Effect of glucose and oxygen deprivation on heme oxygenase expression in human chorionic villi explants and immortalized trophoblast cells

S. D. Appleton, G. E. Lash, G. S. Marks, K. Nakatsu, J. F. Brien, G. N. Smith, and C. H. Graham. Effect of glucose and oxygen deprivation on heme oxygenase expression in human chorionic villi explants and immortalized trophoblast cells. Am J Physiol Regul Integr Comp Physiol 285: R1453–R1460, 2003; 10.1152/ajpregu.00234.2003.—Although hypoxia induces heme oxygenase (HO)-1 mRNA and protein expression in many cell types, recent studies in our laboratory using human placental tissue have shown that a preexposure to hypoxia does not affect subsequent HO enzymatic activity for optimized assay conditions (20% O2; 0.5 mM NADPH; 25 μM methemalbumin) or HO-1 protein content. One of the consequences of impaired blood flow is glucose deprivation, which has been shown to be an inducer of HO-1 expression in HepG2 hepatoma cells. The objective of the present study was to test the effects of a 24-h preexposure to glucose-deprived medium, in 0.5 or 20% O2, on HO protein content and enzymatic activity in isolated chorionic villi and immortalized HTR-8/SVneo first-trimester trophoblast cells. HO protein content was determined by Western blot analysis, and microsomal HO enzymatic activity was measured by assessment of the rate of CO formation. HO enzymatic activity was increased (P < 0.05) in both placental models after 24-h preexposure to glucose-deficient medium in 0.5 or 20% O2. Preexposure (24 h) in a combination of low O2 and low glucose concentrations decreased the protein content of the HO-1 isoform by 59.6% (P < 0.05), whereas preexposure (24 h) to low glucose concentration alone increased HO-2 content by 28.2% in chorionic villi explants (P < 0.05). In this preparation, HO enzymatic activity correlated with HO-2 protein content (r = 0.825). However, there was no correlation between HO-2 protein content and HO enzymatic activity in HTR-8/SVneo trophoblast cells preexposed to 0.5% O2 and low glucose concentration for 24 h. These findings indicate that the regulation of HO expression in the human placenta is a complex process that depends, at least in part, on local glucose and oxygen concentrations.

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REGULATION OF BLOOD FLOW in the placenta is atypical because its blood vessels are not innervated and therefore vascular tone in this organ must be controlled by local soluble factors. CO has been shown to decrease perfusion pressure in an in vitro-perfused placental cotyledon model (3) and has been implicated in placental vascular control.

Heme oxygenase (HO) catalyzes the breakdown of heme in the presence of O2 and NADPH to form 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 (34), and in addition to inducing blood vessel relaxation, CO has been proposed to play a role in cell-cell communication (21, 37, 38). Earlier studies on the stoichiometry of the HO reaction demonstrated that HO consumed three molecules of O2 and two molecules of NADPH for every molecule of heme biotransformed (25, 36). Moreover, we recently demonstrated that placental chorionic villi HO activity is dependent on O2 availability (1).

There are two predominant isoforms of HO; HO-1 is the inducible isoform, whereas the constitutive isoform, HO-2, is responsible for basal HO activity in cells. HO-1 is known to be a stress response enzyme because its synthesis and activity are elevated in response to exposure to heat, heavy metals, and reactive oxygen species (17). Several studies have revealed that exposure to hypoxia increases the levels of HO mRNA and protein in a variety of cells (10, 11, 15, 27–29). Recently, we determined that preexposure of human placental preparations to low levels of O2 does not result in subsequent alterations in total HO enzymatic activity for optimized assay conditions (20% O2; 0.5 mM NADPH; 25 μM methemalbumin). At the same time, Western blot analysis revealed a significant decrease in HO-2 protein content at 5 and 1% O2 vs. 20% O2. HO-1 protein levels were almost undetectable and were not increased after a 24-h exposure to 5 and 1% O2 vs. 20% O2. In that study, the placenta was chosen as a model to study the role of O2 in the regulation of HO expression because this organ is normally exposed to varying O2 concentrations throughout gestation.
Preeclampsia is a pathological condition characterized by compromised uteroplacental blood flow, thereby resulting in a deficit in O2 and glucose delivery to the intervillous spaces of the placenta. Chang et al. (6) demonstrated a 25-fold increase in HO-1 mRNA when HepG2 cells were incubated in glucose-free medium. However, it remains to be determined whether there is a corresponding increase in microsomal HO-1 protein content or HO enzymatic activity after a low-glucose exposure. The first objective of our study was to examine the effects of a glucose-deficient environment on the content of HO-1 protein and HO enzymatic activity in human placental preparations.

Chang et al. (6) demonstrated that glucose availability regulates transcription of the HO-1 gene and postulated that the induction of HO-1 may provide a protective effect in circumstances where cells are deprived of glucose. A link between the regulation of HO expression by hypoxia and glucose deprivation may exist and may be explained, at least in part, through the actions of hypoxia inducible factor-1 (HIF-1), a transcription factor upregulated in response to hypoxia. HIF-1 has been shown to upregulate certain glycolytic enzymes (9, 32), as well as the glucose transporter, GLUT1 (8), thus allowing the cell to compensate for the lack of energy derived from respiration by increasing its capacity to produce energy from glycolysis. In glucose-depleted media, such compensation may not be possible. The second objective was to examine the combined effect of glucose deprivation and hypoxia on HO protein content and in vitro HO enzymatic activity.

MATERIALS AND METHODS

Reagents and solutions. Hemin, ethanolamine, bovine serum albumin (BSA), and NADPH were obtained from Sigma Chemical (St. Louis, MO). All other chemicals were at least reagent grade and were obtained from BDH (Toronto, ON, Canada). The stock solution of methemalbumin (1.5 mM hemin and 0.15 mM BSA) was prepared as previously described (39). 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 adjusted to pH 7.4 with KOH. Further dilutions 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 an ultrasonic probe (Sonic Dismembrator, Fisher Scientific, Toronto, ON, 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 homogenization system. The microsomal fraction was divided into equal aliquots, placed into microcentrifuge tubes, and stored at −20°C and −80°C. Hemin enzymatic activity remains stable under these storage conditions. Total protein concentrations of the microsomal fractions were determined by the Biuret method (12), which was modified as described previously (22).

Preparation of HTR-8/SVneo first trimester human trophoblast cells. HTR-8/SVneo trophoblast cells were obtained from explant cultures of human first-trimester placenta (8–10 wk of gestation) and immortalized by transfection with a cDNA construct that encodes the SV40 large T antigen (13). These cells, although nontumorigenic and metastatic, are highly invasive in vitro and exhibit phenotypic properties of extravillous placental cytotrophoblasts (EVT) in situ. Such EVT are found either lining maternal arterioles, and thus in direct contact with maternal blood, or within the interstitium of the uterine endometrium. Therefore, HTR-8/SVneo cells are a useful model to examine placental trophoblast gene expression in response to changes in the local microenvironment. For incubations under glucose-deprived conditions, cells were cultured in RPMI 1640 medium (glucose-free original stock) and 5% FBS. After serum supplementation, glucose levels in the medium were 0.2 mM as determined with a Radiometer Copenhagen EML 105 (London Scientific, London, ON, Canada). For incubation under standard glucose concentrations (10.8 mM), cells were cultured in regular RPMI 1640 medium supplemented with 5% FBS. Cells were incubated in 0.5 or 20% O2 for 24 h. After the incubation, the culture medium was decanted, and the cells were rinsed with PBS. On 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. Aliquots of the homogenate were placed in 1.5-ml microcentrifuge tubes and stored at −80°C until required for analysis. Total protein concentrations were determined using the Biuret method (12).

Measurement of HO enzymatic activity. HO activity in the microsomal fraction of cultured chorionic villi explant homogenate or sonicate of HTR-8/SVneo cells was determined by measuring the rate of CO formation during the NADPH-dependent oxidation of heme, as originally described by Vreeman and Stevenson (40) and modified by Cook et al. (7). To each of four 3.5-ml amber glass vials (Chromatographic Specialties, Brockville, 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. The samples then
were preincubated for 5 min in the dark at 37°C in a shaking water bath. NADPH (0.5 mM) was added to three of the four vials, the headspace gas was displaced 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. CO production was corrected for the CO produced in the reaction vial that contained no NADPH (blank).

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 Odrcich et al. (26). 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 fraction of chorionic villi homogenate and the homogenate of HTR-8/SVneo cells was expressed as nanomoles CO formed per milligram protein per hour.

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 0.01% Tween-20 (PBS-T) and 10% skim milk powder. The blots were then incubated with a 1:2,000 dilution of the polyclonal anti-HO-1 (SPA-896, StressGen, Victoria, BC, Canada) or anti-HO-2 (SPA-897, StressGen) antibodies. To determine and confirm specificity, the anti-HO antibodies were preincubated for 1 h at room temperature with the specific peptides (1:2,000 dilution) used by the manufacturer (StressGen) as immunogens for the generation of the antisera. This preincubation completely abolished HO immunostaining of placental tissues and eliminated the bands in the Western immunoblots corresponding in molecular weight to HO-1 and HO-2, respectively (18). Blots were subsequently incubated with a peroxidase-labeled goat anti-rabbit IgG secondary antibody (Vector Laboratories, Burlingame, CA). Peroxidase activity was detected using an established method (24). A band corresponding in molecular weight to HO-1 and HO-2 was observed in the Western immunoblots of chorionic villi homogenates and in sonicate of HTR-8/SVneo trophoblast cells preexposed for 24 h to 0.5 or 20% O2 and 10.8 or 0.2 mM glucose was expressed as percent of control (10.8 mM glucose and 20% O2) ± SD. For a statistically significant F statistic of a given set of preexposure conditions (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). Correlational analysis of HO enzymatic activity with each of HO-1 and HO-2 protein content of the individual samples was conducted using an established method (24). Pearson correlation coefficient (r) was determined, and statistical significance was established at P < 0.05.

RESULTS

Effect of glucose deprivation and hypoxia on in vitro HO enzymatic activity. Preexposure of chorionic villi explants from term pregnancies in 20% O2 and glucose-depleted medium for 24 h resulted in a 33.8 ± 1.3% increase in HO enzymatic activity (P < 0.05; Fig. 1). Likewise, preexposure of HTR-8/SVneo first-trimester extravillous trophoblast cells to a glucose-depleted environment and 20% O2 for 24 h increased HO enzymatic activity by 56.2 ± 20.9% relative to control (P < 0.05; Fig. 1). Furthermore, incubation in 0.5% O2 with medium containing 0.2 mM glucose significantly increased the magnitude of in vitro HO enzymatic activity in both biological systems relative to controls incubated in 20% O2 and 10.8 mM glucose (P < 0.05). The magnitude of increased HO enzymatic activity observed in trophoblast cells and chorionic villi incubated in 0.5% O2 combined with 0.2 mM glucose was less than the increase in the in vitro HO enzymatic activity observed in preparations of cells and tissues preexposed to glucose-depleted medium at 20% O2 (P < 0.05). Preexposure of HTR-8/SVneo trophoblast cells or chorionic villi explants for 24 h in 0.5% O2 atmosphere in medium containing 10.8 mM glucose did not change in vitro HO enzymatic activity relative to control.

Data analysis. The HO enzymatic activity in the microsomal fractions of chorionic villi homogenates and in sonicate of HTR-8/SVneo trophoblast cells preexposed for 24 h to 0.5 or 20% O2 and 10.8 or 0.2 mM glucose was expressed as nanomoles CO formed per milligram protein per hour. The data are presented as group means ± SD. Parametric statistical analysis of the HO activity data for the different O2 and glucose 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). The relative optical density for HO-1 and HO-2 protein content in the microsomal fractions of chorionic villi homogenates and in sonicate of HTR-8/SVneo trophoblast cells preexposed for 24 h to 0.5 or 20% O2 and 10.8 or 0.2 mM glucose was expressed as percent of control (10.8 mM glucose and 20% O2) ± SD. For a statistically significant F statistic of a given set of preexposure conditions (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). Correlational analysis of HO enzymatic activity with each of HO-1 and HO-2 protein content of the individual samples was conducted using an established method (24). Pearson correlation coefficient (r) was determined, and statistical significance was established at P < 0.05.

Fig. 1. Heme oxygenase (HO) enzymatic activity in microsomal preparations of chorionic villi explants preexposed to 0.5 or 20% oxygen in the presence of medium containing 10.8 or 0.2 mM glucose for 24 h. Group means with different letters (a, b, and c) are significantly different (P < 0.05). Bars represent mean HO activity ± SD of 4 determinations.
Effect of glucose deprivation and hypoxia on HO-1 and HO-2 protein expression. Western immunoblot analysis revealed that chorionic villi explants and HTR-8/SVneo extravillous trophoblast cells preexposed to 0.2 or 10.8 mM glucose in the presence of 0.5 or 20% O2 expressed detectable amounts of HO-1 and HO-2 protein. Data were normalized to control cells and tissues preexposed to 20% O2 and 10.8 mM glucose for 24 h (Figs. 3 and 4). Densitometric analysis demonstrated a 59.6 ± 8.4% decrease in HO-1 protein content in chorionic villi explants preexposed to 0.5% O2 atmosphere and 0.2 mM glucose in the medium relative to control samples preexposed to 10.8 mM glucose and 20% O2 for 24 h ($P < 0.05$; Fig. 3A). A 28.2 ± 9.4% increase in HO-2 protein content was observed in chorionic villi explants preexposed to 0.2 mM glucose and 20% O2 compared with control ($P < 0.05$; Fig. 3B). Correlational analysis revealed a positive relationship between HO-2 protein content and HO enzymatic activity ($r = 0.825$; Fig. 5). There was a 162.2 ± 78.3% increase in HO-1 protein content relative to control for HTR-8/SVneo trophoblast cells incubated in 0.2 mM glucose and 20% O2 for 24 h ($P < 0.05$; Fig. 4A). In contrast, HO-2 protein content was decreased (83.6 ± 15.3%) in HTR-8/SVneo trophoblast cells incubated in 0.2 mM glucose and 0.5% O2 compared with control cells incubated in 10.8 mM glucose and 20% O2 ($P < 0.05$; Fig. 4B).
DISCUSSION

The present study demonstrates that 24-h preexposure to low glucose concentration, either alone or in combination with low O2 concentration, enhances HO enzymatic activity in microsomal preparations of chorionic villi explants and sonicates of HTR-8/SVneo trophoblast cells. Moreover, incubation in a combination of low O2 and low glucose concentrations decreased the protein content of the HO-1 isoform in both placental biological systems, whereas preexposure to low glucose alone in chorionic villi explants increased HO-2 content. In chorionic villi explants, microsomal HO enzymatic activity correlated positively with HO-2 protein content. However, in HTR-8/SVneo trophoblast cells, there was no correlation between HO-2 protein content and in vitro HO enzymatic activity when the cells were preexposed to 0.5 or 20% O2 and low glucose concentration for 24 h.

Our present observations of increased in vitro HO enzymatic activity in both chorionic villi explants and HTR-8/SVneo trophoblast cells after a 24-h exposure to a glucose-deficient environment indicate that glucose availability is relevant to the potential HO enzymatic activity and CO production in the placenta. Because CO has been shown to modulate placental vascular tone through vasodilatory action (19), we propose that an increase in blood flow to the placenta with glucose deprivation augments the delivery of metabolic substrates to alleviate the glucose-deficient state. Therefore, an increase in HO expression after exposure to glucose deprivation may provide a compensatory mechanism for the placenta to reestablish glucose homeostasis.

In addition, several studies have suggested a physiological role for HO in protecting tissues from oxidative stress (4, 5, 35). Using an in vitro model of human placental reperfusion stress, Hung et al. (16) recently demonstrated that hypoxia followed by reoxygenation induces reactive oxygen species-mediated apoptosis in
chorionic villi. Although low O₂ levels do not appear to stimulate HO enzymatic function (2), it is possible that the low glucose levels resulting from the decreased blood flow may be a stimulus for enhanced HO function and the production of the antioxidants biliverdin and bilirubin. This concept is supported by the fact that the placenta is capable of considerable metabolic adaptations to maintain homeostasis under stable low-O₂ conditions (30). These adaptations include the high capacity of the placenta for anaerobic glycolysis, which may explain the increased expression and function of HO, a protective enzyme, under metabolic constraints.

Our previous observations indicated that preexposure to low O₂ concentration did not exert long-lasting effects on HO enzymatic function (2). The present study examined the hypothesis that preexposure to low levels of O₂ combined with low glucose concentrations affects HO protein content and overall HO activity. It was found that incubation in low levels of O₂ attenuated the glucose-mediated increase in HO catalytic potential in both the chorionic villi explants and HTR-8/SVneo cells. This is likely because ATP-dependent protein synthesis may not occur under the influence of both stresses combined because in the absence of glucose, anaerobic glycolysis is impaired. In support of this is the fact that total protein content was lower in chorionic villi explants and HTR-8/SVneo trophoblast cells exposed to low O₂ and glucose concentrations for 24 h (data not shown).

Previous studies have demonstrated decreased HO-2 protein content and absence of HO-1 protein in placentas from preeclamptic pregnancies (19) with HO enzymatic activity corresponding to HO-2 protein content (18, 24). In this study, HO-2 protein content correlated well with HO enzymatic activity in chorionic villi explants but not in HTR-8/SVneo trophoblast cells. This difference in HO-2 expression between intact chorionic villi and the trophoblast cells in response to O₂ and glucose deprivation may be due to several possibilities. While the HTR-8/SVneo cells do maintain many of the phenotypic properties of the parent cell line, the former were generated after SV40 large T antigen immortalization. Thus it is possible that the lack of increase in HO expression after O₂ and glucose deprivation is a function of their immortal phenotype. Also, intact chorionic villi contain various cell types, such as fibroblasts, endothelial cells, and blood cells, which may not respond to O₂ and glucose deprivation as trophoblast cells. It is interesting that compared with 24-h preexposure to low glucose concentration alone, 24-h preexposure of chorionic villi explants and HTR-8/SVneo trophoblast cells to both hypoxia and low glucose combined resulted in significantly lower HO-1 protein content. Shibahara et al. (33) reported that hypoxia suppresses HO-1 protein expression in various human cell lines. These investigators suggested that this is a mechanism for energy conservation based on the fact that HO-mediated heme catalysis requires two molecules of NADPH for every molecule of heme oxidized. Thus a decrease in HO expression under conditions of low O₂ and low glucose concentrations may conserve energy when the capacity for energy production is compromised.

Although there was a direct relationship between HO-2 protein content and HO enzymatic activity, an explanation for the changes in HO-1 expression is not obvious. The discrepancy between total HO enzymatic activity and HO-1/HO-2 protein expression may be explained, at least in part, by the relative sensitivities of the HO enzymatic assay and the Western immunoblot method. The HO enzymatic assay is among the most sensitive available and has a lower limit of detection of 10 pmol of CO (40). The sensitivity of the Western immunoblot method for detecting HO protein content is limited by the affinity of the anti-HO-1 and anti-HO-2 antibodies, of which there has been some criticism (20).

While HO-2 protein content and in vitro HO enzymatic activity correlated in chorionic villi explants, this was not the case for HTR-8/SVneo trophoblast cells. There appeared to be proteolytic degradation of HO-2 under low glucose and hypoxic conditions, resulting in a protein fragment of ~34 kDa in the Western immunoblot (Fig. 4B). It is possible that this 34-kDa fragment of HO-2 retains enzymatic function, which could explain, at least in part, the increased enzymatic activity in sonicated cells preexposed to hypoxia and low glucose combined. A similar cleavage product has been identified in previous studies (14). While it remains to be determined whether the 34-kDa fragment of HO-2 has enzymatic function, there is evidence that proteolysis of HO-1 results in a functional product (31). A recombinant form of this truncated form of human HO-1 has been expressed (31). This 28-kDa fragment of HO-1 lacks a stretch of 23 hydrophobic amino acid residues that normally anchors it to the endoplasmic reticulum. Also, further proteolytic degradation of this truncated HO-1 produced two major species consisting of residues 1–226 and 1–237.

In the HTR-8/SVneo trophoblast cells, preexposure to low glucose alone resulted in a significant increase in HO-1 protein content. This was not the case for the chorionic villi explants. Again, such differential responses may be due to differences in cellular composition between the two models used. HO activity is limited by the amount of total HO protein levels present as well as by substrate and cofactor availability. The optimized in vitro HO enzymatic activity assay used in this study provides an accurate assessment of the amount of functional HO protein as the assay is conducted in the presence of excess heme and NADPH.

In summary, glucose deprivation appears to stimulate HO expression and is accompanied by increased HO enzymatic activity in chorionic villi explants. Our findings also suggest that O₂ levels may further modulate HO expression and activity in HTR-8/SVneo trophoblast cells and chorionic villi explants.
DISCLOSURES

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REFERENCES


