Acute Hypoxia and Chronic Ischemia Induce Differential Total Changes in Placental Epigenetic Modifications

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Abstract

Preeclampsia is a common obstetrical complication, hallmarked by new-onset hypertension. Believed to result from placental insufficiency and chronic placental ischemia, the symptoms of preeclampsia are caused by release of pathogenic factors from the placenta itself, although the mechanisms of their regulation are in many cases unknown. One potential mechanism is through changes in placental epigenetic chromatin modifications, particularly histone acetylation and DNA methylation. Here, we determined the effects of chronic ischemia on global epigenetic modifications in the rodent placenta *in vivo* and acute hypoxia in BeWo placental trophoblast cells *in vitro*. Placental insufficiency via uterine artery restriction increased maternal blood pressure and fetal demise while decreasing placental and fetal mass. Global placental histone H3 acetylation levels were significantly decreased at H3 K9, K14, K18, K27, and K56. Interestingly, when BeWo-immortalized placental trophoblast cells were cultured in oxygen concentrations mimicking healthy and ischemic placentas, there was a significant increase in acetylated at K9, K18, K27, and K56. This was associated with a small but significant decrease in placental acetyl-CoA, suggesting depletion in the source of acetyl group donors. Finally, while global methylation of cytosine from placental DNA was low in both groups of animals (<1%), there was ~50% increase in 5-mC in response to chronic ischemia. This suggests acute hypoxia and chronic ischemia induce differential global changes in histone acetylation in the placenta and that chronically altered metabolic profiles could affect histone acetylation in the placenta, thereby regulating production of pathogenic factors from the placenta during preeclampsia.

Keywords

preeclampsia, epigenetics, histones, DNA methylation

Introduction

One of the most prevalent complications of pregnancy is preeclampsia (PE), affecting ~5% to 7% of pregnancies in the United States. Traditional diagnostic criteria included new-onset hypertension and proteinuria; however, recently published suggested criteria from the American Congress of Obstetricians and Gynecologists have removed proteinuria as an essential factor and instead couple hypertension with any of a wider array of secondary criteria, potentially increasing the reported incidence of the disease.¹ Although improved prenatal care has significantly decreased maternal mortality due to PE, it remains a leading cause of maternal–fetal morbidity. This is due primarily to the fact that there is a dearth of pharmacological interventions for the disease, and the only effective intervention is induction of labor, often prior to full term.²

Although the earliest initiating events causing the disorder are unclear, it is generally believed that a central feature of the disorder is failure to remodel the maternal vasculature to allow for adequate blood supply to the developing fetal/placental unit.^{3,4} In response, the placenta produces factors that are

believed to cause the maternal symptoms of the disease. Several of these factors have been identified, including the antiangiogenic protein sFlt-1, inflammatory cytokines such as tumor necrosis factor (TNF)- α , and circulating autoantibodies to the angiotensin type 1 receptor.^{5–7} However, the molecular mechanisms that regulate the placenta's response to chronic ischemia and hypoxia remain obscure. One intriguing potential central regulator of the placenta's response is epigenetic modification of chromatin, in particular, histone modification and DNA methylation.⁸

Epigenetics refers to nongenomic modification of DNA, or histones which package DNA, that creates stable, heritable

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changes in gene expression, although RNA-mediated gene regulation (ie, microRNA and long noncoding RNA) is often now lumped into this category. DNA methylation is perhaps the most thoroughly studied chromatin modification that can effect gene expression. DNA methylation levels are regulated by a series of DNA methyltransferases and DNA demethylases, although the modification is generally considered to be relatively stable once in place. Methylation of DNA primarily takes place at the so-called CpG islands, stretches of CpG dinucleotides commonly found in the promoter region of genes. It has long been known that methylation of CpGs in a gene's promoter region was associated with downregulation of the gene, although there are exceptions to this rule. Methylation plays a pivotal role in embryonic development, as the embryo is essentially demethylated prior to implantation and DNA is methylated de novo during the process of cellular and tissue differentiation. Interestingly, cellular lineages that create extraembryonic tissue are also methylated de novo but at significantly lower levels.⁹

More recently, a great deal of attention has focused on the modification of histone proteins, which are responsible for packaging of DNA in metazoans. The smallest repeating subunit of eukaryotic chromatin is the nucleosome, consisting of ~146 bp of DNA wrapped an octamer of histones: 2 molecules each of the core histones H2a, H2b, H3, and H4. A fifth "linker" histone H1 then associates with a further 20 bp of DNA and facilitates higher order chromatin compaction.¹⁰ It is now known that a complex series of histone posttranslational modifications (eg, phosphorylation, acetylation, and methylation) are capable of regulating genes associated with bound histones.¹¹ Similar to DNA methylation, modification of histones is regulated by a complex web of enzymes that work locally to modify specific gene regulatory regions.¹² While the effects of placental ischemia on gene expression have been characterized in detail through high-throughput methods, the role of histone modification in the programming of this response is still unclear.^{13,14} Here, we have utilized an established model of placental ischemia-induced hypertension, the reduced uterine perfusion pressure (RUPP) model, and BeWo-cultured placental trophoblast cells to test the hypothesis that late-term placental ischemia and acute hypoxia exposure would affect global histone acetylation and examine the effects of chronic ischemia on DNA methylation.

Materials and Method

Animal

Sprague-Dawley timed pregnant rats (Charles River Laboratories; Wilmington, MA) arrived at the facility on day 11 of gestation (GD11). The procedures performed were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. They also were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The rats were kept on a 12:12-hour light-dark schedule at a constant temperature of 23°C and given food and water ad libitum.

Reduced Uterine Perfusion Pressure

On GD14, the RUPP procedure was done, restricting blood flow to the aortic and bilateral ovarian arteries as described previously.¹⁵ In short, animals received anesthesia via regulated 3% isoflurane (Henry Schein Animal Health; Dublin, OH), and an incision was made along the midline of the abdomen. Both of the uterine horns were externalized and then a single 0.203-mm silver surgical clip was planted on the abdominal aorta superior to the bifurcation of the iliac. To prevent compensatory blood flow, a 0.100-mm silver surgical clip was placed on the left and right ovarian arteries, which supply the uterus. Sham animals were incised and vessels isolated as above, but no clips were placed. Group size was N = 8 in the sham and RUPP groups.

Mean Arterial Pressure Measurement

On GD18, the rats were anesthetized as discussed earlier (ie, 3% isoflurane) and an indwelling of carotid catheters comprised of V-3 tubing (SCI). Catheters were exteriorized to the back of the neck via subcutaneous channel. The next day, rats were placed in separate restraining cages and given time to acclimatize. Mean arterial pressure was then monitored using a pressure transducer connected to a bridge amp and Power Lab receiver and LabChart version 7 software (ADI; Colorado Springs, CO). The pressure was measured for 20 minutes and mean pressure determined.

Tissue Collection

On GD19, rats were anesthetized as stated earlier. A ventral midline incision was made, and the uterus was externalized. After cardiac excision, the number of both viable and reabsorbed pups, as determined by gross morphology, was recorded in the uterus, and the individual pups and placental weights were additionally recorded. Samples of placentas from each uterine horn were obtained and flash frozen in liquid nitrogen followed by storage at -80°C.

Histone H3 Acetylation Determination

In order to determine the degree of acetylation of histone H3, placenta proteins were isolated utilizing the RIPA lysis buffer system (Santa Cruz Biotechnology, Inc; Dallas, TX) and its associated protocol. The samples in the buffer were loaded into Lysing Matrix D tubes (MPBio; Solon, OH) and FastPrep-24 homogenizer (MPBio; Solon, OH) to assist in breaking up the tissue. With the cells lysed, a quantitative analysis of the protein concentration performed by BCA assay (Thermo Scientific; Rockford, IL) per kit instructions.

For the Western blots, 50 µg of protein were loaded and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using Criterion TGX precast gels (Bio-Rad; Hercules, CA). The membrane was blocked using Odyssey-blocking buffer (LI-COR; Lincoln, NE) at room temperature overnight. Rabbit anti-histone H3 acetyl K9 (Cell Signaling; Danvers, MA, 4499), acetyl K14 (Cell Signaling, 9649), acetyl

K18 (Cell Signaling, 13998), acetyl K27 (Cell Signaling, 8173), acetyl K56 (Cell Signaling, 4243), and mouse anti-total H3 (Abcam; Cambridge, United Kingdom, 6002) were used as primary antibodies. Donkey anti-rabbit IRDye 700DX (Rockland; Limerick, PA) and IRDye 800 donkey anti-mouse (Rockland; Limerick, PA) were used as secondary antibodies in order to detect their respective primary counterpart. Images were obtained with a LiCor Odyssey CLx Fluorescent Imager and band intensity quantitated with NIH ImageJ version 1.51w.¹⁶

DNA Methylation Determination

In order to determine degree of methylation of the DNA, the genomic DNA from the placentas were isolated with the Wizard Genomic DNA purification kit and correlating protocol (Promega Corporation; Madison, WI). The amount of DNA present in the samples was quantified spectrophotometrically using a Nanodrop (Thermo Scientific) to obtain the concentrations. Detection of DNA methylation was done with the 5-mC DNA enzyme-linked immunosorbent assay (ELISA) kit and its respective protocol (Zymo Research Corp; Irvine, CA) with $n = 8$ in each group.

Histone H3 Acetylation in Hypoxic BeWo Cells

After the tests were done from the collected *in vivo* samples, histone H3 acetylation was analyzed in *in vitro* samples. BeWo choriocarcinoma cells (ATCC; Manassas, VA) were cultured and given 24 hours in a hypoxic chamber allowing either 8% or 1% oxygen.¹⁷ The protein isolation was done using solely the RIPA lysis buffering system mentioned above, and BCA assay was carried out as described above. The protocol for the Western blot was followed as stated above. Experiments were performed in duplicate with a total of $n = 8$ in each group.

Acetyl-CoA Determination

Acetyl-CoA was measured using the Abcam Pico Probe assay kit (ab87546), and the protocol associated with the kit was followed. Placentas from normal pregnant and RUPP animals were homogenized in perchloric acid per protocol. The assay was completed as directed, loading 25 μ L of the samples and using a standard range of 100 pmol, and normalized to total starting tissue mass per sample, with $n = 8$ in each group.

Statistical Analysis

All statistical comparisons were performed using Prism 7 software (GraphPad). All comparisons were performed using a Student unpaired *t* test, with $P < .05$ considered statistically significant.

Results

Hypoxia Acutely Increases Histone H3 Acetylation in Trophoblast Cells

We first wished to determine whether acute hypoxia would influence global histone acetylation in a placenta-derived

trophoblast cell line. To that end, we cultured human BeWo immortalized trophoblast cells under conditions simulating the oxygen tensions found either healthy or ischemic placentas (8% and 1% oxygen, respectively) as described previously.¹⁸ Interestingly, in contrast to the results seen in the placental tissue *in vivo*, global levels of all observed H3 acetylation marks were significantly elevated. As seen in Figure 1, acetylation of H3 was significantly increased: H3 at K9 ($100\% \pm 11\%$ vs $175\% \pm 24\%$, $P = .03$), K14 ($100\% \pm 10\%$ vs $164\% \pm 23\%$, $P = .04$), K18 ($100\% \pm 1\%$ vs $176\% \pm 30\%$, $P = .05$), and K56 ($100\% \pm 21\%$ vs $166\% \pm 5\%$, $P = .02$) with a strong trend to increase K27 ($100\% \pm 9\%$ vs $141\% \pm 15\%$, $P = .06$). It is important to note that the relative overall levels of acetylation were near the lower end of the detection limits for the Western assay. While it is not possible to determine overall levels of individual modifications due to varying antibody efficiency, it is possible that this indicated low overall levels in these cells. However, the trends in acetylation seem to indicate that global H3 acetylation is in fact increased in response to acute hypoxia in BeWo cells.

Physiological Characterization of RUPP Dams

To determine the effectiveness of the RUPP procedure, we implanted carotid catheters on GD18 and measured blood pressure on GD19. As seen in Figure 2A, animals that underwent the RUPP procedure exhibited markedly increased blood pressure compared to their sham controls (100 ± 3 mm Hg vs 117 ± 2 mm Hg, $P < .05$). This was associated with a significant decrease in placental mass (650 ± 30 mg vs 530 ± 30 mg, $P < .05$; Figure 2B) and a reduction in fetal weight (3.1 ± 0.3 g vs 2.5 ± 0.1 g, $P = .08$; Figure 2C), although this did not quite reach significance ($P = .08$). Furthermore, there was a dramatic increase in fetal demise in the RUPP group in response to RUPP treatment ($4\% \pm 2\%$ vs $63\% \pm 5\%$, $P < .05$) as seen in Figure 2D. All of these values are in line with previously reported observations.^{15,19}

Global Histone H3 Acetylation is Significantly Decreased in Response to Chronic Ischemia

In order to assess global levels of histone acetylation in response to chronic placental ischemia, we made whole-tissue homogenates from placentas from both sham- and RUPP-treated animals and measured global histone H3 acetylation by Western blotting. As seen in Figure 3, there was a significant decrease in normalized acetylation of H3 at K9 ($100\% \pm 11\%$ vs $56\% \pm 10\%$, $P = .02$), K14 ($100\% \pm 14\%$ vs $56\% \pm 8\%$, $P = .04$), K18 ($100\% \pm 14\%$ vs $60\% \pm 7\%$, $P = .04$), K27 ($100\% \pm 3\%$ vs $76\% \pm 7\%$, $P = .02$), and K56 ($100\% \pm 7\%$ vs $68\% \pm 10\%$, $P = .04$). Together, these data suggest that global histone H3 acetylation is significantly decreased in response to chronic hypoxia/ischemia in placental tissue *in vivo*.

Figure 1. BeWo trophoblast cells increase histone H3 acetylation acutely in response to hypoxia in vitro. After 24 hours culture in oxygen concentration mimicking healthy and hypoxic placentas (8% and 1% oxygen), lysates were analyzed for H3 acetylation (1F). In response to hypoxic treatment, there was significantly ($P < .05$) increased acetylation of H3K9 (~175%)(1A), H3K14 (~164%)(1B), H3K18 (~176%)(1C), and H3K56 (~166%)(1E), with a strong trend for increased acetylation of H3K27 (~141%)(1D) when compared to controls. $n = 8$ per group. Average pup and placental weights were determined for each dam, and averages compared for each. Statistical comparisons with corresponding P values are indicated by connecting lines.

Chronic Placental Ischemia Decreases Placental Acetyl-CoA In Vivo

As histone acetylation requires acetyl-CoA as an acetyl donor, we next measured placental tissue levels of acetyl-CoA in both sham-operated and RUPP-treated rats via a commercial fluorometric assay. As seen in Figure 4, in response to placental ischemia, there was a significant decrease in tissue acetyl-CoA in response to chronic placental ischemia (13.1 ± 0.7 pg/uL/g tissue vs 10.9 ± 0.5 pg/uL/g tissue, $P = .02$).

Global Levels of DNA Methylation are Low in Placenta But are Significantly Increased in Response to Placental Ischemia

We further wished to determine whether chronic ischemia would have any discernable effect on global levels of DNA methylation in placental tissue. To that end, we attempted to measure both the levels of cytosine methylation and 5-hydroxyhydroxymethylcytosine by ELISA. In line with previous reports, overall cytosine methylation in the placenta is exceedingly low, <1% overall. Curiously, and contradicting the

changes in histone acetylation, there was a small but significant increase in 5-methylcytosine levels in response to placental ischemia, approaching a 50% increase ($0.35\% \pm 0.06\%$ vs $0.59\% \pm 0.09\%$, $P < .05$). We attempted to measure levels of 5-hydroxymethylcytosine by ELISA as well, but the levels were below the detection limits (data not shown). Overall, these data indicate that placental DNA methylation remains low in response to chronic ischemia but exhibits a slight but significant increase in cytosine methylation (Figure 5).

Discussion

Despite years of intensive research and the identification of numerous pathogenic pathways believed to be involved the symptomatic phase of PE, the molecular mechanisms underlying the disorder remain obscure. Preeclampsia is sometimes divided into 2 stages: the asymptomatic stage during early defects in placental development and the later stage where placental insufficiency leads to the maternal symptomatic phenotype.²⁰ Unfortunately, a lack of spontaneous animal models has made this earlier stage harder to examine. In contrast, utilization of animal models of placental ischemia with correlative studies in human patients has begun to elucidate effector

Figure 2. Placental ischemia induces significant increases in maternal blood pressure (A) and fetal demise (D), significant decreases in placental mass (B), and a trend for a decrease in viable fetal mass (C). N = 8 per group. Statistical comparisons with corresponding P values are indicated by connecting lines.

pathways important for the maternal syndrome. This has included imbalance of angiogenic factors and production of inflammatory cytokines among others.^{21,22} However, the underlying mechanisms that are responsible for the placental ischemic response have been largely unexamined.

One of the most effective methods to affect gene expression is by modification of chromatin directly, by changes in transcription factor binding, regulatory DNA region methylation, or modification of gene-associated histone proteins.²³ These latter 2 are considered epigenetic mechanisms and refer to nongenomic modifications that can be passed throughout a cellular replication without modification of the underlying DNA sequence. Although early enthusiasm for these mechanisms was for transmission of generational changes in gene expression, there is increasing realization that these regulatory mechanisms are important for shorter term stress responses, such as hypoxia.²⁴ To date, there have been only a few studies that have examined these mechanisms in the context of the placenta during PE.

Perhaps no epigenetic modification has received more attention in recent years than histone acetylation, particularly that of histone H3. Acetylation of lysines on H3 has been extensively studied and is associated generally with increased transcriptional activation of associated genes. Changes in H3 acetylation have been demonstrated in a variety of pathophysiological states.^{25,26} To date, global changes in histone acetylation in response to placental ischemia or PE directly have not been directly addressed. There have, however, been several suggestive studies suggesting an important role for histone

modification in the pathogenesis of PE. Rahat et al found that there was differential methylation of H3K9 and H3K27 at the *c-myc* and *hTERT* genes in PE placentas as well as differential methylation of those genes.²⁷ Interestingly, Munro et al found that inhibition of histone deacetylases by trichostatin A blocked lipopolysaccharide (LPS)-induced TNF- α and interleukin 10 production in placental tissue. However, the relevance of LPS-induced inflammation to PE is not necessarily clear, and whether this was a direct effect on the transcription of the genes or an upstream regulator was not addressed.²⁸

In the present study, we have examined the effects on histone H3 acetylation in both models of in vivo chronic placental ischemia and acute hypoxia on BeWo-immortalized trophoblast cells in vitro. In response to chronic placental ischemia, global levels H3 acetylation are significantly decreased, suggesting an overall increase in chromatin compaction. However, when we exposed placenta-derived immortalized trophoblast cells to an acute hypoxia challenge, we observed an increase in H3 acetylation. This suggests a differential temporal response, an acute increase in histone acetylation in response to acute hypoxia, and a longer term decrease in acetylation in chronic ischemia. In an effort to resolve this apparent contradiction, we examined the tissue levels of acetyl-CoA in the placentas of Sham and RUPP rats. As acetyl-CoA is the substrate utilized by histone acetylases to modify histones, we hypothesized that chronic hypoxia in the placenta could shift cellular bioenergetics to anaerobic glycolysis long term as seen in other tissues.²⁹

Figure 3. Histone H3 acetylation is altered in response to chronic placental ischemia. Whole placental lysates from Sham-operated or RUPP-treated rats were analyzed for global levels of histone H3 acetylation by Western blotting (3F). In response to chronic ischemia, placental histone H3 had significantly lower acetylation at H3K9 (~66%)(3A), H3K14 (~66%)(3B), H3K18 (60%)(3C), H3K27 (~76%)(3D), and H3K56 (~68%)(3E) when compared to control. N = 8 per group. Statistical comparisons with corresponding P values are indicated by connecting lines. NP indicates normal pregnant (sham), RUPP indicates reduced uterine perfusion pressure.

Figure 4. Placental acetyl-CoA is significantly decreased in chronic placental ischemia. Acetyl-CoA was extracted from placental tissue and assayed by fluorimetric assay. In response to reduced placental perfusion, there was a significant decrease (~15%, $P < .05$) in the tissue level of acetyl-CoA in whole placental lysates. N = 8 per group. CoA indicates coenzyme A.

Consistent with this hypothesis, we found a slight but significant and consistent decrease in the levels of placental acetyl-CoA in response to chronic ischemia. This change in the substrate required for histone acetylation could partially

explain the difference observed between the acute and the chronic effects of hypoxia seen in this study. It does not, however, exclude other possible explanations, such as increased activity of histone deacetylase in chronically ischemic tissue. Ongoing studies are examining the expression of various bioenergetics enzymes and histone modifying enzymes to further explore these options.

Perhaps better characterized than histone acetylation is altered DNA methylation in the placenta during PE. Extraembryonic cell lineages such as those forming the placenta are generally hypomethylated when compared to somatic tissues.⁹ Indeed, the preponderance of studies that have examined placental cytosine methylation has found <5% total cytosine methylation.^{30,31} Several studies have found differential methylation of specific loci known to be associated with PE in placental tissues, including pappalysin A, vascular endothelial growth factor, TNF, and FLT-1.³²⁻³⁴ Kulkarni et al found that not only was there significant hypermethylation in PE placentas but that global methylation correlated with maternal hypertension.³⁵ It was unclear, however, whether this was a causative or downstream effect. Here, we demonstrate that global placental methylation levels in the rodent are relatively low, at least to the published values cited above, with control levels at <0.5%. Interestingly, however, we saw

Figure 5. Global 5-Me-cytosine is increased in response to chronic placental ischemia. Whole placentas from sham-operated and RUPP-treated rats were examined for global cytosine methylation by ELISA. In response to placental ischemia, global methylation levels were increased by approximately 2-fold. $N = 8$ per group. Statistical comparisons with corresponding P values are indicated by connecting lines. RUPP indicates reduced uterine perfusion pressure; ELISA, enzyme-linked immunosorbent assay.

~2-fold increase in the global placental methylation in the placentas of RUPP rats, a trend similar to the results seen in PE women.³⁵ This is the first evidence that chronic ischemia in the placenta directly causes alterations in DNA methylation in vivo.

Research into PE has expanded rapidly in the preceding decade, as has research into the basic biology of the placenta, both during normal pregnancy and during pathological states. Indeed, several studies have correlated changes in histone modification and DNA methylation with PE. Here, for the first time, we have utilized a rodent model of placental ischemia to determine the effects of chronic placental hypoxia/ischemia on both histone modification and DNA methylation levels. We have shown that in response to placental ischemia, acetylation of histone H3 is decreased and DNA methylation is significantly increased, suggesting that the results seen in patients with PE are at least a response to ischemia as opposed to a direct cause. It is possible that these changes in epigenetic control could be a significant contributor in the production of any of the various pathogenic factors released into the maternal circulation during placental ischemia. Whether epigenetic modifications also play a role in the establishment of the disorder remains an unanswered question.

Declaration of Conflicting Interests

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