Hypoxia-Reoxygenation
A Potent Inducer of Apoptotic Changes in the Human Placenta and Possible Etiological Factor in Preeclampsia

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Abstract—Preeclampsia is a severe disorder of human pregnancy characterized by generalized activation of maternal endothelial cells. Oxidative stress of the placenta is considered a key intermediary step, precipitating deportation of apoptotic fragments into the maternal circulation, but the cause remains unknown. We hypothesize that intermittent placental perfusion, secondary to deficient trophoblast invasion of the endometrial arteries, leads to an ischemia-reperfusion–type insult. We therefore tested whether hypoxia-reoxygenation (H/R) in vitro stimulates apoptosis in human placental tissues compared with controls kept hypoxic or normoxic throughout. After H/R, release of cytochrome c from mitochondria was significantly increased and was associated with intense immunolabeling for active caspase 3 in the syncytiotrophoblast and fetal endothelial cells. There was also increased labeling of syncytiotrophoblastic nuclei for cleaved poly (ADP-ribose) polymerase (PARP), and higher cytosolic concentrations of cleaved PARP fragment were detected by Western blot. Syncytiotrophoblastic nuclei displayed increased chromatin condensation, and a significantly greater percentage was TUNEL positive. These changes were accompanied by increased lactate dehydrogenase release into the medium. Preadministration of the free radical scavenger, desferrioxamine, reduced cytochrome c release and the TUNEL-positive index, suggesting generation of hydroxyl radicals mediates these processes. By contrast, hypoxia alone caused a smaller increase in the TUNEL-positive index, and the majority of syncytiotrophoblastic nuclei displayed karyolysis, whereas normoxic controls remained euchromatic. We conclude that H/R stimulates apoptotic changes within the syncytiotrophoblast, whereas hypoxia principally induces necrosis. The quality of placental perfusion may therefore be a more important factor in the pathophysiology of preeclampsia than the absolute quantity. (Circ Res. 2002; 90:1274-1281.)

Key Words: preeclampsia ■ oxidative stress ■ apoptosis ■ ischemia-reperfusion injury ■ placenta

Preeclampsia is a disorder unique to human pregnancy characterized by generalized systemic maternal inflammatory response, associated with diffuse endothelial cell dysfunction. It is one of the leading causes of maternal and perinatal morbidity and mortality, affecting 5% to 7% of all pregnancies, yet the etiology and pathogenesis have not been fully defined. The presence of a placenta is both necessary and sufficient to cause the disorder, and recent theories have suggested that deportation of placental debris through the maternal circulation is a key intermediary event in the generation of the syndrome.1–3 One potential source of the debris is turnover of the placental epithelial layer, the syncytiotrophoblast, through apoptosis. The rate of apoptosis is thought to be increased in preeclampsia as a result of placental oxidative stress, which is frequently, although not universally, increased in the condition.

The trigger for the placental oxidative stress has yet to be identified. Defective remodeling of the endometrial spiral arteries is the most widely recognized predisposing factor for preeclampsia.1 As a result, perfusion of the intervillous space is impaired compared with normal pregnancies, leading to the general assumption that the placental changes are induced by hypoxia. However, no direct measurements are available to confirm that this is indeed the case. By contrast, pregnancy at high altitude is one of the few situations in which the oxygen tension in the maternal arterial supply to the placenta is known to be reduced.4 Comparison of the placental histological changes seen in these two situations indicates that many of the features that characterize the preeclamptic placenta, for example, the increased incidence of infarction, cannot be explained on the basis of hypoxia alone.5

Rather, we have proposed that defective remodeling results in the retention of vasoreactivity in the myometrial segments of spiral arteries and that this leads to intermittent perfusion of the intervillous space and hence to fluctuating oxygen concentrations within the placenta. Such fluctuations in oxygen tension could provide the basis for an ischemia-reperfusion–type insult. We have recently demonstrated that oxidative stress occurs when hypoxic placental tissues are...
rexygenated in vitro, which is consistent with an ischemia-reperfusion injury. 
Furthermore, the patterns of immunostaining for 4-hydroxy-2-nonenal adducts and nitrotyrosine residues suggest there are parallels between the resultant oxidative stress and that observed in preeclampsia. Collectively, these findings indicate that hypoxia-reoxygenation (H/R) may represent a suitable model system for investigating the generation of placental oxidative stress in preeclampsia.

The human placenta is of the hemochorial type and consists of an extensively branched fetal villous tree that is bathed by the maternal blood circulating in the intervillous space. The contact surface between the fetal placental tissues and the maternal blood is the syncytiotrophoblast, a multinucleated syncytium that extends without lateral cell boundaries over the entire villous tree. This tissue is responsible for placental transport and hormone synthesis but also acts as the endothelium for the intervillous space. Although it is still unclear how apoptosis can occur regionally within a syncytium, an increased prevalence of syncyial nuclei demonstrating apoptotic appearances has been reported in placentas from pregnancies complicated by preeclampsia compared with normal pregnancies. This may be a primary pathological event or, alternatively, a secondary effect of altered placental oxygenation in preeclampsia. Redman and Sargent proposed that oxidative stress may stimulate syncytotrophoblast apoptosis and so increase shedding of microvillous fragments. The resultant debris is thought to promote an enhanced systemic inflammatory response including endothelial cell dysfunction in the mother. Indeed, oxidative stress can cause apoptosis in numerous cell lines in vitro, and ischemia-reperfusion can also induce apoptosis in the heart and brain in vivo.

The aim of this study was to determine whether H/R exacerbates apoptotic changes in term placental tissues. These changes were assessed by a range of techniques including the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and immunohistochemistry for active caspase 3 and cleavage of poly (ADP-ribose) polymerase (PARP). We also investigated the role of cytochrome c release from mitochondria, because mitochondria are one of the major sources of superoxide anion production and targets of reactive oxygen species (ROS). Furthermore, we tested the effect of desferrioxamine, an effective scavenger of ROS, on the induction of apoptosis in placental tissues during H/R.

**Materials and Methods**

Reagents were purchased from Sigma (Sigma Chemical Co) unless indicated.

**Tissue Collection**

Human term placentas (n=25) were obtained from normal pregnancies with informed consent immediately after elective caesarean deliveries for repeat sections before onset of labor. Samples were taken midway between the chorionic and basal plates, from lobules free of visible calcification or tears. After rinses in cold phosphate-buffered saline (PBS), samples were placed into culture medium (Medium 199 with 25 mmol/L HEPES, Earle’s salts, and 1-glutamine supplemented with 5% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B, all from Life Technologies Ltd) equilibrated with 5% O2/90% N2/5% CO2 in sealed glass bottles on ice.

**Tissue Culture and Conditions**

After dissection, 6 pieces of villous tissue (~5 to 10 mg) were cultured in individual Costar Netwell (24-mm diameter, 500-μm mesh; Corning) supports in 1.5 mL of culture medium. Under normal physiological conditions, the oxygen tension within intervillous space at term is approximately 45 to 50 mm Hg. Therefore, a gas mixture of 5% O2/90% N2/5% CO2 was used as the normoxic condition, and the dissolved oxygen tension in the medium was 45 to 62 mm Hg. Conditions for hypoxia and H/R were established as previously described. For H/R experiments, villous tissues were cultured under hypoxic conditions (Po2 in medium=12 to 16 mm Hg) at 37°C for 3 hours, then transferred to medium equilibrated with air/5% CO2 in a separate humidified chamber continuously flushed with air/5% CO2 for an additional 4 hours (Po2 in medium=143 to 160 mm Hg). In control experiments, villous tissues were kept under either hypoxic or normoxic conditions throughout the 7-hour period with a change of medium at 3 hours.

**Immunofluorescence for Active Caspase 3 and TUNEL Assay**

Dual-color immunofluorescence against the active form of human caspase 3 and for the TUNEL assay was performed sequentially on the same sections from 6 placentas. Formaldehyde-fixed, paraffin-embedded sections were pretreated with 20 μg/mL proteinase-K in 10 mmol/L Tris-HCl for 15 minutes, blocked with 10% normal goat serum, and reacted with a rabbit polyclonal antibody directed against the catalytic subunits without cross-reactivity with the precursor form of human caspase 3 (1 μg/mL, R&D Systems) at 30 minutes, washed again, and incubated with Texas Red streptavidin (10 μg/mL, Vector Laboratories) for 90 minutes. TUNEL assay was performed using a commercial kit that labels DNA strand breaks with FITC (In situ Cell Death Detection Kit, Roche Molecular Biochemicals). Sections were mounted with VECTASHIELD–DAPI (Vector Laboratories) and observed on a Leica TCS-NT-UV confocal microscope. Negative controls were obtained by substitution of the primary antibody with non-immune rabbit IgG or omission of the enzyme TdT.

**Apoptotic Index**

With use of confocal microscopy, the staining for TUNEL (green), anti-human active caspase 3 (red), and DAPI (blue) was examined sequentially at a magnification of ×400. Digital images from 10 randomly selected fields providing a minimum of 1500 nuclei for each section were saved. The percentage of nuclei that were stained by the TUNEL method divided by the total number of DAPI-stained nuclei was assessed, excluding variable of culture condition.

**Transmission Electron Microscopy**

Tissues from a total of 4 placentas were processed for transmission electron microscopy as previously described.

**Immunohistochemical Staining for the p85 Fragment of PARP**

Sections from 5 placentas were heated in 10 mmol/L sodium citrate buffer, using a pressure cooker, for 1 minute before immunohistochemistry. After quenching the endogenous peroxidase activity and blocking the nonspecific binding, slides were reacted with a rabbit polyclonal anti-PARP p85 fragment antibody (1:200, Promega) at 4°C overnight. Further processing for colorimetric detection was made according to the instructions for the Vectastain Elite ABC kit (Vector Laboratories) using diaminobenzidine as the peroxidase substrate.
Semiquantification of PARP p85 Using Western Blot

Western blots were performed as previously described. The membranes were probed with the anti-PARP p85 fragment antibody (1:100) or anti–H9252-actin monoclonal antibody (1:2000, Sigma). The relative intensity of protein signals was normalized to the corresponding H9252-actin density and quantified by densitometric analysis. A total of 4 placentas were studied.

Villous Tissue Integrity Assessment

Release of lactate dehydrogenase (LDH) into the medium was measured in 7 placentas using a commercial kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega).

Mitochondrial Cytochrome c Release

Fresh villous tissues from 4 placentas were used to prepare the mitochondria-enriched fraction from cytosolic fraction with the Mitochondria/Cytosol Fraction Kit (Cambridge Bioscience). Thirty micrograms of either mitochondrial or cytosolic proteins was separated by 15% SDS-PAGE. The membranes were probed with the anti-cytochrome c (4 μg/mL, PharMingen), anti-cytochrome oxidase subunit IV (5 μg/mL, Molecular Probe), or anti–H9252-actin (1:2000, Sigma) monoclonal antibodies.

Effects of Desferrioxamine

Villous tissues were preloaded with desferrioxamine (50 mmol/L, in PBS) before being subjected to H/R. Tissues treated with PBS vehicle were used as controls.

Statistical Analysis

Data are presented as mean±SE or median and interquartile ranges if variances were not homogenous. Statistical analysis was performed with one-way ANOVA or Kruskal-Wallis test and post hoc tests were performed if significant effects were determined. Differences between two groups were evaluated with the Student’s t test or Mann-Whitney U test, as appropriate. Statistical significance was set at a value of P<0.05.

Results

H/R Increased Villous Apoptotic Changes

The percentage of nuclei positively stained for DNA strand breaks by TUNEL was significantly higher within the syncytiotrophoblast and stromal cells of villous tissues subjected to H/R than in the controls maintained under either normoxia or hypoxia throughout (Figures 1 and 2). Hypoxia alone stimulated a small increase in the number of TUNEL-positive nuclei within the trophoblast compared with those fixed immediately after delivery but had no effect on the stromal cells. By contrast, maintenance under normoxic conditions for 7 hours had no significant effect on the apoptotic index in either location.

In parallel with the results of the TUNEL assay, there was a slight increase in immunoreactivity for active caspase 3 in villous tissues kept under hypoxia or normoxia throughout (Figures 1b and 1c). The immunofluorescence was restricted to the trophoblast layer, with only more TUNEL-positive nuclei (arrowheads) present in the syncytiotrophoblast of tissues subjected to H/R than kept under hypoxia or normoxia throughout. Sections were stained with DAPI to show all nuclei. Scale bar=50 μm (a through c and f) and 20 μm (d and e).
occasional stromal cells staining positively. After H/R, the immunofluorescence became both more intense and more generalized around the villous surface (Figures 1d and 1e). A greater number of cells within the stromal core also reacted positively and in particular the endothelial cells were conspicuously labeled (Figure 1f). Immunoreactivity for active caspase 3 and TUNEL positivity was colocalized in the syncytiotrophoblast but not contemporaneously, the labeling for active caspase 3 always preceding the presence of TUNEL-positive nuclei.

By using a polyclonal antibody directed against the 89-kDa caspase-cleaved fragment of human PARP, a small proportion of the nuclei, particularly in the syncytial knots, was stained in villous tissues sampled immediately after delivery (Figure 3a). The immunoreactivity was slightly increased in villous tissues kept under normoxia (Figure 3d) but became more apparent in villous tissues subjected to hypoxia (Figures 3b and 3c) or H/R (Figures 3e and 3f). Compared with hypoxia alone, H/R caused a more intense and diffuse staining, particularly in the syncytiotrophoblast but also in the endothelial cells (Figure 3f). Omission of primary antibodies (anti-active caspase 3 or anti-PARP p85) or substitution of the primary antibodies with non-immune rabbit IgG resulted in the absence of immunostaining (data not shown).

**Semiquantification of Cleaved PARP in Villous Tissues After Hypoxia and H/R**

Because both hypoxia and H/R could significantly induce apoptotic changes, as assessed by either TUNEL or immunohistochemical methods, we compared cytosolic concentrations of the cleaved PARP fragment to provide an estimate of the severity of the apoptosis in these two experimental conditions. Using Western blot as a semiquantitative approach, we found that the cytosolic levels of immunodetectable PARP p85 were significantly higher in homogenates from villous tissues subjected to H/R than hypoxia alone (Figure 3i; \(P<0.05\)).

**Morphological Evidence of Apoptosis**

Further morphological evidence of the above apoptotic changes was obtained from the resin sections, as shown in Figure 4. In villous tissues fixed immediately after delivery, the microvilli on the syncytiot surface were profuse, long, and slender. The syncytioplasm appeared uniformly dense, with nuclei dispersed at intervals throughout (Figure 4a). The syncytial nuclei were often irregular in shape and displayed a small amount of peripheral heterochromatin. Similar appearances were seen in tissues kept under normoxic conditions throughout the culture period (Figure 4c). By contrast, in those tissues kept under hypoxia, the syncytiotrophoblast appeared heavily vacuolated, and the majority of the nuclei were swollen, ovoid, and pale-staining (Figure 4b). After H/R, the syncytiotrophoblast also showed vacuolation, but in addition there was distortion and loss of microvilli over extensive areas. Many nuclei demonstrated apoptotic changes, including peripheral chromatin condensation and clumping, fragmentation, and the formation of apoptotic bodies (Figures 4d through 4f).

**Figure 3.** Immunohistochemistry and Western blot analysis of PARP p85 immediately after delivery (a), hypoxia (b and c), normoxia (d), and H/R (e and f). Immunostaining of PARP p85 was found in a small proportion of nuclei, particularly in the syncytial knots, in villous tissues sampled immediately after delivery (a). The immunoreactivity was slightly increased in villous tissues kept under normoxia (d) but became more apparent in villous tissues subjected to hypoxia or H/R (b, c, e, and f). Compared with hypoxia alone, H/R caused a more intense and diffuse staining, especially in the syncytiotrophoblast and cytotrophoblast cells (f). Scale bars=50 μm. g. Bands shown are representative of immunoblots performed on 4 sets of experiments. H indicates hypoxia; N, normoxia; and H/R, hypoxia-reoxygenation. Villous tissues cocultured with 5 μmol/L of gliotoxin served as a positive control. h, Bands shown are from paired villous tissues from 4 placentas. β-Actin was used to normalize for loading variability. i, Densitometric analysis of the bands shown in panel h. Data are presented as mean±SE. \(P<0.05\), Student’s t test.

These changes were confirmed by transmission electron microscopy. After hypoxia alone, the syncytiot nuclei were swollen, and in the majority there was only a thin peripheral rim of condensed heterochromatin with generalized loss of the euchromatin (Figures 5a and 5c). Occasional nuclei showed a more condensed chromatin pattern. After H/R, the nuclear profiles were of considerably smaller diameter, with dense aggregations of heterochromatin around their peripheries and scattered throughout the nuclei (Figures 5b and 5d). The euchromatin between the aggregations was largely preserved. By contrast, in tissues maintained under normoxia throughout the culture period, the syncytiot nuclei remained...
oval in profile and displayed normal patterns of heterochromatin and euchromatin (Figure 5e).

In both the hypoxia and H/R treatment groups, the syncytial mitochondrial profiles were small and rounded and displayed swelling of the intracristal space and distortion of the cristal architecture (Figure 5f). It was notable that the underlying cytotrophoblast cells and capillaries appeared normal. Their fine structure was well preserved, confirming that the syncytial changes were not the result of fixation artifacts (Figures 5a and 5b).

Loss of Villous Integrity After H/R
Morphologically, breakdown of the apical membrane of the syncytiotrophoblast appeared greatest after H/R (Figure 5d). Release of LDH from tissues subjected to H/R was significantly increased after 4 hours of reoxygenation compared with that from controls kept under normoxia throughout (12.9±2.7% versus 6.1±5.3%, P<0.05) (Figure 6). By contrast, there was no difference in the release of LDH from villous tissues maintained under either hypoxia (6.9±2.8%) or normoxia for 7 hours.

Redistribution of Cytochrome c by H/R
By using Western blot analysis, release of cytochrome c from mitochondria into cytosol was detected, as illustrated in Figure 7. Densitometric analysis showed there was a 3.5-fold increase (P<0.05) in the cytosolic concentration and 2-fold decrease (P<0.001) in the mitochondrial concentration of cytochrome c in villous tissues subjected to H/R compared with villous tissues kept under normoxia for 3.5 hours.
Desferrioxamine Attenuated H/R-Induced Mitochondrial Cytochrome c Release and Apoptosis

Preloading villous tissues with 50 mmol/L of desferrioxamine before being subjected to H/R significantly blocked the release of cytochrome c from mitochondria into cytosol compared with vehicle controls at 3.5 hours of culture (Figures 8a through 8d; \( P < 0.05 \)). Desferrioxamine also significantly reduced the percentage of TUNEL-positive nuclei after H/R (Figure 8e; \( P < 0.05 \)).

Discussion

The syncytiotrophoblast of the placenta is a unique tissue in terms of its epithelial location, its physical extent, and the number of nuclei that it contains. The degree to which classical descriptions of apoptosis apply to such a tissue is therefore uncertain. Nuclei displaying severely condensed chromatin are a normal feature of the syncytiotrophoblast, but their prevalence is increased in preeclampsia.\(^{10,13}\) Therefore, in the present study, we tested whether various key steps in the apoptotic cascade can be activated by H/R. The treatment proved to be a potent inducer of the release of cytochrome c from mitochondria, activation of caspase 3, and cleavage of PARP. These events were associated with an increased number of nuclei displaying condensed chromatin and staining positively by TUNEL. The causal relationship between the generation of ROS and these apoptotic changes was revealed by the fact that preadministration of desferrioxamine, a membrane-permeable iron chelator and powerful scavenger for hydroxyl radicals, attenuated this insult. Taken together with our previous report,\(^{6}\) these findings imply that an ischemia-reperfusion–type injury plays an important role in the pathogenesis of placental oxidative stress and may be critical in stimulating the increased apoptosis seen in preeclampsia. This conclusion is further supported by the observation that although the placenta has a high oxygen consumption in vivo, it is capable of considerable metabolic adaptations so that its structural and functional integrity is well maintained under stable low oxygen conditions.\(^{20}\) These adaptations rely partly on suppression of protein synthesis but also on the high capacity of the placenta for anaerobic glycolysis.

During early pregnancy, a subpopulation of trophoblast cells, the extravillous trophoblast, migrates from the developing placenta into the wall of the uterus. These cells invade both between and down the lumens of the spiral arteries,
Apoptosis and necrosis are common outcomes of ischemia-reperfusion injury. During the reperfusion phase of the injury, ROS are generated both within mitochondria and through the actions of enzymes such as xanthine oxidase, the activity of which is increased in preeclampsia. Imbalances of ROS lead to loss of Ca\(^{2+}\) homeostasis, with release of Ca\(^{2+}\) ions from the endoplasmic reticulum and other intracellular stores. Increased Ca\(^{2+}\) ions act synergistically with oxidation of the thiol groups on proteins in the inner mitochondrial membrane to promote opening of the nonselective mitochondrial permeability transition pore (PTP). Opening of the PTP is associated with release of cytochrome c and other apoptogenic molecules, such as Apaf-1, which activate the caspase pathways leading to the characteristic cleavage of nuclear and cytosolic proteins. However, opening of the PTP is also associated with loss of mitochondrial membrane potential and ATP production. If mitochondria throughout the cell are affected, ATP concentrations fall precipitously, ionic homeostasis is lost, and the cell undergoes primary necrosis. Involvement of a more limited number of organelles or transient opening of the pore may allow ATP to be maintained at levels sufficient to permit apoptosis to occur instead. The balance of ATP is therefore critical to the outcome, and in the present study although the initial stages of apoptosis were activated by H/R in the syncytiotrophoblast, postapoptotic or secondary necrosis intervened and led to loss of tissue integrity.

Breakdown of villous integrity may contribute to the degradation of syncytiotrophoblastic fragments, which is increased in preeclampsia, or the release other fetal products, such as DNA, or asymmetric dimethylarginines. Because of the location of the syncytiotrophoblast, these products will be released directly into the maternal circulation and could therefore lead to activation of the peripheral maternal endothelial cells. It is now generally considered that this activation underlies the clinical manifestations of the syndrome, in particular the cardinal triad of hypertension, proteinuria, and peripheral edema.

The defect in remodeling of the endometrial spiral arteries seen in patients with preeclampsia is, however, only a question of extent, for there is great variation both between individuals and, on a regional basis, within the same individual. Maternal arteries lying beneath the central part of the placenta undergo more extensive remodeling than those at the periphery, and so regional differences in placental perfusion during normal pregnancies might be expected. These may be exacerbated by other factors, such as compression of the endometrial arteries during uterine contractions. Repeated radioarteriographic placentograms conducted on rhesus monkeys demonstrated intermittent functional patency of the entries of spiral arteries to the intervillous space, and the same most likely occurs in humans. The effects of these fluctuations in blood supply on the local oxygen concentration will be exacerbated toward term when placental and fetal oxygen consumption is at the highest. This is consistent with the increased incidence of preeclampsia toward the end of pregnancy.

We speculate, therefore, that all placentas may suffer to some degree from a chronic low-grade ischemia-reperfusion-type injury. In our previous study, we detected varying degrees of oxidative stress within normal non-preeclamptic placentas. The magnitude of the stress will depend on the severity of the insult and on the effectiveness of the placental antioxidant defenses, which may reflect factors such as parental genotypes and maternal diet. The preeclamptic placenta may therefore represent the extreme end of a broad spectrum of placental oxidative stress, explaining the considerable overlap that exists in many markers of the maternal inflammatory response between a normal pregnancy and preeclampsia.

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