Endothelin Receptor A Antagonism Prevents Damage to Glycogen-Rich Placental Cells Following Uterine Ischemia–Reperfusion in the Rat

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Abstract

Fetal growth restriction (FGR) is a common cause of perinatal morbidity and mortality. Suboptimal uteroplacental perfusion is the most commonly identified cause of FGR, and ischemic lesions are often observed in placentas from pregnancies complicated by FGR. Ischemia followed by reperfusion is a strong stimulus to the production of the vasoconstrictor endothelin I (ET-I) which has been implicated in several models of FGR. We sought to investigate oxidative stress and placental morphology in a rat model of ischemia–reperfusion (I/R)-induced FGR and to evaluate the role of ET-I in the observed pathology. Unilateral uterine I/R (30 min) was conducted, with and without simultaneous ET-I receptor A (ET_A) antagonism, on pregnant rats at gestation day 17. Placental tissues collected 24 hours later were evaluated immunohistochemically for oxidative damage. Tissue pathology was studied using quantitative morphometry. Glycogen-rich cellular areas in the placental junctional zone exhibited only 50% intact cells ($P < .001$) in both uterine horns following unilateral I/R, compared to controls. ET_A antagonism prevented damage to the glycogen-rich cellular areas. Oxidative damage in response to I/R was prominent in the labyrinthine layer in both uterine horns and was not affected by ET_A antagonism. We conclude that glycogen-rich cellular areas of the placental junctional zone are particularly vulnerable to damage from uterine I/R in the rat. Nucleic acid oxidative damage in the labyrinth is a prominent effect of uterine I/R. ET_A antagonism protects placental cellular integrity during I/R challenge but does not prevent nucleic acid oxidative damage.

Keywords

endothelin receptor antagonists, ischemia, oxidative stress, placenta, rats

Introduction

Fetal growth restriction (FGR) continues to be a common cause of perinatal morbidity and mortality,^{1–3} affecting 5% to 7% of pregnancies in developed countries.⁴ Fetal growth restriction is of particular concern when it is diagnosed remote from term.⁵ Inadequate uteroplacental perfusion is the most common identifiable cause of human FGR.⁶ Histologic evaluation of placentas from pregnancies complicated by FGR commonly reveals evidence of placental ischemia and/or infarction, the severity of the FGR correlating with the number, and the severity of ischemic lesions observed.⁷ In spite of several studies investigating the relationship between ischemia–reperfusion (I/R) and FGR, the cellular dynamics of I/R in the placenta have not been elucidated.

Various animal models have been developed to evaluate the role of uterine ischemia in FGR. Ligation of the uterine artery in the rat results in FGR.⁸ Subsequently, it has been shown that temporary uterine ischemia by unilateral occlusion of uterine perfusion followed by reperfusion of the ischemic horn result in FGR in both uterine horns, not just the one subjected to

ischemic insult.⁹ This is more analogous to the pathophysiology of human FGR than the previous uterine artery ligation model, particularly as it reveals the impact of I/R on regional blood flow in the placenta. The occurrence of FGR even after relatively brief (5–60 minutes) occlusion of uterine blood flow suggests that it is not only the restriction of blood flow per se that results in a compromise of fetal growth but also the long-term consequences of I/R that are equally if not more

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important. Further, the restriction of fetal growth in the contralateral nonischemic uterine horn suggests that circulating mediators are involved in the resultant pathology. Notably, FGR produced by brief uterine I/R in pregnant rodents has not been observed to result in the symptoms of preeclampsia. However, longer term ischemia using the reduced uterine perfusion pressure model in rats does result in hypertension along with FGR.¹⁰

Endothelin 1 (ET-1), one of the most potent endogenous vasoconstrictors¹¹ and a proinflammatory hormone,^{12,13} is produced primarily by endothelial cells. Along with its receptors, it is expressed in the placenta as well as in many other tissues.¹⁴⁻¹⁷ Specific cells in the placenta are equipped to respond to ET-1. Both of the 2 types of ET-1 receptors have been identified in the rat placental labyrinth (ET_A and ET_B), in the cytotrophoblasts and trophoblastic giant cells of the junctional zone (ET_B), and in the decidua and vascular endothelium (ET_A)¹⁷ but not in the glycogen-rich cells of the junctional zone. ET-1 infusion in maternal rats results in FGR independent of maternal hypertension.¹⁸ ET-1 also has a central role in the pathophysiology of hypoxia-induced FGR in the rat, producing FGR that is completely ameliorated by ET_A antagonism.¹⁹ Ischemia followed by reperfusion has been shown to be a potent stimulus to ET-1 production in rat renal medullary interstitial cells²⁰ and in myocardial cells (along with increased plasma ET-1)²¹ as well as in rat uterus and placenta.²² Placental levels of ET-1 have also been shown to be elevated in hypoxia-induced²³ and uterine ischemia-induced²⁴ models of rat FGR. In a separate study from the one reported herein, unilateral uterine I/R produced FGR in both uterine horns, and when an ET_A antagonist was administered following I/R, FGR was prevented in both uterine horns,²² implicating circulating factors in the pathophysiology of the FGR. Ischemia-induced hypertension and increased uterine vascular resistance in pregnant rats have also been shown to be reduced by an ET_A antagonist.¹⁰ Consequently, we hypothesized that ET-1 may have a significant impact on the placental pathology of I/R-induced FGR.

Ischemia-reperfusion can cause oxidative damage to DNA, and this damage to nucleic acids can be detected by the presence of 8-hydroxyguanine (8-HG) as has been shown in I/R-injured rat kidneys.²⁵ The type and extent of placental tissue damage that occurs in response to I/R has not been described. Our objective was to evaluate whether the oxidative stress of I/R causes placental nucleic acid alteration and/or changes in placental morphology. If such changes were identified, we sought to evaluate the role of ET-1 in this process.

Materials and Methods

Animals

Nonpregnant Sprague-Dawley female and male rats were purchased from Harlan Laboratories (Madison, Wisconsin), housed in the NorthShore University HealthSystem Research Institute (NorthShore RI) Center for Comparative Medicine,

maintained in 12-hour light-dark cycles, and allowed free access to a standard laboratory rodent diet and water. Animal care and the conduct of all experiments were approved by the NorthShore RI Animal Care and Use Committee. Rats were bred at an age of 11 to 20 weeks and a weight of 225 to 250 g. Three experimental groups were studied, including Sham-operated, I/R, and I/R plus ET_A antagonist. Eighteen maternal rats were used for this study, 6 in each experimental group. Because only 2% of the delivered ET_A antagonist crosses the placenta and no abnormalities have been observed in rat fetuses following maternal treatment with the antagonist alone between gestational days 14 and 21,²⁶ and because the antagonist maintained the integrity of the target cells during ischemia and did not produce any discernible placental pathology, a separate group treated with antagonist alone was not included.

Unilateral Uterine I/R

On day 17 of gestation (term = 22 days), a laparotomy was performed under general anesthesia consisting of a single intraperitoneal (IP) injection of xylazine, ketamine, and acepromazine in combination (8, 40, and 1.3 mg/kg, respectively). Unilateral uterine I/R was carried out as described previously.²⁷ A midline abdominal incision was utilized to expose both uterine horns. Vascular clamps were then applied to both the uterine and the ovarian arteries of the right (ipsilateral, ischemic) uterine horn to totally occlude perfusion, while the vasculature of the left (contralateral, nonischemic) horn was not clamped. The uterine horns were replaced into the abdominal cavity, with the occlusion clamps in place. After 30 minutes, the vascular clamps were removed, and the abdominal incision was closed. Rats in the sham-operated group underwent the same procedure except that no vascular clamps were applied.

Endothelin Receptor A (ET_A) Antagonist Administration

ET_A antagonist ABT-546 (20 mg/kg/day, Abbott Laboratories, Abbott Park, Illinois), or its vehicle (20% ethyl alcohol, 40% propylene glycol, in 0.04 M NaOH), was administered intraperitoneally for 24 hours, beginning on day 17 and ending on day 18 of gestation, via an Alzet osmotic pump #2001D (Durect Corp., Cupertino, California) placed into the abdomen during I/R surgery. The IP route of administration has been demonstrated to completely ameliorate FGR in I/R-treated rats.²² This ET_A antagonist is a nonpeptide compound with a high affinity for ET_A receptors ($K_i = 0.46$ nmol/L) and a low affinity for ET_B receptors ($K_i = 13\,000$ nmol/L), thus it has an ET_A selectivity of approximately 28 000-fold.²⁸

Placental Tissue Analysis

Because oxidative damage occurs quickly, and long-term effects on rat pregnancy have been previously described,²² we chose in this study to examine the placentas at 24 hours following I/R, in order to observe early effects of I/R insult. On

gestation day 18, hysterotomies were performed under surgical anesthesia and, after removal of the fetuses (all conceptuses were alive and fetuses were euthanized at hysterotomy), both ipsilateral ischemic and contralateral nonischemic uterine horns with attached placentas were removed. Half of the uteroplacental units from each horn were fixed in 4% neutral-buffered formalin for 24 hours and then embedded in paraffin for sectioning and hematoxylin and eosin (H&E) staining. The remaining uteroplacental units were embedded in optimal cutting temperature compound and frozen for cryosectioning and immunohistochemical staining.

Placental morphology was examined after H&E staining. Intact and damaged cellular areas were quantified by digital morphometry in the following manner. Placentas from each uterine horn from each maternal rat were examined. Extensive I/R-induced cellular damage was observed in the junctional zone; therefore, photomicrographs of the entire junctional zone, across the full width of the placental cross-section, in each of 2 H&E-stained sections from each placenta were recorded at a magnification of $25\times$. Forty-eight placental profiles were analyzed from each experimental group of rats (using 4 placentas per rat, therefore 24 placentas per experimental group). Intact and damaged glycogen-rich cellular areas were identified (nuclei present and organized vs few or no visible nuclei; loss of cellular integrity and few identifiable cell borders in degraded cellular areas) and their absolute areas separately quantified in each photomicrograph using IPLab software version 4.08 (Scanalytics, Rockville, Maryland). Regions of intact and degraded glycogen cells were outlined manually using the IPLab Segment tool and then the area of each segment and the total area of segments representing either intact or damaged glycogen cells, respectively, were calculated via the Quantify Segments tool. This analysis was conducted with the observer blinded to treatment group. Composite areas of intact glycogen-rich cells, expressed as a percentage of the combined absolute areas of intact and damaged glycogen-rich cells from each animal (for an n of 6 for each experimental group), were compared statistically among the groups. There was no treatment-induced change in the area occupied by each of the placental zones, among the experimental groups.

Frozen placental sections were labeled immunohistochemically for 8-HG. After immersion in 70% ethyl alcohol and rinsing in phosphate-buffered saline (PBS), DNA was denatured in 4 N HCl for 7 minutes followed by neutralization in 50 mmol/L Tris base for 5 minutes and then reequilibrated in PBS. Sections were treated with 1% nonfat dry milk in PBS for 10 minutes to block nonspecific background. Anti-8-HG (Trevigen, Gaithersburg, Maryland) at a dilution of 1:300 in 10 mmol/L Tris-HCl, pH 7.5, was applied and incubated for 2 hours at 25°C followed by PBS here and between all subsequent steps. Control sections were incubated in PBS in place of the primary antibody. Endogenous peroxidase was blocked using the EnVision Endogenous Enzyme Block (Dako, Carpinteria, California) for 10 minutes. This step was performed after primary antibody incubation because hydrogen peroxide in the blocking solution can damage DNA. Incubation in secondary

Figure 1. Morphology of the rat placental layers and attachment to uterine tissue. Inset is whole placenta; bar = 500 μm . The decidua (D) is adjacent to maternal endometrium (E). The junctional zone (J) shows large areas of glycogen-rich cells, while the labyrinth (L) is the primary layer in which maternal and fetal vasculature are in close proximity for nutrient and gas exchange. Sham surgery with vehicle treatment; hematoxylin & eosin stain; bar = 250 μm .

antimouse antibody was followed by Mach 4 HRP-polymer (Biocare, Concord, California) and then EnVision Chromogen-DAB (diaminobenzidine). Sections were counterstained in 1% methyl green, dehydrated, and mounted in Permount for microscopy. The same number of placentas was used for this analysis as for the morphometric analysis.

Statistical Analysis

Results are presented as mean $\pm$ standard error of the mean. Statistical analysis was by analysis of variance (ANOVA) with post hoc Newman-Keuls and with significance at $P < .05$.

Results

Figure 1 shows a section through the layers of the rat uteroplacental unit. The junctional zone, primarily an endocrine compartment,²⁹ exhibited cellular damage in response to I/R, as observed morphologically, and was the focus of the morphometric analysis. The labyrinth, the site of maternal–fetal gas and nutrient exchange, exhibited I/R-induced nucleic acid oxidation and was the primary layer examined in the immunohistochemical analysis reported herein. The decidua, at the maternal–placental interface, did not exhibit any morphologic or oxidative change in response to I/R.

Figure 2. Morphology of the placental junctional zone as affected by ischemia–reperfusion (I/R) and ET_A antagonism. Sham-operated animals showed intact glycogen-rich cellular areas (recognizable by their very light staining except for the numerous darkly stained nuclei) in both uterine horns. The I/R-exposed animals exhibited large areas of cellular disintegration within the glycogen-rich cellular component in both uterine horns. The ipsilateral horns were subjected to I/R except in the sham group, while contralateral horns were not made ischemic. ET_A antagonism prevented the I/R-induced injury in the glycogen-rich areas in both uterine horns. Arrows identify the following structures: GC, glycogen cell area; SP, spongiotrophoblast cellular area. Hematoxylin & eosin stain; bar = 250 μ m. Insets are details of the intact and degraded glycogen cells. Inset bar = 50 μ m. ET_A indicates ET-1 receptor A.

The general morphology of the placental junctional zone in the different treatment groups is shown in Figure 2. In response to I/R, the glycogen-rich cellular areas exhibited extensive cellular injury, possibly secondary to apoptotic cell death, in these glycogen-rich cells. This injury was prevented by maternal treatment with ET_A antagonist. The placentas from ipsilateral ischemic and contralateral nonischemic uterine horns exhibited similar morphology within a given animal and treatment group. The spongiotrophoblast cells in the same placental zone as the glycogen-rich cells did not appear to be adversely affected by I/R exposure.

In order to determine the extent of the cellular injury observed in response to I/R, and its prevention with an ET_A antagonist, we chose to quantify the relative areas exhibiting intact and disintegrating cells. Figure 3 shows an example of the identification of intact cellular areas and damaged areas within the placental junctional zone. These were quantified as described in the Methods section. As shown in Figure 4, in response to I/R, only about 50% of cells in the total glycogen-rich area remained intact. This result was virtually identical for placentae from both the ischemic and the nonischemic uterine horns. ET_A antagonist preserved the glycogen-rich cells and maintained similar areas of intact glycogen-rich cells, as a percentage of the combination of intact and injured cellular areas, as observed in the sham animals.

Oxidative damage to nucleic acids was prominent in the labyrinthine layer but was not observed in other layers of the

Figure 3. Morphometric identification and analysis of glycogen-rich areas in the placental junctional zone. Areas of intact glycogen-rich cells are outlined in red and injured areas are outlined in green. This representative photomicrograph is of a placental section from an ET_A antagonist-treated, ischemia–reperfusion-exposed rat. Hematoxylin & eosin stain shown in grayscale to make the outlined areas more clearly visible; bar = 250 μ m. ET_A indicates ET-1 receptor A.

placenta. In response to I/R, 8-HG immunolabeling was extensive throughout the labyrinth (Figure 5). ET_A antagonism did not prevent or reduce the extent of 8-HG following I/R

Figure 4. Percentage of intact glycogen-rich cellular area. Graphed bars represent morphometric quantification of glycogen-rich areas in the placental junctional zone of rats following sham or ischemia-reperfusion (I/R) surgery and treated with vehicle or ET_A antagonist ABT-546. The data are presented as percentage of intact cellular area relative to the combined total area occupied by intact and injured cells. Placentas from both the ipsilateral ischemic and the contralateral non-ischemic uterine horns yielded very similar results. Bars represent mean $\pm$ SEM; * P < 0.05; ** P < .01; *** P < .001 between the indicated experimental groups, by ANOVA. ET_A indicates ET-1 receptor A; ANOVA, analysis of variance; SEM, standard error of the mean.

exposure (data not shown). Nitrotyrosine labeling, to detect protein oxidation, was also conducted on these placentas, but there was virtually no immunolabeling observed in response to 30-minute I/R compared with controls (data not shown) in any of the placental cell types.

Discussion

In this study, we demonstrated that unilateral I/R results in damage of the glycogen-rich cells in the junctional zone of the rat placenta. This occurs in both the ipsilateral ischemic and the contralateral nonischemic horns of the I/R-exposed rat. We also demonstrated that ET_A antagonism prevents these I/R-induced changes in both horns. This corresponds with our previous results showing that ET_A antagonism prevents I/R-induced FGR.²² Fetal and placental weight differences among the experimental groups in the present study were not compared because of the short time of experimental treatment (24 hours). However, fetal and placental growth data in a separate longer term study using this I/R model of FGR have been reported in detail, showing I/R-induced FGR that is completely ameliorated by ET_A antagonism.²²

Implications of compromised glycogen cell integrity for fetal and placental well-being may include any or all of several possible outcomes. The primary source of energy within the placenta is glucose from the breakdown of glycogen, a process that begins with glucagon binding its receptors on glycogen cells. The glycogen cell population increases within the junctional zone which reaches its maximum volume at

approximately gestation day 18.5 in the rat, similar timing to our study, and this follows closely the end of glycogen cell proliferation (corresponding approximately to mouse gestation day 16.5).²⁹ This accumulated glycogen is then available to provide energy to meet the increasing demands of the developing fetus and placenta. But the glycogen cell injury we have reported herein is not simply a normal energy release because the cells within the glycogen cell clusters exhibit a loss of integrity, not simply a reduction in volume. Differences in glycogen storage (abundance) among murine placentas have been demonstrated,³⁰ but this does not explain our data because we have shown rapid glycogen cell degradation in response to an ischemic insult, while any inherent differences among placentas would affect all experimental groups. Since glycogen cells provide an essential energy source in the placenta, it is possible that ischemia-induced FGR produced by limited perfusion from excess ET-1 and the resultant inadequate gas exchange and delivery of nutrition is compounded by an inadequate supply of available glucose due to damaged glycogen cells. In addition, if an I/R insult occurs early, reduced glycogen in the glycogen cell clusters could also result in metrial gland hypoplasia, as has been shown for failed glycogen cell island development,³¹ producing its own sequelae of inadequate maternal arterial modification and reduced perfusion. Finally, in addition to providing an energy source, the glycogen cells normally influence differentiation of other placental cell types by producing retinoic acid.³² Extensive glycogen cell injury, then, has negative implications for overall placental development.

The results of the morphometric study suggest a particular vulnerability of the glycogen-rich cells to the stress caused by I/R. The reason for this vulnerability in these particular cells remains to be determined. The injured areas cannot be normal glycogen breakdown by the placenta near term because only the ischemic group exhibited this pattern, and all placentas were examined at the same gestational age. The protection of the glycogen-rich cells by ET_A antagonism, which leads to vasodilation and increased blood flow, suggests that the primary stress to which these cells are subjected during I/R is the result of limited blood flow caused by increased production of ET-1. Since there are no ET receptors in the glycogen-rich cells,¹⁷ the effect of ET_A antagonism on these cells is necessarily indirect. Whether the actual cause of the damage to the glycogen-rich cells is oxidative in nature has not been determined. While the impact of reduced and restored blood flow on these cells indicates that an oxidative process may be involved, the fact that ET_A antagonism alleviates this damage without reducing nucleic acid oxidation in the labyrinthine cells suggests the possibility that other mechanisms may be involved.

We have also shown that I/R causes nucleic acid damage in both horns of the uterus, but in contrast to placental morphology, ET_A antagonism does not prevent the oxidative alteration of nucleic acids in the placental labyrinth following I/R. This is true in spite of the fact that ET_A antagonism completely ameliorates I/R-induced FGR.²² This finding corresponds with a previous report that ET_A antagonism did not produce, prevent,

Figure 5. 8-Hydroxyguanine immunohistochemistry of the placental labyrinth. Extensive nucleic acid oxidation is apparent in response to ischemia–reperfusion (dense brown nuclei). This damage was not prevented or reduced by ET_A antagonism (data not shown). Immunolabeling was conducted as described in “Methods” section; bar = 250 μ m. ET_A indicates ET-1 receptor A.

or reduce oxidative stress-induced 8-HG in the rat renal cortex.³³ It is possible that nucleic acid oxidation happens very quickly, before ET_A antagonist is at adequate levels; however, it has been shown that even after antioxidant treatment of I/R-induced FGR in the rat, fetal weights remain below those from nonischemic controls.²⁷ The results of our 8-HG immunohistochemistry, along with our previously reported data showing that ET_A antagonism prevents I/R-induced FGR in the rat,²² suggest that placental oxidative damage has limited impact on fetal growth following I/R.

The relationship between oxidative stress and human FGR has also been tested by several investigators. Human placental oxidative DNA damage has been shown to be higher in pregnancies complicated by FGR, with and without preeclampsia, compared to normal pregnancies.^{34,35} Antioxidation, an avenue to the prevention of nucleic acid damage, has been reported not to prevent FGR in human pregnancy.³⁶ A recent report that included an analysis of several oxidant and antioxidant factors showed that umbilical cord blood levels of 8-HG were significantly higher in neonates from pregnancies complicated by preeclampsia or eclampsia compared to controls.³⁷ The authors concluded that there is an increased free radical generation and a decreased antioxidant state in fetal circulation in preeclampsia and eclampsia, and they hypothesized that this leads to placental vascular insufficiency and FGR.

Oxidative stress has a measurable impact on the placenta in humans as well as in animal models which can be measured by histochemical analysis as we have shown in this study.

However, evidence would suggest that it is more the response to the oxidative stress that has a physiologic impact rather than the oxidative stress per se. This is evidenced in our study by the fact that 8-HG was present in both the contralateral nonischemic and the ipsilateral ischemic horn of the I/R-exposed rat uterus. The fact that administration of an ET_A antagonist prevented the histologic damage from I/R in both horns while having no impact on the presence of 8-HG is further evidence. In humans, the fact that administration of antioxidants did not prevent FGR in at-risk pregnancies also suggests that other mechanisms are in play.

The human placenta consists of multiple cotyledons that are in immediate juxtaposition. The ischemic damage that is seen in placentas from pregnancies complicated by FGR is not necessarily evenly distributed among the cotyledons. However, if the oxidative stress results in the production of circulating mediators such as endothelin, those mediators may have an impact on nearby cotyledons, analogous to what we observed in placentas from the contralateral horn of our I/R-exposed rats. In the human placenta, the cytotrophoblasts are the primary glycogen-rich cells³⁸ and could conceivably have similar vulnerabilities to I/R as the glycogen-rich cells of the rat placenta. It may be the response to the oxidative stress rather than the stress itself that more significantly compromises placental function.

In conclusion, the glycogen-rich cellular areas of the placental junctional zone are particularly vulnerable to damage from uterine I/R in the rat. Nucleic acid oxidative damage is

a prominent effect of uterine I/R, primarily in the placental labyrinth. ET_A antagonism protects placental cellular integrity but does not prevent nucleic acid oxidation during I/R challenge in the pregnant rat.

Declaration of Conflicting Interests

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