Elevated Adenosine Induces Placental DNA Hypomethylation Independent of A2B Receptor Signaling in Preeclampsia

Aji Huang, Hongyu Wu, Takayuki Iriyama, Yujin Zhang, Kaiqi Sun, Anren Song, Hong Liu, Zhangzhe Peng, Lili Tang, Minjung Lee, Yun Huang, Xin Ni, Rodney E. Kellems, Yang Xia

Abstract—Preeclampsia is a prevalent pregnancy hypertensive disease with both maternal and fetal morbidity and mortality. Emerging evidence indicates that global placental DNA hypomethylation is observed in patients with preeclampsia and is linked to altered gene expression and disease development. However, the molecular basis underlying placental epigenetic changes in preeclampsia remains unclear. Using 2 independent experimental models of preeclampsia, adenosine deaminase–deficient mice and a pathogenic autoantibody-induced mouse model of preeclampsia, we demonstrate that elevated placental adenosine not only induces hallmark features of preeclampsia but also causes placental DNA hypomethylation. The use of genetic approaches to express an adenosine deaminase minigene specifically in placentas, or adenosine deaminase enzyme replacement therapy, restored placental adenosine to normal levels, attenuated preeclampsia features, and abolished placental DNA hypomethylation in adenosine deaminase–deficient mice. Genetic deletion of CD73 (an ectonucleotidase that converts AMP to adenosine) prevented the elevation of placental adenosine in the autoantibody-induced preeclampsia mouse model and ameliorated preeclampsia features and placental DNA hypomethylation. Immunohistochemical studies revealed that elevated placental adenosine–mediated DNA hypomethylation predominantly occurs in spongiotrophoblasts and labyrinthine trophoblasts and that this effect is independent of A2B adenosine receptor activation in both preeclampsia models. Extending our mouse findings to humans, we used cultured human trophoblasts to demonstrate that adenosine functions intracellularly and induces DNA hypomethylation without A2B adenosine receptor activation. Altogether, both mouse and human studies reveal novel mechanisms underlying placental DNA hypomethylation and potential therapeutic approaches for preeclampsia. (Hypertension. 2017;70:209-218. DOI: 10.1161/HYPERTENSIONAHA.117.09536.)

Key Words: adenosine ■ DNA hypomethylation ■ epigenetics ■ placenta ■ preeclampsia

Preeclampsia is a dangerous pregnancy complication characterized by hypertension and impaired renal function.1-5 The condition affects ≈8% of first pregnancies and accounts for >80,000 premature births each year in the United States (=15% of total premature births), >$4 billion in medical costs, and immeasurable human suffering. Preeclampsia adversely affects the mother’s kidneys, heart, liver, and other vital organs and, if undetected or untreated, can lead to seizures (eclampsia), cerebral hemorrhage, failure in vital organs (eg, kidney and heart), and death. By conservative estimates, each year this disease is responsible for >75,000 maternal deaths worldwide. Thus, understanding the pathogenesis of preeclampsia is extremely important for developing effective therapeutic strategies to treat this disease that is dangerous to both moms and babies.

A different gene expression profile is observed between normal and preeclampsia placentas, and the differences in gene expression are widely considered to contribute to the pathophysiology of preeclampsia.6-7 Emerging evidence suggests that these differences in gene expression are caused, in part, by epigenetic changes, especially differences in placental DNA methylation.8 Initial studies determined that preeclampsia placentas are characterized by widespread DNA hypomethylation when compared with placental DNA from normotensive pregnancies.9,10 Subsequent studies focused on CpG dinucleotide repeats (CpG islands) located in the promoter region of many genes.11-13 DNA methylation at these promoter-specific CpG islands is frequently associated with the repression of gene expression, and conversely, hypomethylation is frequently associated with gene activation.14 Consistent with this, detailed analysis of DNA from preeclampsia placentas showed that hypomethylation at specific promoter regions was associated with increased expression of specific genes that may contribute to the pathophysiology of...
preeclampsia. However, the factors responsible for widespread placental DNA hypomethylation in preeclampsia have not been identified.

Because of decreased trophoblast invasion and lack of appropriate remodeling of maternal spiral arteries, preeclampsia placentas are characterized by ischemia, hypoxia, and inflammation. Adenosine is a key signaling molecule that orchestrates the cellular response to hypoxia, energy depletion, and tissue damage by the activation of G-protein–coupled cell surface receptors. Recently, we demonstrated that placental adenosine is elevated in patients with preeclampsia. Using genetic and pharmacological approaches, we demonstrated that elevated placental adenosine is sufficient to induce preeclampsia features via activation of A2B adenosine receptors (ADOR2B) in adenosine deaminase (ADA)–deficient mice (Ada−/−) and in a pathogenic autoantibody–induced preeclampsia model. Among the 4 genes encoding adenosine receptors, only Adora2b gene expression was elevated in these models. Adora2b gene expression is also elevated in the placentas of women with preeclampsia relative to normotensive pregnancies. We have provided both mouse and human evidence that elevated CD73 (an ectonucleotidase that converts AMP to adenosine) is a key enzyme responsible for increased placental adenosine and subsequent disease development. Thus, our prior findings revealed the pathogenic consequences of chronically elevated placental adenosine, the enzymatic basis for its elevation (increased CD73), and the specifically elevated ADORA2B responsible for clinical features of preeclampsia.

Besides functioning as a ligand to activate adenosine receptors, a potential metabolic consequence of elevated adenosine is cellular uptake via equilibrative nucleoside transporters (ENTs), leading to formation of increased levels of intracellular S-adenosylhomocysteine (SAH), a potent inhibitor of many cellular methyltransferase enzymes, including those that methylate DNA. DNA methylation requires the donation of a methyl group from S-adenosylmethionine catalyzed by various DNA methyltransferases. The resulting product, SAH, is further converted to adenosine and homocysteine by the action of SAH hydrolase. The equilibrium constant of this reaction favors the accumulation of SAH and will only proceed when adenosine and homocysteine are constantly removed. Thus, if adenosine accumulates, SAH levels will rise and inhibit the methyltransferases through product inhibition. For this reason, elevated adenosine is associated with DNA hypomethylation. However, the function of elevated placental adenosine in DNA hypomethylation in preeclampsia remains unknown largely because of the lack of in vivo experimental models. Here, we report the use of 2 independent preeclampsia models, both characterized by elevated placental adenosine, that led us to discover that elevated placental adenosine contributes to the placental DNA hypomethylation in preeclampsia. Mechanistically, we demonstrated that placental DNA hypomethylation mainly occurs in trophoblasts and is dependent on intracellular adenosine and independent of ADORA2B activation.

Materials and Methods

Animals

Wild-type (WT) mice were purchased from Harlan Laboratories (Indianapolis, IN). Global ADA-deficient mice die late in gestation from impaired liver function. To circumvent this complication and to produce viable embryos with excessive placental adenosine, we genetically rescued ADA-deficient embryos from perinatal lethality by introducing an ADA minigene that is only expressed in the fetal liver under control of the α-fetoprotein gene regulatory elements. These fetal liver–rescued ADA-deficient mice are designated as Ada−/−/fLi-Tg+. We also generated fetal liver–rescued ADA-deficient mice that were deficient in the A2B adenosine receptor. These mice are designated as Ada−/−/fLi-Tg+/Adora2b−/−. We have previously rescued ADA-deficient mice from perinatal lethality using an ADA minigene that is exclusively expressed in the trophoblast cell lineage. The placental-rescued ADA-deficient mice are designated as Ada−/−/fLi-Tg−/−. The mice were housed in the animal care facility of the University of Texas Health Science Center at Houston. All protocols involving animal studies were reviewed and approved by the Institutional Animal Care and Use Committee.

Mating Strategy of ADA-Deficient Mice

As indicated above, we had 3 strains of ADA-deficient mice: Ada−/−/fLi-Tg+ mice, Ada−/−/PL-Tg+ mice, and Ada−/−/fLi-Tg+/Adora2b−/− mice. Briefly, we crossed 8- to 10-week-old Ada−/− females with Ada−/− males from each of the 3 genetic backgrounds. By this mating strategy, we generated pregnant mice in which half of the placentas were homozygous for the null Ada allele and half were heterozygous. For liver-rescued ADA-deficient mice, all fetuses expressed the ADA transgene in the fetal liver. For placenta-rescued ADA-deficient mice, all placentas expressed the ADA transgene instead of fetuses. For the Adora2b−/− mice, all fetuses and placentas were ADA2B deficient.

Maintenance of ADA-Deficient Mice

ADA-deficient mice (both placental-rescued and fetal liver–rescued Ada−/− mice) die within 3 to 4 weeks after birth because of the ADA deficiency. To avoid post-natal lethality, we provide ADA enzyme replacement therapy in the form of polyethylene glycol–modified ADA (PEG-ADA) based on a previous report. The maintenance of ADA-deficient mice was described previously. Briefly, Ada−/− mice were identified on day 5 after birth by screening for ADA enzymatic activity in the tail blood using zymogram analysis and were maintained by intraperitoneal or intramuscular injections of PEG-ADA. The day when the copulation plug was detected was designated as E0.5. DNA was extracted from tail biopsies of pups by the proteinase K/phenol/chloroform method, and polymerase chain reaction was conducted with primers for the genotyping of each mouse.

Introduction of Pathogenic Autoantibody Into Pregnant Mice

Briefly, IgGs were purified from preeclampsia or normotensive patient serum as described previously. Pregnant mice were injected with normotensive-IgG or preeclampsia-IgG (0.8 mg) on E13.5 and E14.5 by retro-orbital sinus injection. Mouse blood and organs were collected on E18.5 before delivery.

Measurement of Blood Pressure in Mice

The systolic blood pressure of mice was measured at the same time daily by a carotid catheter–calibrated tail-cuff system (CODA, Kent Scientific), and the mice were kept warm using a warming pad (AD Instruments Co.).

Adenosine Measurement

Adenosine was isolated from frozen placentas (which were collected in liquid nitrogen) and quantified by reverse phase high-performance liquid chromatography as described previously.

Measurement of Adenosine in Preeclampsia Placentas

In a separate set of experiments, we measured adenosine in placentas from women with preeclampsia (n = 20) and gestational hypertension (n = 10) and compared these results to those from normotensive control placentas (n = 10). Preeclampsia placentas were characterized by dilated spiral arteries and thickened basalis layers as previously described. Adenosine was measured by HPLC using the technique described previously.

Maintenance of Global ADA-Deficient Mice

Global ADA-deficient mice die late in gestation because of the ADA deficiency. For liver-rescued ADA-deficient mice, the liver was perfused with 4% paraformaldehyde. The liver was cryoprotected using a 20% sucrose solution. The liver was then sectioned using a cryostat, and sections were stained with hematoxylin and eosin. The liver sections were imaged using an Axio Observer Z1 microscope (Carl Zeiss AG). The percentage of liver sections with a normal lobular structure was determined using ImageJ software.

Maintenance of ADA-Deficient Mice

ADA-deficient mice die late in gestation because of the ADA deficiency. For liver-rescued ADA-deficient mice, the liver was perfused with 4% paraformaldehyde. The liver was cryoprotected using a 20% sucrose solution. The liver was then sectioned using a cryostat, and sections were stained with hematoxylin and eosin. The liver sections were imaged using an Axio Observer Z1 microscope (Carl Zeiss AG). The percentage of liver sections with a normal lobular structure was determined using ImageJ software.

Conclusion

In conclusion, adenosine is a key mediator of preeclampsia. It is elevated in the placenta and is dependent on intracellular adenosine. It contributes to placental DNA hypomethylation, which is associated with preeclampsia. The elevation of placental adenosine is caused by increased CD73 expression, which converts adenosine to adenosine. The accumulation of adenosine leads to increased S-adenosylhomocysteine, which inhibits DNA methyltransferases. This results in decreased DNA methylation, which is associated with preeclampsia.
Quantification of Global DNA Methylation Levels Using ELISA
DNA was extracted from frozen placenta or cells using the DNeasy Blood and Tissue Kit (Qiagen). Global DNA methylation was measured using the MethylFlash Methylated DNA Quantification Kit (Epigentek).

Quantification of Global DNA Methylation Levels Using Dot Blot
DNA was extracted from frozen placenta using the DNeasy Blood and Tissue Kit (Qiagen). Global DNA methylation was quantified by 5-methylcytosine (5 mC) using dot blot as described previously.

Quantification of DNA Methylation Levels Using Immunofluorescence
Formalin-fixed placenta blocks were cut into 4-μm thick slides and subjected to immunofluorescence. Briefly, antigen retrieval was conducted by incubating slides in sodium citrate buffer (pH=6.0) at 95°C for 15 minutes. After blocking, slides were incubated with antibody against 5 mC (1:250, ab10805, Abcam) in a humidified chamber at 4°C overnight. After incubation with secondary antibody, slides were sealed by ProLong Gold Antifade Mountant with 4′,6-diamidino-2-phenylindole (P36935, Thermo Fisher Scientific). The exposure time for 5 mC was 1/7 seconds and for 4′,6-diamidino-2-phenylindole was 1/60 seconds. The positive staining for 5 mC in nuclei was quantified by ImageJ software. The intensity of positive staining was obtained from 10 fields in each section under x40 magnification and averaged to get mean values.

HTR-8 Cell Culture
Human trophoblast cells, HTR-8, were cultured at 37°C with 5% CO2 in a cell incubator. Culture medium contained RPMI-1640 (Sigma-Aldrich), 15% fetal bovine serum (Sigma-Aldrich), and 1% penicillin-streptomycin (Gibco).

Statistical Analysis
All data are expressed as the means±SEM. GraphPad Prism 5 software was used to analyze the data for statistical significance. Nonparametric equivalent Wilcoxon rank-sum test and Student t test were applied in 2-group analysis. Differences between the means of multiple groups were compared by 1-way ANOVA followed by a Tukey multiple comparisons test. *P<0.05 was considered significant.

Results
Mice With Elevated Placental Adenosine Display Global Placental DNA Hypomethylation and Preeclampsia
To examine the consequences of excessive placental adenosine on DNA methylation, we needed an experimental approach to generate pregnant mice in which adenosine levels are elevated in the placenta. We have recently achieved this using a genetic approach to generate pregnant mice with a deficiency in placental ADA, the enzyme that irreversibly degrades adenosine to inosine. ADA levels are relatively high in mouse placentas, and in the absence of ADA, adenosine accumulates to high levels. Global ADA-deficient fetuses die late in gestation because of impaired fetal liver function resulting from the metabolic consequences of ADA deficiency. We have recently used a transgenic approach to successfully rescue ADA-deficient fetuses from prenatal lethality by restoring ADA expression to the fetal liver (using an ADA minigene under control of α-fetoprotein gene regulatory elements, Ada−/−/Li-Tg+; Figure 1A). When homozygous ADA-deficient males (Ada−−/Li-Tg+) were crossed with heterozygous ADA-deficient females (Ada−+/Li-Tg+), half of the placentas were ADA deficient and half were heterozygous for the null Ada−− allele. All fetuses expressed the ADA transgene in the fetal liver (Figure 1A), a feature that rescued otherwise ADA-deficient fetuses from perinatal lethality. In agreement with our recently published results, these dams displayed features of preeclampsia, including hypertension on gestational day 18.5 (Figure 1B). As expected, we found that adenosine was significantly elevated in the placentas that were ADA deficient compared with WT ADA-positive mouse placentas (Figure 1C). Thus, fetal liver–rescued ADA-deficient mice provided us a genetic investigative model to assess the impact of elevated placental adenosine on DNA hypomethylation under preeclampsia conditions.

Next, we measured 5 mC to quantify global DNA methylation levels in E18.5 placentas from Ada−/−/Li-Tg+ females mated with Ada−−/Li−/Tg− males as illustrated in Figure 1A. As a result of these matings, half of the placentas are ADA deficient and half are ADA positive (heterozygous for the null allele). We also determined 5 mC levels in placentas from homozygous ADA-positive WT matings. ELISA and dot blot experiments showed reduced global DNA methylation levels in Ada−−/Li−/Tg− placentas compared with homozygous WT placentas (Figure 1D). These studies demonstrate that elevated adenosine in ADA-deficient placentas is associated with placental DNA hypomethylation and preeclampsia features in the dams.

Elevated Placental Adenosine Is Responsible for Placental DNA Hypomethylation in ADA-Deficient Mice
To determine whether elevated placental adenosine contributes to placental DNA hypomethylation in ADA-deficient mice, we used a transgenic approach to genetically restore ADA exclusively to the placentas of ADA-deficient mice to lower placental adenosine levels (genotype Ada−/+/PL-Tg+). Specifically, we crossed placental-rescued homozygous ADA-deficient males (Ada−−/PL-Tg+) with heterozygous ADA-deficient females (Ada−+/−/PL-Tg+) as illustrated in Figure 1A. By this mating strategy, half of the placentas (and fetuses) were heterozygous for the mutant Ada allele (ie, Ada−+) and half were homozygous deficient (Ada−−). However, all placentas expressed the ADA transgene (PL-Tg+). Consistent with our published studies, expression of ADA in placentas not only rescued ADA-deficient fetuses but also abolished maternal hypertension (Figure 1B). Moreover, restoring ADA to placentas completely normalized placental adenosine levels and DNA methylation to that seen in WT placentas on E18.5 (Figure 1C through 1E). Altogether, using both fetal liver–rescued and placental-rescued ADA-deficient mice, we provide strong genetic evidence that elevated placental adenosine contributes to placental DNA hypomethylation and maternal features of preeclampsia in mice.

Elevated Placental Adenosine Induces DNA Hypomethylation in Spongiotrophoblasts and Labyrinthine Trophoblasts in ADA-Deficient Mice
To determine the specific cell types in which elevated adenosine induces DNA hypomethylation in ADA-deficient
placentas, we conducted immunofluorescence analysis in WT, Ada−/−/fLi-Tg+, and Ada−/−/PL-Tg+ mouse placentas using a 5-mC–specific antibody. We found that Ada−/−/fLi-Tg+ placentas had lower 5 mC levels in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A). In contrast, Ada−/−/PL-Tg+ placentas (which produced ADA encoded by the placental minigene) displayed significantly increased 5 mC staining in spongiotrophoblasts and labyrinthine trophoblasts (Figure 2A).

Figure 1. Elevated placental adenosine caused by placental-specific deficiency of adenosine deaminase (ADA) promotes global DNA hypomethylation in the mouse placentas of Ada−/−/fLi-Tg+ mice and Ada−/−/fLi-Tg+/Adora2b−/−. Samples were collected on E18.5. A, Mouse models and mating strategy to generate pregnant mice with preeclampsia features in Ada−/−/fLi-Tg+. B, Change of mean systolic pressure in pregnant mice on E18.5 compared with E12.5. C, Placental adenosine levels were quantified by reverse phase high-performance liquid chromatography. D–E, Global DNA methylation levels were determined by quantification of 5-methylcytosine (5 mC) using ELISA and dot blot. All data are expressed as mean±SEM. *P<0.05 vs wild type (WT), **P<0.05 vs Ada−/−/fLi-Tg+ (n=5). Wilcoxon rank-sum test was applied in (B) and (C); Student t test was applied in (D)–(E).

Figure 2. Elevated placental adenosine causes global DNA hypomethylation in the spongiotrophoblasts and labyrinthine trophoblasts of mouse placentas of Ada−/−/fLi-Tg+ mice and Ada−/−/fLi-Tg+/Adora2b−/−. Arrows indicate a nucleus with positive staining. Positive staining for 5-methylcytosine (5 mC) in nuclei was quantified by ImageJ software. n=10 fields per placenta under ×40 magnification. The exposure time for 5 mC was 1/7 seconds and for 4′,6-diamidino-2-phenylindole (DAPI) was 1/60 seconds. Relative pixel intensity refers to the ratio of mean intensity of positive 5 mC staining and mean intensity of DAPI staining. All data are expressed as mean±SEM. *P<0.05 vs wild type (WT). Statistics were analyzed by 1-way ANOVA, followed by a Tukey multiple comparisons test.
trophoblasts, achieving levels close to those seen in WT mice (Figure 2). Thus, these findings revealed that elevated placental adenosine in Ada−/−/fLi-Tg+ placentas induced DNA hypomethylation in spongiotrophoblasts and labyrinthine trophoblasts.

**Elevated Placental Adenosine, Independent of ADORA2B, Induces DNA Hypomethylation in Spongiotrophoblasts and Labyrinthine Trophoblasts in ADA-Deficient Mice**

We have recently shown that elevated placental adenosine signaling via ADORA2B is responsible for maternal preeclampsia features. However, whether elevated placental adenosine–induced placental DNA hypomethylation is dependent on ADORA2B signaling remains unknown. To test this possibility, we generated ADORA2B-deficient mice on the background of fetal liver–rescued ADA-deficient mice (Ada−/−/fLi-Tg+/Adora2b−/−) by crossing Ada−/−/fLi-Tg+/Adora2b−/− males with Ada−/−/fLi-Tg+/Adora2b−/− females (Figure 1A). Consistent with our previously published results, we found that genetic deletion of ADORA2B resulted in loss of maternal hypertension (Figure 1B), without affecting the levels of elevated placental adenosine (Figure 1C). Analysis of placental DNA showed reduced global DNA methylation levels in the placentas of both Ada−/−/fLi-Tg+ mice and Ada−/−/fLi-Tg+/Adora2b−/− mice compared with the WT mice and Ada−/−/PL-Tg− mice (Figure 1D and 1E). Immunofluorescence experiments showed a similar result that Ada−/−/fLi-Tg+ mice and Ada−/−/fLi-Tg+/Adora2b−/− mice had lower 5 mC levels in both spongiotrophoblast and labyrinthine trophoblasts (Figure 2). Our findings demonstrated that reduced global DNA methylation levels in spongiotrophoblasts and labyrinthine trophoblasts of Ada−/−/fLi-Tg+ mouse placentas resulted from elevated placental adenosine and was independent of ADORA2B signaling.

**Increased Placental Adenosine Contributes to Placental DNA Hypomethylation in an Experimental Model of Preeclampsia**

To test the generality of the intriguing hypothesis that placental DNA hypomethylation in preeclampsia patients is because of elevated placental adenosine, we took advantage of an established experimental model of preeclampsia in mice induced by transfer of preeclampsia patient–derived IgG known to contain the pathogenic autoantibodies, AT1-AA, that activate the angiotensin II type 1 receptor. Briefly, we transferred IgGs isolated from patients with preeclampsia or normotensive pregnant women to WT or CD73-deficient pregnant mice on E13.5 and E14.5 as before. All mice were euthanized on E18.5 before delivery. Consistent with published studies, preeclampsia-IgG, but not normotensive-IgG, induced hypertension in WT dams (Figure 4A). Preeclampsia-IgG also induced increased placental adenosine levels in WT dams (Figure 4B). However, preeclampsia-IgG–induced hypertension and elevated placental adenosine were significantly attenuated in CD73-deficient dams (Figure 4A and 4B).

Next, we determined whether elevated placental adenosine causes placental DNA hypomethylation in the preeclampsia-IgG–induced experimental model. ELISA analysis showed that the 5 mC levels in placentas of preeclampsia-IgG–injected WT dams with elevated adenosine levels were significantly reduced compared with normotensive-IgG–injected WT dams (Figure 4C). Similar results were observed when using ADORA2B-deficient dams (Figure 4C). However, DNA hypomethylation and increased placental adenosine were significantly ameliorated in preeclampsia-IgG–injected CD73−/− dams (Figure 4C). We also determined DNA methylation by immunohistochemical determination of 5 mC levels in the spongiotrophoblasts and labyrinthine trophoblasts as described above. The results (Figure 5) show that 5 mC levels were reduced in spongiotrophoblasts and labyrinthine trophoblasts in preeclampsia-IgG–injected dams relative to those injected with normotensive-IgG. Furthermore, the reduction in 5 mC in preeclampsia-IgG–injected dams was not dependent on ADORA2B signaling. However, the reduction in 5 mC levels resulting from preeclampsia-IgG injection did not occur in CD73-deficient animals (Figure 5). These results indicate that preeclampsia-IgG–induced placental adenosine contributes to placental DNA hypomethylation and is independent of ADORA2B signaling but requires CD73.

**Adenosine Functions Intracellularly to Induce DNA Hypomethylation in Cultured Human Trophoblasts**

As shown above, elevated adenosine induces placental DNA hypomethylation in spongiotrophoblasts and...
labyrinthine trophoblasts independent of ADORA2B signaling. To test the hypothesis that elevated adenosine functions intracellularly to induce DNA hypomethylation, we used cultured HTR-8 cells, a commonly used immortalized human trophoblast cell line. Specifically, HTR cells were treated with adenosine for 24 hours with and without MRS1706...
At the end of these experiments, we compared 5 mC levels in these cells to quantify global DNA methylation. We found that adenosine treatment alone, or combined treatment of adenosine with MRS1706, induced DNA hypomethylation compared with the controls (Figure 6A). In contrast, we found that blocking exogenous adenosine uptake by dipyridamole, a specific ENT inhibitor, significantly reduces exogenous adenosine-mediated DNA hypomethylation (Figure 6B). These results demonstrate that adenosine-mediated DNA hypomethylation depends on intracellular adenosine and is independent of ADORA2B signaling.

**Discussion**

Using a combination of genetic and biochemical approaches, we demonstrated that elevated placental adenosine contributes to the induction of maternal preeclampsia features through A2B signaling and also causes placental DNA hypomethylation.

**Figure 5.** Global 5-methylcytosine (5 mC) in the placentas of preeclampsia (PE)-IgG–induced PE mouse models detected by immunofluorescence. Arrows indicate a nucleus with positive staining. The positive staining of 5 mC in nuclei was quantified by ImageJ software. n=10 fields per placenta under ×40 magnification. The exposure time for 5 mC was 1/7 seconds and for 4′,6-diamidino-2-phenylindole (DAPI) was 1/60 seconds. Relative pixel intensity refers to the ratio of mean intensity of positive 5 mC staining and mean intensity of DAPI staining. All data are expressed as mean±SEM. *P<0.05 vs wild-type (WT) normotensive (NT)-IgG. Statistics were analyzed by 1-way ANOVA, followed by a Tukey multiple comparisons test.

**Figure 6.** Elevated intracellular adenosine directly leads to DNA hypomethylation in cultured human trophoblast cells. **A,** Outline of adenosine, dipyridamole (equilibrative nucleoside transporter [ENT]–specific inhibitor), and MRS1706 (ADORA2B inhibitor) treatments in cell culture. **B,** Global DNA methylation levels were determined by quantification of 5-methylcytosine (5 mC) by ELISA (n=3). All data are expressed as mean±SEM. Statistics were analyzed by Student t test. *P<0.02 vs control. **C,** Working model. CD73-dependent production of elevated extracellular adenosine leads to accumulation of intracellular adenosine. Meanwhile, intracellular accumulation of adenosine because of placental-specific deficiency of adenosine deaminase (ADA) leads to elevation of extracellular adenosine. As such, increased placental adenosine underlies preeclampsia by dual mechanisms: elevated extracellular adenosine–mediated ADORA2B activation and intracellular adenosine–mediated DNA hypomethylation collaboratively work together to alter gene expression associated with preeclampsia. SAH indicates S-adenosylhomocysteine; and SAM, S-adenosylmethionine.
Emerging evidence shows that global DNA hypomethylation occurs in the placentas of patients with preeclampsia, in particular early-onset preeclampsia. These epigenetic changes are believed to affect placental gene expression and in this way contribute to the pathophysiology of preeclampsia. However, specific molecules and cell types underlying placental DNA hypomethylation in preeclampsia remain unclear. Forty years ago, in vitro studies showed that elevated intracellular adenosine leads to DNA hypomethylation in immune cells. More recent mouse studies showed that increased intracellular adenosine, resulting from genetic deletion of adenosine kinase, reverses elevated global DNA hypermethylation in specific neuronal cells of mouse models of epileptic disease.

We demonstrated that genetic deletion of placental ADA specifically in placentas of ADA-deficient fetuses restored placental adenosine to normally low levels, attenuated pathological consequences of excessive ADORA2B signaling, and restored placental DNA hypomethylation. We therefore believe that elevated placental adenosine–mediated DNA hypomethylation and how adenosine-mediated DNA hypomethylation collaboratively work together to alter placental gene expression associated with preeclampsia. Our findings support a novel working model: CD73-mediated elevated extracellular adenosine leads to increased intracellular adenosine mediated through uptake by ENTs. Moreover, elevation of adenosine mediated by placental-specific deficiency in ADA leads to increased intracellular and extracellular adenosine via bidirectional transport across ENTs. Escudero et al. reported that ADORA2B receptor activation may down-regulate hENT1 activity in human placental microvascular endothelial cells. However, our early studies showed no difference in mRNA levels of either Ent1 or Ent2 between normal and preeclamptic human and mouse placentae. This difference is likely because placental microvascular endothelial cells studied by Escudero et al. presumably represent a much smaller population of cells in the placenta compared with the trophoblast cells. Extracellular adenosine signaling via trophoblast ADORA2B contributes to pathophysiology of preeclampsia by promoting increased expression of genes encoding HIF-1α (hypoxia inducible factor-1 alpha), Flt-1 (fms like tyrosine kinase-1), ADORA2B, and CD73. Meanwhile, increased intracellular adenosine promotes DNA hypomethylation in the preeclampsia placentas, and this effect is independent of ADORA2B signaling. In particular, adenosine-mediated DNA hypomethylation in CpG islands of promoter regions likely causes altered placental gene expression associated with preeclampsia (Figure 6C). Thus, our findings raise a novel but compelling concept that elevated placental extradstitial adenosine–mediated ADORA2B activation and intracellular adenosine–mediated DNA hypomethylation collaboratively work together to alter placental gene expression associated with preeclampsia. Our findings stimulate multiple important future research directions, including identification of specific genes whose expression is altered by elevated adenosine-mediated DNA hypomethylation and how adenosine-mediated DNA hypomethylation contributes to the pathophysiology of preeclampsia.

**Perspectives**

Our current findings add significant new insight to poorly understood epigenetic changes associated with preeclampsia by showing that elevated placental adenosine functions intracellularly causing DNA hypomethylation in spongiotrophoblasts and labyrinthine trophoblasts in distinct experimental models of preeclampsia. Our previous studies revealed the detrimental consequences of excess placental adenosine via ADORA2B activation. Thus, excess placental adenosine associated with preeclampsia may be harmful in 2 ways: DNA hypomethylation with associated changes in gene expression and the detrimental consequences of excessive ADORA2B signaling. Our findings suggest that the use of PEG-ADA to lower adenosine, CD73 inhibitors to reduce adenosine synthesis, and nosine, CD73 inhibitors to reduce adenosine synthesis, and
and selective ADORA2B antagonists to reduce receptor signaling may provide helpful pharmacological intervention for preeclampsia.

Sources of Funding
This work was supported by National Institute of Health Grants 1R01DK096566 and R01DK094722. Additional support was provided by the Vascular Biology Training Program Grant T32HL007336 to N. Siddiqui and P. Mylonakis. The content of this publication does not reflect the views of the National Institutes of Health. This work was supported by National Institute of Health Grants R01DK094722 and T32HL007336.


---

**Novelty and Significance**

**What Is New?**
- Elevated placental adenosine underlies DNA hypomethylation in spongiosotrophoblasts and labyrinthine trophoblasts in 2 distinct preeclampsia models and in cultured human trophoblasts. This effect is independent of ADORA2B activation.

**What Is Relevant?**
- Lowering elevated placental adenosine by polyethylene glycol–modified adenosine deaminase, a Food and Drug Administration approved drug, is a potential treatment for preeclampsia by lowering extracellular adenosine–induced ADORA2B activation and intracellular adenosine–induced placental DNA hypomethylation and preeclampsia features. However, the use of adenosine lowering strategies should be used with caution because the reduction of circulating adenosine, a well-known vasodilator, may have hypertensive consequences.

---

**Summary**

Elevated intracellular adenosine–mediated DNA hypomethylation and extracellular adenosine–mediated A2B adenosine receptor activation collaboratively and cooperatively work together to promote preeclampsia. Targeting adenosine-mediated A2B adenosine receptor activation and intracellular adenosine–mediated DNA hypomethylation are likely effective therapeutic strategies for preeclampsia.
Elevated Adenosine Induces Placental DNA Hypomethylation Independent of A2B Receptor Signaling in Preeclampsia

Aji Huang, Hongyu Wu, Takayuki Iriyama, Yujin Zhang, Kaiqi Sun, Anren Song, Hong Liu, Zhangzhe Peng, Lili Tang, Minjung Lee, Yun Huang, Xin Ni, Rodney E. Kellems and Yang Xia

Hypertension. 2017;70:209-218; originally published online May 15, 2017;
doi: 10.1161/HYPERTENSIONAHA.117.09536

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2017 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/70/1/209

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/