Elevated Placental Adenosine Signaling Contributes to the Pathogenesis of Preeclampsia

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Background—Preeclampsia is a prevalent hypertensive disorder of pregnancy and a leading cause of maternal and neonatal morbidity and mortality worldwide. This pathogenic condition is speculated to be caused by placental abnormalities that contribute to the maternal syndrome. However, the specific factors and signaling pathways that lead to impaired placentas and maternal disease development remain elusive.

Methods and Results—Using 2 independent animal models of preeclampsia (genetically engineered pregnant mice with elevated adenosine exclusively in placentas and a pathogenic autoantibody-induced preeclampsia mouse model), we demonstrated that chronically elevated placental adenosine was sufficient to induce hallmark features of preeclampsia, including hypertension, proteinuria, small fetuses, and impaired placental vasculature. Genetic and pharmacological approaches revealed that elevated placental adenosine coupled with excess A2b adenosine receptor (ADORA2B) signaling contributed to the development of these features of preeclampsia. Mechanistically, we provided both human and mouse evidence that elevated placental CD73 is a key enzyme causing increased placental adenosine, thereby contributing to preeclampsia.

Conclusions—We determined that elevated placental adenosine signaling is a previously unrecognized pathogenic factor for preeclampsia. Moreover, our findings revealed the molecular basis underlying the elevation of placental adenosine and the detrimental role of excess placental adenosine in the pathophysiology of preeclampsia, and thereby, we highlight novel therapeutic targets. (Circulation. 2015;131:730–741. DOI: 10.1161/CIRCULATIONAHA.114.013740.)

Key Words: adenosine ■ hypertension ■ models, animal ■ preeclampsia ■ pregnancy

Preeclampsia is a gestation-specific syndrome with a high incidence of mother and infant morbidity and mortality worldwide.1 For years, the diagnosis has been made solely by the detection of sudden-onset hypertension and proteinuria. Despite intensive research efforts, current strategies for managing preeclampsia are inadequate and limited to symptomatic therapy or the termination of pregnancy, because the pathogenesis of the disease remains elusive.2

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The placenta is a newly formed organ that links mother and fetus throughout pregnancy.3 It plays an important role to support intrauterine fetal growth by facilitating the transfer of nutrients and oxygen from the mother to the fetus and by removing fetal waste products.4,5 Additionally, the placenta is an endocrine organ that synthesizes and secretes multiple hormones, neurotransmitters, and vasoactive factors.5,6 Impairment in placental development and function is considered to contribute to the pathogenesis of preeclampsia.1,7 Considerable evidence indicates that dysregulation of cytoprotective pathways6,10 and an increase in antiangiogenic growth factors11,12 complement activation,13 and autoantibodies14 contribute to placental damage and the progression of the disease. However, the placenta-specific molecular basis responsible for placental impairment that leads to preeclampsia has not been fully understood. Here, we sought to identify...
novel pathogenic factors linking placental pathology to the development of preeclampsia.

Adenosine is a key signaling molecule that orchestrates the cellular response to hypoxia, energy depletion, and tissue damage by activation of G-protein–coupled receptors on multiple cell types. Extracellular adenosine levels are tightly regulated by multiple factors involved in the synthesis from ATP by the sequential action of 2 ectonucleotidases (CD39 and CD73), degradation by adenosine deaminase (ADA), and cellular uptake by equilibrative nucleoside transporters (ENTs). Extracellular adenosine exerts its function through the activation of 4 G-protein–coupled cell surface receptors: ADORA1, ADORA2A, ADORA2B, and ADORA3. Acutely elevated adenosine signaling is intended to be brief and beneficial. The response is normally time limited because of the short half-life of adenosine. In contrast, chronically elevated adenosine is detrimental and is associated with multiple pathological conditions, including chronic kidney disease, pulmonary fibrosis, priapism, and sickle cell disease. Intriguingly, previous studies have reported that levels of adenosine are elevated in the maternal or fetal circulation of patients with preeclampsia compared with normal pregnant women and are correlated to disease severity. Another earlier study showed that elevated adenosine in patients with preeclampsia is correlated to Th1/Th2 imbalance. In vitro studies indicate that elevated adenosine is related to increased platelet aggregation and P-selectin expression. More recent reports demonstrate that adenosine is capable of inducing production of soluble fms-like tyrosine kinase-1 (sFlt-1) in rat villous explants. However, the role of elevated adenosine in the pathophysiology of preeclampsia remained unknown and cannot be fully understood using in vitro cell and organ culture systems. Thus, in vivo animal studies are needed to accurately and fully understand whether elevated adenosine signaling contributes to the pathogenesis of preeclampsia.

To address this question, we sought to (1) generate pregnant animals specifically with elevated placental adenosine, (2) determine the pathophysiological roles of elevated placental adenosine in preeclampsia, and (3) delineate the molecular basis for its elevation in preeclampsia in mice and humans. Here, we provide both mouse and human evidence that excess placental adenosine coupled with the enhanced ADORA2B signaling contributes to the pathogenesis of preeclampsia. Mechanistically, we discovered that elevated placental CD73 is a key enzyme responsible for increased placental adenosine production and thereby contributes to the development of preeclampsia.

**Methods**

For an expanded Methods sections, please refer to the online-only Data Supplement.

**Animals**

Fetal liver rescued ADA-deficient mice (Ada−/−/fLi-Tg+ mice) were generated by introducing an ADA minigene (fLi-Tg) that is only expressed in the fetal liver under the control of α-fetoprotein (Figure 1 in the online-only Data Supplement). Placental rescued ADA-deficient mice (Ada−/−/Pl-Tg+ mice) equipped with an Ada minigene (Pl-Tg) that is expressed exclusively in the trophoblast cell lineage were generated and genotyped as described previously. Details are provided in the online-only Data Supplement.

**Patients**

Patients admitted to Memorial Hermann Hospital were identified by the obstetrics faculty of the University of Texas Medical School at Houston. Preeclampsia patients diagnosed based on the definition set by the National High Blood Pressure Education Program Working Group were included in the study. Human subject data were summarized and included in Table I in the online-only Data Supplement. The research protocol was approved by the Institutional Committee for the Protection of Human Subjects, and informed consent was obtained from all the patients.

**Statistical Analysis**

All data are expressed as mean±SEM. Mann-Whitney U test was applied in 2-group analysis. Differences among multiple groups were compared by the Kruskal-Wallis test, followed by a Dunn post hoc test. Comparisons of the data obtained at different time points were analyzed by the Fisher exact test. Statistical significance was set as P<0.05 and analyzed by GraphPad Prism 5 (GraphPad).

**Results**

**Pregnant Mice With Elevated Placental Adenosine Display Spontaneously Developed Hallmark Features of Preeclampsia**

To examine the pathological consequences of excessive placental adenosine, we needed an experimental approach to generate pregnant mice with placenta with elevated adenosine. One approach is to generate placentas that lack ADA, the enzyme that irreversibly degrades adenosine to inosine. However, global ADA-deficient mice die late in gestation as a result of impaired liver function. To circumvent this complication and to produce viable embryos with excessive placental adenosine, we genetically rescued ADA-deficient embryos from prenatal lethality by introducing an ADA minigene that is only expressed in the fetal liver (for details, see Methods and Figure 1A and 1B in the online-only Data Supplement). Next, we took advantage of fetal liver rescued ADA-deficient mice (Ada−/−/fLi-Tg+) to assess the impact of elevated placental adenosine throughout pregnancy in vivo. Specifically, we designed a mating strategy by crossing Ada−/−/fLi-Tg+ males with Ada++/fLi-Tg+ females to generate pregnant mice in which half of the placentas were expected to be ADA deficient and have elevated adenosine (dams with elevated placental adenosine; Figure 1A). Dams were positive for ADA, and fetuses expressed ADA activity only in the fetal liver (Figure 1A; Figure 1C and 1D in the online-only Data Supplement). In control crosses of Ada++/fLi-Tg+ females with Ada−/− males, all placentas were ADA positive (control dams). We found that placental adenosine was significantly elevated in the ADA-negative placentas compared with the ADA-positive placentas on embryonic day 12.5 (E12.5) and remained elevated through E18.5 (Figure 1B). In contrast, the placentas in control dams with either Ada−/− or Ada++ genotype contained similar levels of adenosine in the normal range on E18.5 (Figure 1B, open bar). Correspondingly, we confirmed that ADA protein
and enzymatic activity were not detected in Ada−/− placentas, whereas ADA-positive placentas with Ada+/− or Ada+/+ genotype displayed ADA enzymatic activity (Figure IC and ID in the online-only Data Supplement). Additionally, because dams with elevated placental adenosine and control dams were both positive for ADA expression (maternal genotype ADA+/−), there was no increase in adenosine levels in the maternal circulation of pregnant mice harboring ADA-negative placentas compared with control dams with all ADA-positive placentas (Figure 1C). Thus, fetal liver rescued ADA-deficient mice provided us a genetic investigative tool to assess the impact of elevated placental adenosine throughout pregnancy in vivo.
We first monitored hallmark features of preeclampsia in dams with elevated placental adenosine and control dams and found that dams with elevated placental adenosine displayed a significant increase in mean systolic blood pressure late in pregnancy beginning on E15.5, which remained significantly elevated through E17.5 compared with the control dams. Elevated systolic blood pressure returned to normal by day 5 postpartum (Figure 1D). To validate the tail cuff measurement of blood pressure, we conducted invasive measurement of mean arterial pressure of the carotid artery. We found that mean arterial pressure was significantly elevated on E18.5 in dams with elevated placental adenosine compared with the control dams (Figure 1E). Additionally, we found that urinary protein (ratio of albumin/creatinine) was not increased significantly in the dams with elevated placental adenosine on E12.5, whereas it was significantly elevated on E18.5 (Figure 1F). By postpartum day 7, proteinuria was reduced to levels similar to that of the nonpregnant state (Figure 1F). Interestingly, Ada<sup>−/−</sup>/fLi-Tg<sup>+</sup> dams crossed with Ada<sup>−/−</sup>/fLi-Tg<sup>+</sup> males, in which 25% of the placentas are expected to be ADA negative and have elevated adenosine, did not display an increase in blood pressure or proteinuria (data not shown), which suggests that there is a threshold for the number of ADA-deficient placentas with excess placental adenosine to trigger maternal features of preeclampsia. Histological studies revealed pathological changes in the kidneys including swollen glomeruli with narrowed capillary and Bowman’s spaces in the pregnant mice with elevated placental adenosine (Figure IIA and IIB in the online-only Data Supplement). Furthermore, electron microscopic studies revealed that glomeruli in dams with elevated placental adenosine showed the typical pathological change “glomerular endotheliosis” as seen in patients with preeclampsia (Figure 1G). Thus, our findings showed that elevation of placental adenosine occurs before the onset of maternal preeclampsia features and suggest that elevated placental adenosine contributes to the pathogenesis of preeclampsia.

### Mice With Elevated Placental Adenosine Present With Impaired Placental Vasculature and Fetal Growth Restriction

In addition to maternal preeclampsia features, we found that ADA-deficient placentas with elevated adenosine were smaller and weighed significantly less than ADA-positive placentas with normal levels of adenosine from dams with preeclampsia features (Table II in the online-only Data Supplement). Likewise, the fetuses associated with ADA-deficient placentas with elevated placental adenosine were smaller and weighed significantly less than fetuses associated with ADA-positive placentas without morphological abnormalities (Figure 2A; Table II in the online-only Data Supplement). To further validate our findings, we compared fetal and placental weights with those observed in control dams. We found that ADA-negative placentas and their associated fetuses from the preeclampsia dams with elevated placental adenosine were significantly smaller and weighed less than those from the control dams.

Histological analysis of placentas with CD31 staining revealed that ADA-negative placentas showed disorganized and impaired vasculature in the labyrinthine zone compared with ADA-positive placentas (Figure 2B). Semiquantification of CD31 staining demonstrated the CD31-positive vessels were significantly reduced in Ada<sup>−/−</sup> placentas with elevated adenosine (Figure 2C). Supporting this finding, we found that ADA-deficient placentas with elevated adenosine...
contained significantly elevated Flt-1 mRNA compared with ADA-positive placentas (Figure 2D). Accordingly, we found that maternal circulating sFlt-1 levels in dams with elevated placental adenosine were significantly higher than those of the control dams (Figure 2E). These studies provide genetic evidence that increased placental adenosine is associated with small fetuses and small placentas that feature impaired vasculature and increased Flt-1 gene expression.

Elevated Placental Adenosine Contributes to the Onset of Maternal Preeclampsia Features, Impaired Placentas, and Small Fetuses in Pregnant Mice

Next, to determine whether elevated placental adenosine causes placental impairment, small fetuses, and maternal preeclampsia features, we used a transgenic approach to genetically restore ADA exclusively to the placentas of ADA-deficient mice to lower placental adenosine (genotype Ada−/−/PL-Tg+; Figure 3A).26,27 We found that ADA enzyme activity was only observed in the placentas and was absent in fetal organs of Ada−/−/PL-Tg+ mice (Figure 3B). More importantly, the restoration of ADA to placentas completely restored placental adenosine levels to the normal range (Figure 3C, gray bar) compared with the placenta from fetal liver rescued dams with elevated placental adenosine (Figure 3C, black bar) on E18.5. Consequently, placental and fetal weights were increased significantly compared with the dams with elevated placental adenosine (Table II in the online-only Data Supplement). Moreover, when ADA was expressed only in the placentas, it restored the normal placental vasculature and returned placental Flt-1 gene expression to levels found in controls (Figures III and IV A in the online-only Data Supplement) and abolished elevated maternal circulating sFlt-1 levels (Figure IVB in the online-only Data Supplement), hypertension, proteinuria (Figure 3D and 3E), and kidney histopathological changes (Figure V in the online-only Data Supplement). Altogether, these results provide strong genetic evidence that elevated placental adenosine is associated with impaired placental vasculature, fetal growth restriction, and maternal features of preeclampsia.

Next, we performed pharmacological studies using polyethyleneglycol-ADA (PEG-ADA) enzyme therapy to prevent the accumulation of adenosine in Ada−/− placentas. We found that PEG-ADA treatment significantly reduced placental adenosine levels in these mice (Figure 3C). PEG-ADA treatment significantly ameliorated all the features observed in dams with elevated placental adenosine, including impaired placentas (Figure III in the online-only Data Supplement), maternal preeclampsia features (Figure 3D and 3E; Figure IV in the online-only Data Supplement), and renal pathological changes (Figure V in the online-only Data Supplement). Taken together, we provide both genetic and pharmacological evidence that elevated placental adenosine underlies impaired placental vasculature, fetal growth restriction, and maternal preeclampsia features in mice.

Genetic Deletion of the A2B Adenosine Receptor (ADORA2B) Prevents Elevated Adenosine-Induced Placental Impairment, Fetal Growth Restriction, and Maternal Features of Preeclampsia

To determine which adenosine receptor may be responsible for elevated adenosine-induced placental impairment, small fetuses, and
maternal preeclampsia features, we determined the gene expression profiles of the 4 adenosine receptors in placentas with normal and elevated adenosine. We found that among adenosine receptor genes, only Adora2b gene expression was increased significantly in ADA-deficient placentas with elevated adenosine compared with ADA-positive placentas (Figure 4A). This result suggests that elevated ADORA2B signaling may mediate the development of preeclampsia features in dams with elevated placental adenosine. To test this possibility, we generated mice deficient in Adora2b on the background of fetal liver rescued ADA-deficient mice (Ada−/−/fLi-Tg+/Adora2b−/− mice; Figure 4B; Methods in the online-only Data Supplement). We found that genetic deletion of Adora2b did not affect the levels of elevated placental adenosine compared with controls (Figure 4C). However, genetic deletion of Adora2b resulted in a significant increase in placental and fetal weight (Table II in the online-only Data Supplement). CD31 staining showed that the placental vasculature was more organized and uniform in Adora2b−/− placentas with elevated adenosine (Figure III in the online-only Data Supplement). Additionally, all the preeclampsia features observed in dams with elevated placental adenosine, including hypertension and proteinuria (Figure 4D and 4E), increased in maternal circulating sFlt-1 (Figure IVB in the online-only Data Supplement), and renal pathological changes (Figure V in the online-only Data Supplement) were ameliorated by the genetic deletion of Adora2b. Thus, we provided genetic evidence that excess placental adenosine coupled with enhanced ADOAR2B signaling is responsible for maternal preeclampsia features, impaired placentas, and subsequent fetal growth restriction.

Adenosine Levels and ADORA2B Expression Are Significantly Increased in the Placentas of Patients With Preeclampsia, and Elevated ADORA2B Signaling DirectlyInduces sFlt-1 Production

To examine the translational relevance of our mouse findings to human pregnancy, we first measured adenosine levels in
the placentas of normotensive pregnant women and patients with preeclampsia. We found that adenosine levels were significantly elevated in the placentas of women with preeclampsia compared with placentas from normotensive pregnant women (Figure 5A). Similarly, ADORA2B gene expression and protein level were significantly elevated in the preeclampsia placentas compared with the normotensive placentas (Figure 5B and 5C).

To test whether elevated ADORA2B signaling may contribute to features of preeclampsia in humans, we conducted experiments using human placental villous explants cultured from normotensive placentas. First, we found that NECA (5′-N-ethylcarboxamide adenosine), a nonmetabolized adenosine analogue, significantly induced FLT-1 gene expression and that the stimulation of FLT-1 gene expression was attenuated by an ADORA2B-specific antagonist (MRS1754; Figure 5D). Moreover, we found that the ADORA2B-specific agonist, BAY60-6583, significantly induced FLT-1 gene expression (Figure 5E) and sFlt-1 secretion (Figure 5F) under normoxic conditions in the villous explants. These results translate our mouse findings to human pregnancy by showing that (1) placental adenosine levels are increased in patients with preeclampsia, and (2) elevated adenosine signaling via ADORA2B contributes to elevated Flt-1 gene expression and subsequent sFlt-1 secretion from human villous explants.

**CD73 Is Elevated in Placentas From Patients With Preeclampsia**

Next, to examine the molecular mechanisms underlying the elevation of placental adenosine in patients with preeclampsia, we initially assessed the possible involvement of ADA, in contrast to mouse placentas, ADA enzyme activity was extremely low in the normal human placentas, with no significant difference in enzyme activity between the placentas of normotensive pregnant women and those of patients with preeclampsia (Figure 6A). Thus, we concluded that reduction in ADA levels is not a major factor contributing to increased placental adenosine in patients with preeclampsia.

In an effort to identify the factors causing elevated adenosine in the placentas of patients with preeclampsia, we conducted expression profiling of genes encoding purinergic proteins. Among the genes screened, we found that mRNA encoding CD73, a key ectonucleotidase producing extracellular adenosine from AMP, was significantly elevated in placentas of patients with preeclampsia (Figure 6B). Additionally, we found that the protein level and enzyme activity of CD73 were significantly increased in placentas of patients with preeclampsia (Figure 6C and 6D). In contrast, the mRNA levels encoding CD39 (an ectonucleotidase that converts ATP to AMP) and ENT1 and ENT2 (adenosine transporters) showed no significant difference between normotensive and preeclampsia placentas (Figure VI in the online-only Data Supplement). Thus, analysis of human placentas indicated that elevated CD73 is responsible for increased placental adenosine and subsequent disease development.

**Elevated CD73 Underlies Increased Placental Adenosine and Contributes to Pathophysiolog of Preeclampsia via ADORA2B Activation in an Experimental Model of Preeclampsia**

To test the hypothesis that elevated placental adenosine in patients with preeclampsia is caused by elevated
CD73-mediated production of adenosine, we took advantage of an established experimental mouse preeclampsia model induced by transfer of preeclampsia patient–derived IgG (PE-IgG) known to contain the pathogenic autoantibodies (termed AT1-AA) that activate the angiotensin II type 1 receptor. As with patients with preeclampsia, we found that placental CD73 activity and adenosine levels were significantly increased in PE-IgG–injected dams compared with normotensive pregnant women–derived IgG (NT-IgG)–injected dams (Figure 7A and 7B). In contrast, adenosine levels were not increased in the maternal circulation nor in the maternal kidneys of PE-IgG–injected dams compared with dams injected with NT-IgG (Figure VIIA and VIIIB in the online-only Data Supplement). Similar to human studies, gene expression analysis revealed that Cd73 mRNA and protein levels were increased significantly in the placentas of PE-IgG–injected dams (Figure 7C, Figure VIII in the online-only Data Supplement). Additionally, we found that among adenosine receptors, only Adora2b mRNA was significantly elevated in the placentas of PE-IgG–injected mice (Figure 7D). Placental expression of Ada, Cd39, and Ent genes showed no significant difference between NT-IgG– and PE-IgG–treated dams (Figure VI in the online-only Data Supplement).

Next, to determine whether the increased expression of Cd73 was responsible for PE-IgG–induced placental adenosine production and the pathogenesis of preeclampsia, we injected PE-IgG into Cd73-deficient dams (Cd73−/− females mated with Cd73−/− males). Five days after PE-IgG injection, we found that CD73 deficiency resulted in significantly reduced PE-IgG–induced production of adenosine in the placentas (Figure 7A), which indicates that elevated CD73 was required for the increase of placental adenosine induced by PE-IgG. Additionally, we found that the key diagnostic features of preeclampsia, hypertension and proteinuria, were significantly attenuated in Cd73−/− dams injected with PE-IgG compared with PE-IgG–injected wild-type dams (Figure 7E and 7F; Figure IX in the online-only Data Supplement). In addition, we found that levels of maternal circulating sFlt-1 induced by PE-IgG were significantly suppressed in Cd73−/− dams compared with those in PE-IgG–injected wild-type dams (Figure 7G). Thus, this study revealed that elevated CD73 is essential for increased placental adenosine and that excess adenosine is responsible for disease development in the pathogenic autoantibody-induced animal model of preeclampsia.

Finally, to determine whether elevated Adora2b expression in the placentas has pathophysiological significance, we injected PE-IgG into Adora2b−/− dams (Adora2b−/− females mated with Adora2b−/− males). We found that all the preeclampsia features, including hypertension, proteinuria, and elevation of circulating sFlt-1, that were observed in PE-IgG–injected wild-type dams were significantly suppressed in Adora2b−/− dams (Figure 7E, 7F, and 7G; Figure IX in the online-only Data Supplement). These results provide genetic evidence that CD73-mediated chronically elevated adenosine in placentas exerts its detrimental effects in PE-IgG–injected pregnant mice thorough enhanced ADORA2B signaling.

**Discussion**

The involvement of adenosine signaling in the pathogenesis of preeclampsia was unknown before the present study. Here, we report the use of genetic approaches to successfully generate pregnant mice with elevated adenosine only in placentas. We demonstrated the pathogenic role of elevated placental adenosine signaling via excessive ADOAR2B signaling, which induces placental impairment associated with sFlt-1 induction, small fetuses, and features of maternal preeclampsia. Moreover, we have provided both mouse and human findings that elevated CD73 is a key enzyme underlying increased placental adenosine.
and subsequent disease development. Overall, our findings reveal the pathogenic consequences of chronically elevated placental adenosine, the molecular basis for its elevation, and the specific signaling pathways leading to clinical features of preeclampsia.

Preeclampsia is commonly considered to result from impaired placental development secondary to hypoxia or other hypoxia-independent mediators, including cytokines, complement, and autoantibody. Adenosine is a key signaling molecule that orchestrates a rapid multicellular...
physiological response to hypoxia or tissue damage. However, certain disease states are associated with chronically elevated adenosine, and the resulting persistent adenosine signaling is detrimental. A potentially detrimental role for adenosine signaling in preeclampsia placentas was suggested by a recent study showing that adenosine stimulates increased sFlt-1 production in cultured rat placental villous explants. To the best of our knowledge, in vivo evidence for a role of elevated placental adenosine signaling in preeclampsia had not been reported previously until we observed hallmark features of preeclampsia in pregnant mice with excessively accumulated placental adenosine attributable to an absence of placental ADA. It is interesting to note that elevated placental adenosine occurs on E12.5, before the maternal symptoms (ie, hypertension and proteinuria), which develop around E15.5 and disappear postpartum. Thus, our studies provide strong in vivo evidence that elevated placental adenosine is a causative factor to induce maternal preeclampsia features. The detrimental role of elevated placental adenosine in preeclampsia is also supported by another animal model of preeclampsia based on the injection of pathogenic autoantibodies purified from patients with preeclampsia. Altogether, in 2 independent animal models of preeclampsia, one with placental ADA deficiency and another with injection of pathogenic autoantibodies, we demonstrated the common pathogenic role of chronically elevated placental adenosine in preeclampsia in vivo. Our mouse findings were further validated in humans by showing that placental adenosine levels were also significantly elevated in women with preeclampsia. Thus, we have provided significant mouse and human evidence that elevated placental adenosine contributes to the pathogenesis of preeclampsia.

Extracellular adenosine levels are regulated by multiple factors involved in the synthesis (CD73), degradation (ADA), and cellular uptake (ENTs) of adenosine. Our initial mouse studies created placentas with elevated adenosine using genetic strategies to create ADA-deficient placentas. Previous studies demonstrated that placental and circulating ADA activities were quite low and that slightly higher levels of circulating and placental ADA activity were observed in patients with preeclampsia. Altogether, in 2 independent animal models of preeclampsia, we found that expression and activity of CD73, a key enzyme responsible for the extracellular synthesis of adenosine from AMP, were significantly elevated in the placentas of women with preeclampsia. Similar to these findings in women with preeclampsia, we found that an autoantibody-injection model of preeclampsia in mice also displayed increased levels of placental adenosine and CD73. The pathological significance of increased placental CD73 and adenosine was demonstrated by experiments showing that genetic deletion of Cd73 prevented PE-IgG–induced placental adenosine production and attenuated hypertension, proteinuria, and sFlt-1 secretion in the pregnant mice. Thus, elevated CD73, not reduced ADA, underlies increased placental adenosine production and subsequent disease development.

In contrast to the work of Espinoza et al, which focuses on elevated fetal plasma adenosine, and the previous work of others who demonstrated elevated adenosine in the maternal circulation, our studies reported here focus on the detrimental consequences of locally elevated adenosine in the placenta. Additionally, our results with 2 mouse models of preeclampsia (genetically engineered mice with ADA-deficient placentas and PE-IgG-injected pregnant mice) show that the elevation of placental adenosine was not accompanied by an elevated level of maternal circulating adenosine. Thus, our findings indicate that a local increase in adenosine in the placenta is sufficient to trigger features of preeclampsia in 2 different mouse models of preeclampsia. As a limitation of human evidence for elevated placenta adenosine, the determination of placental adenosine levels during human pregnancy before the onset of disease is not feasible. Nevertheless, our finding imply that elevated circulating adenosine observed in patients with preeclampsia may be secondary to an initial increase in placental adenosine and in this way may serve as a presymptomatic biomarker for preeclampsia.

The metabolic consequences of elevated adenosine include intracellular accumulation of S-adenosylhomocysteine, a potent inhibitor of many cellular methyltransferase enzymes, including those that methylate DNA. For this reason, elevated adenosine is associated with DNA hypomethylation. It is likely that the elevated placental adenosine in women with preeclampsia, and in placentas from our mouse models of preeclampsia, contributes to DNA hypomethylation and changes in gene expression. However, the pathophysiological consequences of elevated placental adenosine that we report here are mediated through ADORA2B receptor signaling, a process distinct from the metabolic consequences of elevated adenosine on methyltransferase reactions. Adenosine is a signaling nucleoside that activates 4 receptors: ADORA1, ADORA2A, ADORA2B, and ADORA3. In 2 independent animal models of preeclampsia, we demonstrated that excess placental adenosine signaling via elevated ADORA2B underlies the pathophysiology of preeclampsia. Moreover, the significance of ADORA2B signaling in humans was evident from translational studies showing that ADORA2B levels were elevated in placentas of patients with preeclampsia relative to control subjects and that enhanced ADORA2B signaling contributed to elevated sFlt-1 production in human placental villous explants.

Our studies support a novel but compelling concept of the pathogenesis of preeclampsia: (1) Elevated CD73 underlies increased placental adenosine; and (2) chronic excess placental adenosine preferentially signaling via elevated ADORA2B induces sFlt-1 production, impaired placentas, small fetuses, and maternal preeclampsia features. Without interference, placental damage caused by elevated sFlt-1–mediated impaired vasculature leads to further elevation of adenosine. As such, elevated CD73-mediated elevated placental adenosine, enhanced ADORA2B activation, and placental vasculature...
impairment function as a malicious cycle to promote the progression of maternal disease development by continuously inducing local placental accumulation of adenosine (Figure 7H). These findings suggest multiple possible therapeutic options for the treatment of preeclampsia, including the use of PEG-ADA to reduce adenosine levels, an ADORA2B antagonist to specifically inhibit the detrimental adenosine signaling, or a CD73 inhibitor to prevent the excess production of adenosine. In particular, PEG-ADA is a US Food and Drug Administration–approved drug that has been used successfully to treat ADA-deficient patients for several decades. It is our hope that the use of adenosine-based therapies to prevent features of preeclampsia that result from excessive placental adenosine signaling will reduce the morbidity and mortality of preeclampsia in humans in the future.

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CLINICAL PERSPECTIVE

Preeclampsia is a gestation-specific hypertensive syndrome with a high incidence of mother and infant morbidity and mortality worldwide. Despite intensive research efforts, current strategies for managing preeclampsia are inadequate and limited to symptomatic therapy or the termination of pregnancy, because the pathogenesis of the disease remains elusive. Impairment in placental development and function has been considered to contribute to the pathogenesis of preeclampsia; however, the placenta-specific molecular basis responsible for placental impairment leading to preeclampsia has not been fully understood. Here, we report the use of genetic approaches to successfully generate pregnant mice with elevated adenosine only in placentas. We demonstrate the pathogenic role of elevated placental adenosine signaling via excessive A2B adenosine receptor (ADORA2B) signaling, which induces placental impairment associated with induction of soluble fms-like tyrosine kinase-1 (sFlt-1), small fetuses, and maternal preeclampsia features. Moreover, we have provided both mouse and human findings that elevated CD73 is a key enzyme underlying increased placental adenosine and subsequent disease development. Overall, our findings reveal the pathogenic consequences of chronically elevated placental adenosine, the molecular basis for its elevation, and the specific signaling pathways leading to clinical features of preeclampsia. It is our hope that the use of adenosine-based therapies to prevent features of preeclampsia resulting from excessive placental adenosine signaling will reduce the morbidity and mortality of preeclampsia in humans in the future.
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Elevated placental adenosine signaling contributes to the pathogenesis of preeclampsia


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Supplemental Methods

**Generation and characterization of fetal liver rescued ADA-deficient mice.** *Construction of transgene.* The use of an ADA minigene under control of α-fetoprotein gene regulatory elements was attempted based on the assumption that expression of ADA in the fetal liver would also protect ADA-deficient fetuses from perinatal lethality. For this purpose the 3.2-kb mouse alpha-fetoprotein promoter (AFP), containing enhancer I, which confers tissue- and developmental stage-specific expression of alpha-fetoprotein, was excised from pAFP-CAT\(^1\) by AccI/PstI digestion. This restriction fragment was ligated to SmaI and PstI sites of pBluescript II KS. The 1.5-kb human wild-type ADA cDNA was removed from pADA211\(^2\) by EcoRI digestion, and ligated downstream from the AFP promoter at the EcoRI site of pBluescript II KS (Supplemental Figure 1A).

**Generation of fetal liver rescued ADA-deficient mice by a two stage genetic approach.** First stage, the recombinant plasmid was purified by equilibrium centrifugation on a cesium chloride density gradient. The SacI/ApaI fragment containing the transgene was fractionated on an agarose gel, electroeluted and purified using a Qiagen (Valencia, CA) mini-plasmid kit. The DNA fragment was injected into (C57BL/6J x C3H/HeJ) F1 eggs for transgenic mouse production using standard procedures. Ten founder mice, designated as fLi-Tg\(^+\), were derived. The mouse showing highest fetal liver expression of human ADA was selected. At the second stage, the selected fLi-Tg\(^+\) mice were mated with Ada\(^{+/−}\) mice, which are heterozygous for the null Ada allele and transgene-positive heterozygous mice were backcrossed to the Ada\(^{+/−}\) mice or intercrossed to derive Ada\(^{−/−}\)/fLi-Tg\(^+\) offspring (Supplemental Figure 1B). Under control of α-fetoprotein gene regulatory elements, the hepatic production of ADA subsides during the first week following birth and the resulting ADA-deficient mice die by three weeks of age if not
treated by adenosine deaminase enzyme replacement therapy as described below.

**Polymerase chain reaction for genotyping.** DNA was extracted from tail biopsies by the proteinase K/phenol/chloroform method. Primers for polymerase chain reaction (PCR) for detection of the transgene were 5’GAGCGGCATTACCCGTACTG and 5’TGACTGCATGACTCCGTGTCC, and for positive identification of the null and wild-type Ada alleles were 5’ACTAGTGAGACGTGCTACTT and 5’AGATCCACAACGTCATCAGG, and 5’AAGTGCGCTATAGCCCACAC and 5’AGATCCACAACGTCATCAGG, respectively. PCR reactions were carried out using the GeneAmp kit from PE BioSystems (Foster City, CA), according to the manufacturer’s instructions. PCR products were analyzed on a 2% NuSieve 3:1 gel (Schleicher and Schuell, Keene, NH).

**Western blot analysis for ADA proteins and zymogram for ADA activities.** The presence or absence of ADA in the fetal organs was readily determined by immunoblotting to detect ADA protein or zymogram analysis to detect ADA enzymatic activity. As shown in Supplemental Figure. 1C-D, ADA protein (Supplemental Figure 1C) and enzymatic activity (Supplemental Figure 1D) were only detected in fetal livers and not in any other fetal organs tested, including brain, kidney and skin (genotype Ada−/fLi-Tg+). Significantly, production of ADA exclusively in fetal liver was sufficient to rescue the fetuses from perinatal lethality and allowed viable fetuses to be delivered at term. Thus, we conclude that the AFP-driven human ADA transgene was able to provide sufficient ADA activity in fetal liver to prevent the perinatal lethal phenotype of the ADA-deficient mice. More importantly, fetal liver rescued ADA-deficient mice provided us a genetic investigative tool to produce pregnancies with ADA-deficient placentas allowing us to assess the impact of elevated placental adenosine throughout pregnancy in vivo.
Placental rescued ADA-deficient mice. ADA-deficient mice equipped with an Ada minigene that is expressed exclusively in the trophoblast cell lineage were generated and genotyped as previously described 3,4. Mice homozygous for the null Ada allele were designated as Ada+/Pl-Tg+ mice. The rationale for placenta specific ADA expression to rescue ADA-deficient mice from prenatal lethality is based on the fact that the highest levels of ADA during prenatal development are found in the trophoblast cells of the placenta 4. The perinatal lethality and associated liver impairment of the global Ada knockout is prevented by genetically restoring ADA production to the trophoblast cell lineage of the placenta 3. Following birth, and removal of the placenta, the resulting mice are completely ADA deficient and die within three weeks of age from severe pulmonary impairment due to excessive accumulation of adenosine. However, it is possible to keep the mice alive indefinitely by the use of ADA enzyme replacement therapy that prevents the cytotoxic accumulation of adenosine 5 (for details see in following section).

Maintenance of ADA-deficient mice by ADA enzyme replacement therapy. To prevent postnatal lethality ADA-deficient mice (both placental rescued and fetal liver rescued Ada−/− mice) were provided ADA enzyme replacement therapy in the form of polyethylene glycol-modified ADA (PEG-ADA) based on a previous report 5. Ada−/− mice were identified at birth by screening for ADA enzymatic activity in the blood by zymogram analysis and were maintained on i.m. injections of PEG-ADA on postnatal days 1, 5, 9, 13, and 17 (1.25, 2, 2.5, 3.75, and 5 U, respectively) as previously described 6. Beginning with postnatal day 21 Ada−/− mice were treated with 7.5 U PEG-ADA weekly by intraperitoneal injection.

Generation of ADA and ADORA2B double-deficient mice. Adora2b−/− mice were generated and genotyped as previously described 7. And then, mice deficient in ADORA2B on the background of fetal liver rescued ADA-deficient mice were generated by mating Adora2b+/− mice
with Ada⁺/fLi-Tg⁺ mice.

**Mating strategy of fetal liver rescued ADA-deficient mice, ADA and ADORA2B double-deficient mice, and placental rescued ADA-deficient mice.** To generate pregnant mice with elevated placental adenosine, we crossed 8-10 week-old Ada⁺/fLi-Tg⁺ females with Ada⁻/fLi-Tg⁺ males. By this mating strategy, we generated pregnant mice in which half of the placentas were ADA-deficient and half were ADA-positive. All fetuses expressed the ADA transgene in the fetal liver. In control crosses of Ada⁺/fLi-Tg⁺ females with Ada⁺/+ males, all placentas were ADA-positive. Similarly, Ada⁺/fLi-Tg⁺/Adora2b⁺/⁻ female mice and Ada⁺/+/PL-Tg⁺ female mice were mated with Ada⁺/fLi-Tg⁺/Adora2b⁺/⁻ male mice and Ada⁺/+/PL-Tg⁺ male mice, respectively. The day when the copulation plug was detected was designated as E0.5. DNA was extracted from tail biopsies of fetuses by the proteinase K/phenol/chloroform method and PCR was conducted with primers for the genotyping of each mice. The mice were housed in the animal care facility of the University of Texas, Houston and had access to food and water ad libitum. All the protocols involving animal studies were reviewed and approved by the Institutional Animal Welfare Committee of the University of Texas Houston Health Science Center.

**Introduction of human autoantibody (AT1-AA) into pregnant mice**

PE mouse models induced by AT1-AA were conducted as previously described⁸. Briefly, purified IgGs were isolated from PE patients or NT pregnant women sera (PE-IgG, NT-IgG respectively). And then, 8 to 10 week-old timed-pregnant wild type dams mated with wild type males or CD73-deficient (Cd73⁻⁻) dams mated with Cd73⁻⁻ males which were generated and genotyped as previously reported ⁹ or Adora2b⁻⁻ dams mated with Adora2b⁻⁻ males were treated
with NT-IgG or PE-IgG (0.8mg) on E13.5 and E14.5 by retro-orbital sinus injection. All mice were sacrificed on E18.5 prior to delivery, and their blood and organs were collected.

**The measurement of blood pressure and proteinuria in mice**

The systolic blood pressure of all 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). Mice were trained several times for at least one week prior to the actual measurement in the non-pregnant state. The intracarotid mean arterial blood pressure (MAP) was also measured on E18.5 as previously described \(^{10}\). MAP was monitored from the right carotid artery with a mouse jugular catheter connected to a pressure transducer and an amplifier unit. The amplifier was linked to a data acquisition module and MAP was recorded on a personal computer by Chart 5 Software (AD Instruments Inc). Blood pressure was recorded and averaged over a 10-min period. For the measurement of proteinuria, urine was collected for analysis using metabolic cages (Nalgene). Total microalbumin and creatinine in the urine were determined by using ELISA kit (Exocell) and the ratio of urinary albumin to creatinine was calculated as an index of proteinuria as previously described \(^{8,10}\).

**Measurement of adenosine levels**

Samples were rapidly removed and collected in liquid nitrogen. Adenosine was extracted from frozen placenta tissues or plasma using 0.6 N perchloric acid, separated and quantified using reversed phase HPLC as described previously \(^{10,11}\).

**Zymogram analysis of tissue ADA activity**

ADA activity detection in mouse tissue samples were performed by zymogram analysis as
previously described with several modifications. Briefly, tissue samples were lysed in lysis buffer containing 20 mM Tris, 1mM EDTA, 0.03% 2-mercaptoethanol and adjusted the protein concentration. 2 µl lysate was loaded to the G493 thin agarose gel films (Authentifilm thin agarose gel films, Innovative Chemistry, Marshfield, Massachusetts, USA) and subjected to electrophoresis at 70 V for 30 min in the running buffer (0.5 M Tris, 16 mM EDTA, 0.65 M boric acid, pH8) in a temperature-controlled electrophoresis chamber (Innovative Chemistry, Marshfield, Massachusetts, USA). Then, gel films were overlaid with a 10 ml solution containing adenosine (42.5 mg, Sigma), sodium arsenate (38 mg, Sigma), nitro blue tetrazolium (13 mg), Tris-HCl (pH 8, 0.68 ml of 1 M stock), purine nucleoside phosphorylase (2.4 units, CalBiochem), xanthine oxidase (0.2 unit, Sigma), phenazine methosulfate (0.8 mg, Sigma), and melted agar (9.2 ml of a 1% solution, Sigma) in water. The gels were allowed 30 min at 37ºC to develop intense purple bands signaling the presence of ADA activity.

**Spectrophotometric assay for the measurement of placental ADA activity**

Small pieces of mouse and human placentas were homogenized in ice-cold PBS with proteinase inhibitor cocktails (Roche Diagnostics) by sonication. Then, ADA activity was measured in the supernatant obtained from the high-speed centrifugation under saturating substrate conditions using a spectrophotometric assay as previously described. Briefly, approximate 990 µl assay buffer (12.5ml 0.1M K₂HPO₄, 5ml 0.1M KH₂PO₄, added distilled water to 50 ml) was added to 3 µl supernatant (30µg protein) and 10 µl 10mM adenosine, and was mixed immediately. The decrease in absorbance at 265 nm resulting from deamination of adenosine to inosine was continuously monitored in a Beckman DU-50 spectrophotometer and the rate of inosine production was calculated at linearity. Specific activities are presented as nanomoles of adenosine converted to inosine per min per mg of protein.
Real-time RT-PCR analysis

RNA isolation and real-time RT-PCR were conducted as previously described. Syber green was used for the analysis of all genes measured using the following primers: Mouse *Adora1*: forward; 5’-TGTGCCCGAAATGTACTGG-3’ and reverse; 5’-TCTGTGGCCCCAATGTGTAAG-3’, Mouse *Adora2a*: forward; 5’-GCCATCCCATTCCCATCA-3’ and reverse; 5’-GCAATAGCCAAGAGGCTGAAGA-3’, Mouse *Adora2b*: forward; 5’-GCGAGAGGATCATTGCTG-3’ and reverse; 5’-CAGGAACCGAGGTCAATCCAA-3’, Mouse *Adora3*: forward; 5’-ATACCAGATGTCGGAATGTGC-3’ and reverse; 5’-GCAGGCGTAGACAAATAGGGTT-3’, Mouse *Cd73*: forward; 5’-CAGATCCGCAAGGAAGAACC-3’ and reverse; 5’-ATGATGCCTTGACTTGTC-3’, Mouse *Cd39*: forward; 5’-GCAAGCAGAGACAAAAAC-3’ and reverse; 5’-GCAAATCTCTCTCACCTTAAGAATCC-3’, Mouse *Ent-1*: forward; 5’-CAGGCTCAGGACAGGTATAAGG-3’ and reverse; 5’-GTTTTGTGAAATACCTTTGGTTGCCG-3’, Mouse *Ent-2*: forward; 5’-TCATTACGCACATCCCGTGACT-3’ and reverse; 5’-CCCAGTGTGTGAAGTGAAGGTGTA-3’, Mouse *Ada*: forward; 5’-ACCCGCACTTCACAAAACCCA-3’ and reverse; 5’-AGGGCGATGCCTCTCTTCTTCT-3’, Mouse *Flt-1*: forward; 5’-CCACCTCTCTATCCGCTGG-3’ and reverse; 5’-ACCAATGTGCTAACCCTCTTATT-3’, Mouse *Gapdh*: forward; 5’-TGACCTCAACTACATGGTTCACA-3’ and reverse; 5’-CTTCCCATTCTCGGCCCTTG-3’, Human *ADOR2B*: forward; 5’-TGCACTGACTTCTACGCTTG-3’ and reverse; 5’-GGTCCCCGTGACAAACTT-3’, Human *CD73*: forward; 5’-ACCACGTATCCATGTGCATT-3’ and reverse; 5’-AAAGGGCAATACACAGCCAG-3’,
Human \textit{CD39}: forward; 5’- AGCAGCTGAAATATGCTGGC-3’ and reverse; 5’- GAGACAGTATCTGCCGAAGTCC-3’, Human \textit{GAPDH}: forward; 5’- TGCACCACCAA GTGCTTAGC-3’ and reverse; 5’-ACAGTCTTCTGGGTGCGAGT-3’.

**Measurement of circulating sFlt-1 levels**

sFlt-1 levels in mouse plasma and supernatants from human villous explants were quantitatively determined by using ELISA kits which are commercially available (Qantikine ELISA) (R&D Systems) as previously described

**Electron Microscopy of glomeruli**

Cortical tissue from the mouse kidneys was harvested for electron microscopy in order to assess glomerular ultrastructure. The tissue was fixed in glutaraldehyde and processed for plastic block embedding. Semi-thin sections were performed and stained with toluidine blue fuchsin in order to assess adequacy of the cortical renal tissue for electron microscopic examination. Thin sections were then performed and stained with lead citrate and uranyl acetate and transmission electron microscopy was performed to assess glomerular ultrastructure. Digital images were taken at standard magnifications (1000x and 4000x) and qualitative comparisons were made among the experimental groups by an American Board of Pathology certified anatomic pathologist.

**Assessment of histopathologic changes in kidneys by hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) staining**

Kidneys were dissected from the mice on E18.5, fixed in 4% formaldehdye and embedded in paraffin. Tissue blocks were cut into 4 μm and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) by standard procedures. The extent of renal damage was assessed by
quantifying the glomeruli that showed characteristic features of damage in PE such as decreased Bowman’s space and occlusion of capillary loop spaces. To examine those features, the glomeruli were counted in 5 fields of randomized and blinded slides (10x magnification), with each field having at least 10 glomeruli. A highest score of 5 was accorded to glomeruli with a normal amount of capillary space within Bowman’s capsule. A score of 1 was assigned to the glomeruli that showed complete loss of capillary space and an intermediate score of 3 was assigned to the glomeruli that displayed reduced, but not completely obliterated, capillary space.

**Immunohistochemistry**

Formalin fixed tissue blocks were cut into 4-μm thick sections and subjected to immunohistochemistry. Briefly, endogenous peroxidase activity was quenched by 10 min of incubation in a 3% hydrogen peroxide/methanol buffer. Antigen retrieval was conducted by incubating slides in sodium citrate buffer (pH 6.0) at 89°C for 15 min. After blocking with the normal goat serum, the slides were then incubated with antibody against CD31 (1:200, ab124432, abcam) or human CD73 (1:30, 2B6, Lifespan Biosciences) or mouse CD73 (1:50, H-300, Santa Cruz) in a humidified chamber at 4°C overnight. After the primary antibody incubation, ABC staining system kit (VEACTASTAIN ABC-AP, VECTOR LAB) was used according to the protocol. Antigen-antibody reactions were visualized with alkaline phosphatase substrate kit (VECTOR Red Substrate Kit, VECTORLAB) and then nuclei were counterstained with Mayer's hematoxylin.

**Immunoblotting**

Tissues were homogenized and lysed with RIPA lysis buffer (Santa Cruz) in the presence of proteinase inhibitor cocktail (Roche Diagnostics). Lysates were resolved on SDS-PAGE and
electroblotted onto polyvinylidene difluoride membranes. After blocking with Odyssey Blocking Buffer (LI-COR), the membranes were probed with an antibody against ADA which was purified from sheep anti-ADA antiserum\textsuperscript{12} or human ADORA2B (1:1000, ab40002, abcam) and then probed with secondary antibodies labeled with IRDye fluorophores (LI-COR). The antibody/antigen complexes were scanned and detected using the ODYSSEY infrared imaging system and software (LI-COR).

**Human Placental Villous Explant Culture**

Human placentas were obtained from normotensive patients who delivered at term at Memorial Hermann Hospital in Houston. The explant culture system was conducted as described previously\textsuperscript{13}. On delivery, the placentas were placed on ice and submerged in phenol red–free DMEM containing 10% BSA and antibiotics. Villous explant fragments weighing 50 mg were dissected from the placenta and transferred to 12-well plates at 37°C under 5% CO\textsubscript{2}. The explants were incubated for 24 hours and then pretreated with or without ADORA2B antagonist (10 nM MRS1754) (Tocris Biosciences) for 30 min and then treated with a non-metabolized adenosine analogue (1μM NECA) (Sigma-Aldrich) or an ADORA2B agonist (1 μM BAY60-6583) (Tocris Biosciences) for 24 hours, followed by the collection of medium and cells.
### Table S1 Patient characteristics

<table>
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<th>NT (n=13)</th>
<th>PE (n=13)</th>
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<tr>
<td>Age - yr</td>
<td>27.2±1.7</td>
<td>27.6±2.2</td>
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<tr>
<td>Primigravida - no. (%)</td>
<td>5 (38.4%)</td>
<td>6 (46.2%)</td>
</tr>
<tr>
<td>Race - no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>4 (30.8%)</td>
<td>4 (30.8%)</td>
</tr>
<tr>
<td>African American</td>
<td>5 (38.5%)</td>
<td>6 (46.2%)</td>
</tr>
<tr>
<td>Hispanic</td>
<td>1 (7.7%)</td>
<td>2 (15.4%)</td>
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<tr>
<td>Other or unknown</td>
<td>3 (23.1%)</td>
<td>1 (7.7%)</td>
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<tr>
<td>Body-mass index</td>
<td>30.5±1.3</td>
<td>35.8±2.0*</td>
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<tr>
<td>Systolic blood pressure - mmHg</td>
<td>117.8±1.9</td>
<td>171.5±4.6**</td>
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<tr>
<td>Diastolic blood pressure - mmHg</td>
<td>72.4±1.2</td>
<td>107.9±3.8**</td>
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<tr>
<td>Proteinuria - mg/24h</td>
<td>N/A</td>
<td>1784±420.9</td>
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<tr>
<td>Gestational age at delivery - week</td>
<td>37.8±1.2</td>
<td>36.1±2.7</td>
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<tr>
<td>Infant’s birth weight - g</td>
<td>3157±111.7</td>
<td>2277±230.5**</td>
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<td>Small-for gestational age infant - no. (%)</td>
<td>0 (0%)</td>
<td>4 (30.8%)</td>
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Data presented as mean ± SEM were statistically analyzed by the Mann-Whitney’s U test.

Data shown as number of cases and percentage were statistically analyzed by the Fisher’s exact test.

(*P<0.05, **P<0.01 vs NT)
Table S2 Summary for the weights of fetuses and placentas

<table>
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<tr>
<th>Mating pair and Placental adenosine</th>
<th>( Ada^{+/+} fLi-Tg^* ) ( \varphi ) ( X ) ( Ada^{+/+} ) ( \varphi )</th>
<th>( Ada^{+/+} fLi-Tg^* ) ( \varphi ) ( X ) ( Ada^{+/+} ) ( \varphi )</th>
<th>( Ada^{+/+} fLi-Tg^* (Adora2b^{+/+}) ) ( \varphi ) ( X ) ( Ada^{+/+} ) ( \varphi )</th>
<th>( Ada^{+/+} (Adora2b^{-/-}) ) ( \varphi )</th>
<th>( Ada^{+/+} (PL-Tg^{+/+}) ) ( \varphi )</th>
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<tr>
<td>No. of moms</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>5</td>
<td></td>
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<tr>
<td>Average No. of fetuses / mom</td>
<td>8.0±0.71</td>
<td>8.3±0.47</td>
<td>8.0±1.1</td>
<td>7.4±0.93</td>
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<tr>
<td>Embryonic genotype</td>
<td>( Ada^{+/+} )</td>
<td>( Ada^{+/+} )</td>
<td>( Ada^{+/+} )</td>
<td>( Ada^{+/+} )</td>
<td>( Ada^{+/+} )</td>
</tr>
<tr>
<td>No. of fetuses</td>
<td>21 (52.5%)</td>
<td>19 (47.5%)</td>
<td>27 (46.5%)</td>
<td>31 (53.4%)</td>
<td>12 (50.0%)</td>
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<tr>
<td>Fetal weight (g)</td>
<td>0.95±0.02</td>
<td>0.96±0.02</td>
<td>0.72±0.02</td>
<td>0.89±0.05</td>
<td>1.01±0.02</td>
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<td>Placental weight (g)</td>
<td>0.095±0.003</td>
<td>0.094±0.003</td>
<td>0.076±0.002</td>
<td>0.093±0.004</td>
<td>0.098±0.003</td>
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</table>

Data of weights in fetuses and placentas are expressed as mean ± SEM. (**\( P<0.01 \) vs other groups)
Supplemental References


Supplemental Figure 1 Generation of ADA-deficient mice harboring the transgene to produce ADA only in the liver during the fetal period (Ada⁺/-/fLi-Tg⁺) (A) Schema of transgene harboring the human ADA cDNA and alpha-fetoprotein (AFP) promoter and enhancer elements to induce the transcription of ADA minigene exclusively in the fetal liver (fLi-Tg). (B) Schematic drawing of generation of fetal liver rescued ADA-deficient mice (Ada⁺/-/fLi-Tg⁺) by two stage genetic approaches. 1) generation of transgenic mice containing a transgene expressing human ADA cDNA only in fetal liver by alpha fetal protein (AFP) promoter (fLi-Tg⁺); 2) generation of fetal liver rescued Ada-deficient mice (Ada⁺/-/fLi-Tg⁺) by mating Ada⁺/-/fLi-Tg⁺ to each other. (C) Expression levels of ADA protein in the liver, brain, kidney, skin, and placenta from fetal liver rescued ADA-deficient fetus (Ada⁺/-/fLi-Tg⁺), and the placentas from Ada⁺⁺, Ada⁺/-/fLi-Tg⁺ fetuses were detected by immunoblotting (20 µg of protein from each tissue was loaded). (D) ADA enzymatic activity in tissues determined by ADA zymogram analysis. ADA zymogram analysis was performed on 8 µg of protein from each tissue.
Supplemental Figure 2  Renal histology was assessed by hematoxylin and eosin staining (H&E) and periodic acid-Schiff staining (PAS)  (A) Pathologic changes in kidneys (swollen glomeruli with narrowed capillary loops and Bowman’s spaces) were observed in dams with elevated placental adenosine (Ada^{+/−}/Li-Tg^{+} females mated with Ada^{−/−}/Li-Tg^{+} males). Scale bar; 200µm (upper panel), 50µm (lower panel).  (B) The glomerular damage among each group is quantified. (n=4 each), (\*P<0.05 vs control dams).
### Supplemental Figure 3 Placental vasculature was assessed by CD31 immunostaining

In the ADA-deficient placentas from dams with elevated placental adenosine, positive CD31 staining (red) was found to be disorganized and did not localize along vascular structures in the labyrinth zone compared with the ADA-deficient placentas with PEG-ADA treatment, ADA and ADORA2B double-deficient placentas, and placentas from placental rescued ADA-deficient mice. Scale bar; 200 µm.
Supplemental Figure 4  Expression levels of Flt-1 gene in the mouse placentas and maternal circulating levels of sFlt-1  (A) Flt-1 mRNA levels in the mouse placentas of indicated genotypes from dams described above were determined by real-time RT-PCR. (n=5-6 per group), (*P<0.05, **P<0.01 vs Ada+/− placenta from dams with elevated placental adenosine). (B) Maternal circulating sFlt-1 levels were determined by ELISA (n=4 per group), (*P<0.05, **P<0.01 vs Dams with elevated placental adenosine).
Supplemental Figure 5  Renal histology was assessed by hematoxylin and eosin staining (H&E) and periodic acid-Schiff staining (PAS) Scale bar; 200µm (upper panel), 50µm (lower panel). The glomerular damage among each group was quantified. (n=4 each), (*P<0.05 vs Dams with elevated placental adenosine).
Supplemental Figure 6  Placental expression profiling of purinergic molecules in human and mouse placenta. mRNA expression levels of purinergic molecules in human placentas from normotensive pregnant women (NT) or preeclampsia patients (PE) (n=10 per group), or placentas of mice injected with NT- or PE-IgG (NT-IgG:n=6, PE-IgG:n=6) were determined by real-time RT-PCR. mRNA expression level was determined as a relative value to GAPDH, and each value was expressed as fold induction relative to placentas of NT or NT-IgG-treated mice. No significant difference was observed between groups.
Supplemental Figure 7  Adenosine levels in the circulation and the kidneys of pregnant mice treated with PE patient-derived IgG (PE-IgG) or normotensive pregnant women-derived IgG (NT-IgG) (A and B) Adenosine levels in the plasma (A) or kidneys (B) of pregnant mice treated with NT- or PE-IgG were determined by HPLC on E18.5. (n=4 each), No significant difference was detected.
Supplemental Figure 8  Expression of CD73 protein in mouse placentas detected by immunohistochemistry. PE-IgG treatment induced the elevation of placental CD73 expression. The staining in the placenta from PE-IgG-injected Cd73^{-/-} dam was displayed as a negative control. Scale bar, upper panels: 1.0mm, lower panels: 200μm
Supplemental Figure 9  Intra carotid mean arterial blood pressure of WT, Cd73\(^{-/-}\), and Adora2b\(^{-/-}\) dams treated with NT- or PE-IgG was measured on E18.5  Increase of intra carotid mean arterial blood pressure (MAP) seen in PE-IgG-treated WT dams was significantly suppressed in Cd73\(^{-/-}\) or Adora2b\(^{-/-}\) dams. (n=3-5 per group), (*P<0.05 vs NT-IgG-treated WT dams, †P<0.05, †† P<0.01 vs PE-IgG-treated WT dams)