-2 expression in the endothelial cells of fetal vessels and little or nondetectable HO-1 protein. It would be important to determine whether a decrease in HO-2 protein content of similar magnitude results in a decrease in HO enzymatic function. The fact that, in our study, exposure to hypoxia for 24 h had little (17% decrease) or no effect on HO-2 protein content in trophoblast cells and chorionic villi, respectively, suggests that factors (e.g., ischemia-reperfusion, humoral factors) other than hypoxia contribute to the decreased expression of HO-2 protein in pathological pregnancies. Alternatively, it is possible that more prolonged episodes of placental hypoxia result in greater decrease in HO-2 protein content and, consequently, decreased HO enzymatic function. In support of this concept are the findings of Lyall et al. (17), who reported significantly decreased HO-2 protein levels in placentas of women who reside at high altitudes and are thus exposed to chronic hypoxia.

There are several possible explanations for the differential effect of hypoxia on HO-2 protein expression by the HTR-8/SVneo trophoblast cells versus the term CVE. The HTR-8/SVneo cell line used in this study was generated after SV40 large T antigen immortalization of trophoblast cells that had migrated out of 8- to 10-wk-old CVE (11). These cells are highly invasive and maintain many of the phenotypic properties of the parent cells, including the expression of several markers of extravillous cytotrophoblasts (11, 12, 14, 15). In contrast, intact chorionic villi contain multiple types of nonimmortal cells such as villous syncytiotrophoblast, villous cytotrophoblast, fibroblasts, endothelial cells, Hofbauer cells, and blood cells. It is possible that these cells do not respond to hypoxia in the same way as the HTR-8/SVneo cells. However, the fact that both the cell line and the intact chorionic villi responded to hypoxia with increased PROXY-1 expression indicates that these two biological systems maintained the capacity to respond to a hypoxic stimulus. In addition, the cell line exhibited increased HO enzymatic activity under optimized conditions after administration of methemalbumin, a known inducer of HO-1 expression. These findings indicate that, whereas 24-h incubation with 1% O2 did not increase HO enzymatic function in the HTR-8/SVneo cells, these cells maintained their ability to regulate HO protein expression. While a single 24-h episode of hypoxia (1% O2) may exert little or no effect on HO protein content in trophoblast cells and term chorionic villi, the present findings support our previous study (2) in which we demonstrated that HO activity is dependent on O2 availability during the enzymatic reaction. Thus, despite the fact that an episode of up to 24 h of severe hypoxia does not affect substantially HO protein expression and does not alter HO enzymatic function, lack of available O2 during the enzymatic reaction itself decreased HO-catalyzed oxidation of heme to CO, biliverdin, and iron.

In summary, the data of our study do not support the hypothesis that hypoxia in the human placenta substantially changes both HO protein content and HO enzymatic function. The fact that HO-2 protein content can be altered in human trophoblast cells without a corresponding alteration in HO enzymatic function reinforces the necessity to complement data on HO protein expression with data on HO enzymatic activity.

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H858


