Adiponectin supplementation in pregnant mice prevents the adverse effects of maternal obesity on placental function and fetal growth

Irving L. M. H. Aye[a,1], Fredrick J. Rosario[a], Theresa L. Powell[a,b], and Thomas Jansson[a,c]

[a]Division of Reproductive Sciences, Department of Obstetrics and Gynecology, University of Colorado Anschutz Medical Campus, Aurora, CO 80045; [b]Section of Neonatology, Department of Pediatrics, University of Colorado Anschutz Medical Campus, Aurora, CO 80045; and [c]Center for Pregnancy & Newborn Research, Department of Obstetrics and Gynecology, University of Texas Health Science Center, San Antonio, TX 78229

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Mothers with obesity or gestational diabetes mellitus have low circulating levels of adiponectin (ADN) and frequently deliver large babies with increased fat mass, who are susceptible to perinatal complications and to development of metabolic syndrome later in life. It is currently unknown if the inverse correlation between maternal ADN and fetal growth reflects a cause-and-effect relationship. We tested the hypothesis that ADN supplementation in obese pregnant dams improves maternal insulin sensitivity, restores normal placental insulin/mechanistic target of rapamycin complex 1 (mTORC1) signaling and nutrient transport, and prevents fetal overgrowth. Compared with dams on a control diet, female C57BL/6J mice fed an obesogenic diet before mating and throughout gestation had increased fasting serum leptin, insulin, and C-peptide, and reduced high-molecular-weight ADN at embryonic day (E) 18.5. Placental insulin and mTORC1 signaling was activated, peroxisome proliferator-activated receptor-α (PPARα) phosphorylation was reduced, placental transport of glucose and amino acids in vivo was increased, and fetal weights were 29% higher in obese dams. Maternal ADN infusion in obese dams from E14.5 to E18.5 normalized maternal insulin sensitivity, placental insulin/mTORC1 and PPARα signaling, nutrient transport, and fetal growth without affecting maternal fat mass. Using a mouse model with striking similarities to obese pregnant women, we demonstrate that ADN functions as an endocrine link between maternal adipose tissue and fetal growth by regulating placental function. Importantly, maternal ADN supplementation reversed the adverse effects of maternal obesity on placental function and fetal growth. Improving maternal ADN levels may serve as an effective intervention strategy to prevent fetal overgrowth caused by maternal obesity.

adipokines | maternal-fetal exchange | amino acids | glucose | insulin resistance

Obesity and the metabolic syndrome are major risk factors for a wide array of diseases, including type 2 diabetes mellitus, cardiovascular disease, and cancer (1, 2). Compelling evidence shows that metabolic syndrome is caused, in part, by a suboptimal intrauterine environment (3). The strong association between maternal obesity during pregnancy and metabolic syndrome in childhood is of particular concern because almost two-thirds of American women now enter pregnancy either overweight or obese (4). Obesity during pregnancy therefore creates a vicious, detrimental cycle of intrauterine transmission of metabolic disease from the mother to her children (5). Intervention strategies involving lifestyle changes or antiobesity drugs remain largely unsuccessful, and it is therefore urgent to explore the possibility of intervening in utero to prevent the development of obesity and metabolic syndrome.

Obesity in pregnant women is associated with activation of placental insulin and mechanistic target of rapamycin complex 1 (mTORC1) signaling, up-regulation of specific placental amino acid transporters, and fetal overgrowth (6, 7). In addition, circulating levels of adiponectin (ADN) are decreased in obese pregnant women (8, 9). The ADN protein is synthesized in adipose tissue and undergoes tightly regulated multimerization involving chaperone proteins, including disulfide-bond A oxidoreductase-like protein (DsbA-L), resulting in the assembly of oligomeric ADN proteins of different molecular weight (10). Multimerization into the high-molecular-weight (HMW) form increases the $t_{1/2}$ of ADN (11), and the insulin-sensitizing effect of ADN can largely be attributed to the HMW form (12). Low circulating levels of HMW ADN strongly predict the development of gestational diabetes mellitus (GDM) independent of maternal adiposity (13, 14).

We recently reported that ADN, in contrast to its well-known insulin-sensitizing effects in skeletal muscle and liver, inhibits insulin and mTORC1 signaling and amino acid transport in cultured primary human trophoblast (PHT) cells (15) and in pregnant mice in vivo (16). This effect is mediated by activation of trophoblast peroxisome proliferator-activated receptor-α (PPARα) signaling and increased ceramide synthesis, resulting in inhibition of IRS-1 (17). Thus, low circulating ADN in maternal obesity may be causally linked to changes in placental function and increased fetal growth. These findings, together with the recent discovery of an orally active ADN receptor agonist (AdipoRon) (18), provide the rationale for exploring the possibility that maternal ADN supplementation may prevent the adverse fetal outcomes in maternal obesity.

We recently established a mouse model of obesity in pregnancy, which shows extensive similarities to the human condition, including low maternal ADN and glucose intolerance, increased placental nutrient transport, and fetal overgrowth (19). In this study, we used this model to test the hypothesis that ADN

Significance

Obesity and metabolic syndrome may, in part, originate in fetal life. In particular, babies of mothers with obesity and/or gestational diabetes mellitus (GDM) are often large at birth and have increased adiposity, which predisposes them to the development of metabolic disease later in life. Maternal obesity and GDM are typically associated with low circulating levels of adiponectin (ADN), and we found that ADN supplementation to pregnant obese mice completely normalized the changes in placental function and prevented fetal overgrowth caused by maternal obesity. These findings suggest that strategies to increase ADN levels in maternal obesity and GDM may alleviate the adverse effects of these pregnancy complications on the fetus.

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[1]To whom correspondence should be addressed. Email: irving.aye@ucdenver.edu.

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Table 1. Maternal and fetal characteristics

Parameter	C/PBS	OB/PBS	OB/ADN	*P* value
Maternal				
Initial body weight, g	23.4 ± 0.7	22.9 ± 0.8	22.6 ± 0.6	NS
Body weight at mating, g	23.3 ± 0.4	28.7 ± 1.0*	28.4 ± 0.6*	*$P < 0.001$
Body weight at E18.5, g	31.5 ± 1.0	38.2 ± 0.9*	36.9 ± 1.0*	*$P < 0.01$
Fetal				
Litter size	7.6 ± 0.4	7.6 ± 0.3	7.3 ± 0.3	NS
Placental weight, mg	91.6 ± 5.5	100.6 ± 4.9	95.4 ± 3.5	NS
Fetal weight, g	0.93 ± 0.06	1.20 ± 0.03*	0.99 ± 0.04†	*,†$P < 0.01$
Fetal/placental weight ratio	10.4 ± 0.96	12.2 ± 0.57	10.6 ± 0.63	NS

Fetal values were obtained at E18.5. Data are mean ± SEM [$n = 7$ (C/PBS), $n = 10$ (OB/PBS), and $n = 9$ (OB/ADN)]. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. NS, not significant. * indicates that the values are significantly different from C/PBS and † indicates that the values are significantly different from OB/PBS.

supplementation in obese pregnant dams improves maternal insulin sensitivity, restores normal placental insulin signaling and nutrient transport, and prevents fetal overgrowth.

Results

Effect of Maternal Obesity and ADN Supplementation on Maternal and Fetal Characteristics. Fig. S1 shows an overview of the experimental paradigm for the allocation of the diets, treatments, and experimental analyses. Female C57BL/6J mice fed an obesogenic (OB) diet were mated after they had gained 25% of their initial body weight, which occurred after ∼6 wk on the diet (Table 1). After mating, three groups of pregnant animals were studied: lean mice on a control diet infused with vehicle (C/PBS), obese mice infused with vehicle (OB/PBS), and obese mice with infusion of ADN (OB/ADN) (Fig. S1). To target placental function during the period of rapid fetal growth rather than placental development, all infusions were performed for the last 4 d of pregnancy [embryonic day (E)14.5–E18.5], a period that accounts for 70% of the total fetal growth in C57/Bl6 mice (20). Fig. S2 illustrates the daily total caloric and macronutrient intake in pregnant mice at different gestational intervals. Compared with mice on the control diet, mice fed the OB diet consumed more calories in the form of carbohydrate and fat throughout gestation (Fig. S2).

At E18.5, the difference in maternal body weights between lean mice on the control diet and obese mice receiving PBS or ADN infusion was maintained (Table 1). However, there were no differences in maternal body weight between obese mice receiving PBS compared with obese mice receiving ADN infusion, suggesting that 4 d of ADN supplementation did not reduce maternal fat mass. However, supplementation of ADN in obese mice modestly reduced food intake during the period of infusion (Fig. S2). At E18.5, the fetuses of obese dams were 29% heavier than the fetuses of control mice, and this effect was completely reversed by maternal ADN infusion (Table 1). These changes in fetal weight were not associated with differences in the litter size, placental weight, or fetal/placental weight ratio (Table 1).

ADN Supplementation Improves Maternal Hormonal Changes Associated with Obesity in Pregnancy. The increase in fasting serum leptin, insulin, and C-peptide in obese dams compared with normal dams was normalized by ADN infusion (Table 2). Although there were no significant changes in total ADN with maternal obesity, HMW ADN was markedly reduced in obese dams. Supplementation with ADN increased both total and HMW ADN. Consequently, the HMW-to-total ADN ratio was reduced in OB/PBS mice compared with C/PBS mice, and ADN infusion in obese mice normalized this ratio to control levels (Table 2). Interestingly, ADN supplementation also increased maternal adipose tissue expression of the ADN multimerization protein DsbA-L (Fig. S3).

Maternal glucose levels were not influenced by obesity or ADN infusion; however, fetal blood glucose was increased in the obese mice compared with control dams (Table 2). Importantly, fetal hyperglycemia in maternal obesity was normalized by maternal ADN infusion (Table 2). Maternal cholesterol and phospholipids were not significantly different between control and obese dams. However, ADN infusion decreased maternal cholesterol and phospholipids in the obese dams by ∼50%. Maternal triglycerides

Table 2. Maternal and fetal serum hormones and metabolites

Serum analyte	C/PBS	OB/PBS	OB/ADN	*P* value
Maternal HMW ADN, μg/mL	11.0 ± 1.4	5.7 ± 0.8*	14.5 ± 1.5†	*,†$P < 0.05$
Maternal total ADN, μg/mL	29.0 ± 2.3	28.6 ± 2.0	42.5 ± 4.2*,†	*,†$P < 0.05$
Maternal HMW/total ADN ratio	0.38 ± 0.04	0.19 ± 0.03*	0.37 ± 0.04†	*,†$P < 0.01$
Maternal insulin, ng/mL	0.083 ± 0.01	0.187 ± 0.02*	0.112 ± 0.019†	*,†$P < 0.05$
Maternal C-peptide, pM	591.0 ± 35.2	804.5 ± 75.1*	534.1 ± 26.6†	*,†$P < 0.05$
Maternal leptin, ng/mL	3.4 ± 0.9	6.2 ± 0.8*	2.1 ± 0.5†	*,†$P < 0.05$
Maternal glucose, mg/dL	150.3 ± 7.3	145.3 ± 7.9	136.4 ± 6.7	NS
Fetal glucose, mg/dL	56.7 ± 10.6	95.9 ± 22.7*	56.2 ± 4.3†	*,†$P < 0.05$
Maternal triglycerides, mg/dL	52.1 ± 3.8	57.8 ± 4.0	53.2 ± 5.8	NS
Maternal cholesterol, mg/dL	78.0 ± 12.8	94.6 ± 13.3	48.5 ± 8.7†	*,†$P < 0.05$
Maternal phospholipids, mg/dL	67.5 ± 7.6	94.7 ± 15.0	43.05 ± 7.5†	*,†$P < 0.05$
Maternal NEFAs, mEq/L	0.56 ± 0.02	0.58 ± 0.04	0.66 ± 0.10	NS

Serum hormones and metabolites were measured at E18.5. Data are mean ± SEM [$n = 7$ (C/PBS), $n = 10$ (OB/PBS), and $n = 9$ (OB/ADN)]. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. NEFAs, nonesterified fatty acids. * indicates that the values are significantly different from C/PBS, and † indicates that the values are significantly different from OB/PBS.

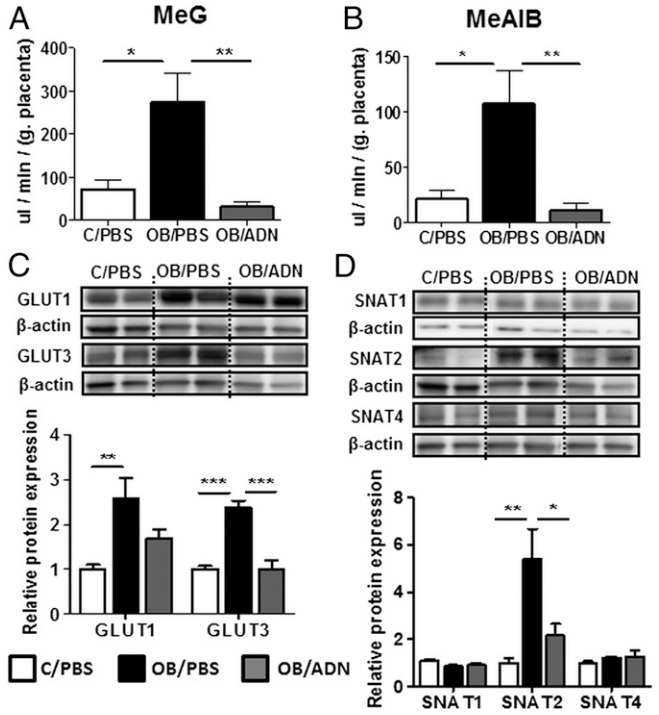

Fig. 1. ADN infusion in OB pregnant mice decreases placental nutrient transporter expression and function. Placental nutrient transport capacity in vivo was measured at E18.5. Unidirectional maternal-fetal clearances for MeG (*A*) and MeAIB (*B*) were measured in anesthetized dams. Protein expression of glucose (*C*) and system A amino acid (*D*) transporter isoforms was determined in isolated TPMs. Data are mean + SEM ($n = 5$ per group). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

and nonesterified fatty acids were not significantly altered by obesity or ADN treatment (Table 2).

ADN Infusion Reverses the Increased Placental Nutrient Transport Associated with Maternal Obesity. To examine whether the effects of maternal ADN infusion on fetal growth were due to effects on placental nutrient transport, the in vivo maternal-fetal transfer of radiolabeled nonmetabolizable glucose (3-O-methyl-D-1-^{3}H-glucose; MeG) and neutral amino acid (α-[1-^{14}C]-methylaminoisobutyric acid; MeAIB) was measured (Fig. 1 *A* and *B*). Similar to our previous report (21), maternal obesity increased transplacental transport of MeG by 3.8-fold (Fig. 1*A*) and MeAIB by fivefold (Fig. 1*B*). ADN infusion reversed the effects of maternal obesity on placental MeG and MeAIB transport to the fetus (Fig. 1 *A* and *B*).

To determine if the marked changes in placental transport with maternal obesity and in response to ADN infusion were associated with changes in nutrient transporter abundance in the placental barrier, amino acid and glucose transporter isoform protein expression was measured in isolated trophoblast plasma membranes (TPMs). TPMs constitute the maternal-facing plasma membrane of trophoblast layer II of the mouse placenta, and they are believed to be functionally analogous to syncytiotrophoblast microvillous plasma membranes of the human placenta (22). The protein expression of glucose transporters GLUT1 and GLUT3 was increased by 2.6-fold and 2.4- fold, respectively, and the expression of the system A amino acid transporter isoform SNAT2 was increased by 5.4-fold in the TPMs of obese mice compared with controls (Fig. 1 *C* and *D*). ADN infusion in obese dams decreased GLUT3 and SNAT2 TPM expression to the levels in control dams but did not significantly reduce GLUT1 TPM levels (Fig. 1 *C* and *D*). TPM SNAT1 and SNAT4 protein expression was not significantly different between the groups (Fig. 1*D*).

Maternal ADN Supplementation Normalizes the Increased Placental Insulin and mTORC1 Signaling in Maternal Obesity. To examine the mechanisms associated with the effects of ADN on placental nutrient transport and fetal growth, we determined placental insulin and mTORC1 signaling activity. TPM expression of insulin receptor-β (IR-β) was significantly increased with maternal obesity, which was normalized by ADN infusion (Fig. 2*A*). Insulin receptor substrate-1 (IRS-1) is known to be activated by phosphorylation at Tyr608 and inhibited by phosphorylation at Ser307 (23). Compared with controls, placentas from obese dams exhibited IRS-1 activation as indicated by increased Tyr608 and decreased Ser307 phosphorylation, and these changes were reversed by ADN supplementation (Fig. 2*A*).

Next, we determined whether the changes in IRS-1 activity were reflected by the corresponding changes in the activation of downstream proteins in the insulin signaling pathway. Consistent with increased IRS-1 activity, Akt Thr308 phosphorylation, but not total Akt expression, was significantly increased by maternal obesity, and this effect was mitigated by ADN treatment (Fig. 2*B*). However, PI3K/p85 expression was not altered by obesity or ADN treatment. Similarly, Grb2 and Erk (Thr202/Tyr204) signaling was not significantly different between the three groups (Fig. 2*C*).

Placental mTORC1 is activated by insulin signaling (24) and is a key regulator of placental amino acid transporters (25). Consistent

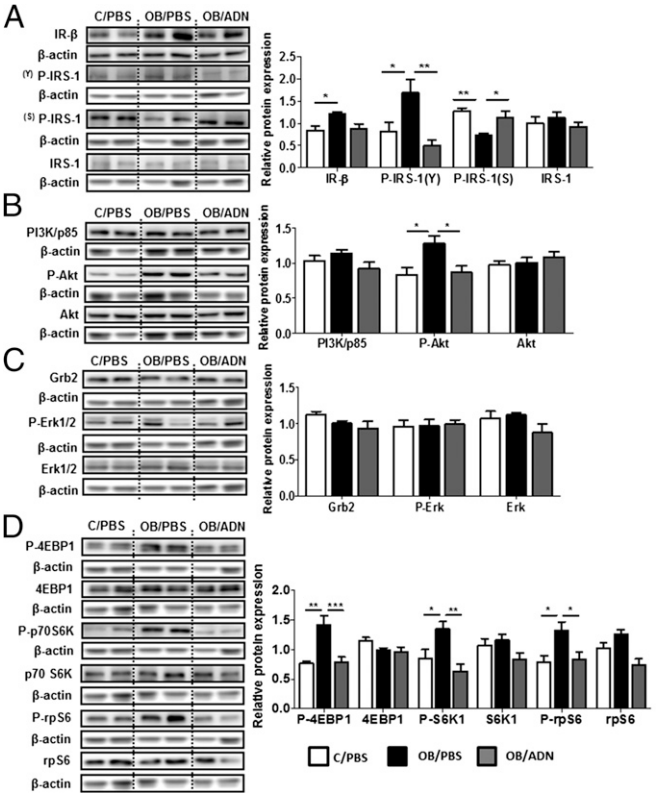

Fig. 2. Impact of maternal obesity and ADN infusion on placental insulin and mTORC1 signaling. Placental insulin signaling as determined by the expression and/or phosphorylation of IR-β and IRS-1 (*A*) and downstream activation of the PI3K/Akt (*B*) and Grb/Erk (*C*) pathways. (*D*) mTORC1 signaling was determined by the expression and phosphorylation of 4EBP1, S6K1, and rpS6. Data are mean + SEM [$n = 7$ (C/PBS), $n = 10$ (OB/PBS), and $n = 9$ (OB/ADN)]. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. S, Ser 307; Y, Tyr 608.

with increased Akt signaling, we observed increased phosphorylation of p70S6K (Thr389) and 4EBP1 (Thr37/46), two well-established functional readouts of mTORC1, in the obese mice compared with controls (Fig. 2*D*). In addition, the p70S6K target rpS6 (Ser235/236) was activated in the placentas of obese mice (Fig. 2*D*). The increased phosphorylation of p70S6K, 4EBP1, and rpS6 in obese dams was completely reversed by ADN infusion (Fig. 2*D*). Collectively, these data demonstrate that maternal ADN supplementation normalizes placental mTORC1 activation in response to maternal obesity.

Maternal ADN Infusion Increases Placental PPARα Phosphorylation. Our previous work in PHT cells has established a role for PPARα activation in mediating the effects of ADN on trophoblast insulin signaling and nutrient transport (17). Hence, we determined whether placental PPARα activity was regulated by maternal ADN in mice. Remarkably, maternal obesity decreased placental PPARα Ser21 phosphorylation (Fig. 3). This effect was reversed by ADN infusion in obese dams. Total placental PPARα expression was increased in the obese dams compared with controls, whereas placental PPARα expression in ADN-supplemented animals was similar to the expression of PPARα in placentas of control mice.

In skeletal muscle, liver, and adipose tissues, ADN mediates its cellular effects, in part, through 5′ adenosine monophosphate-activated protein kinase (AMPK) activation. However, in mouse placenta, ADN infusion decreased both total expression and phosphorylation of AMPK Thr172 (Fig. 3). Maternal obesity did not influence either phosphorylated or total AMPK expression. Moreover, placental p38 MAPK phosphorylation and total expression were not altered by maternal obesity or ADN treatment (Fig. 3).

Discussion

We demonstrate that ADN supplementation in pregnancy prevents fetal overgrowth caused by maternal obesity. Moreover, we provide evidence that ADN functions as an endocrine link between maternal adipose tissue and fetal growth by regulating placental function. These findings suggest that improving ADN levels in women with obesity and/or GDM may serve as an effective intervention strategy to prevent the intrauterine transmission of obesity and metabolic disease.

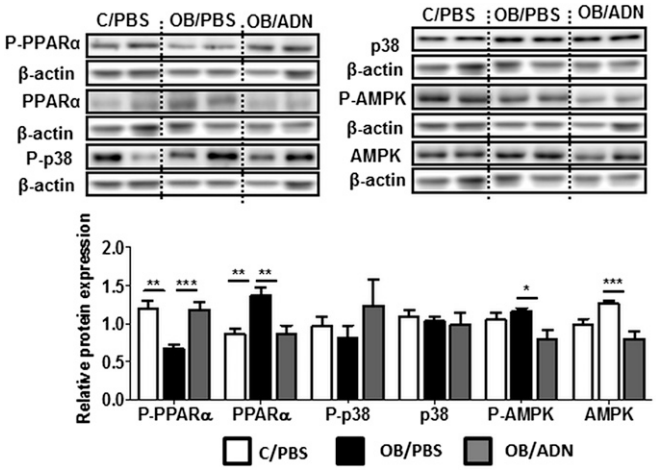

Fig. 3. Regulation of placental PPARα, p38 MAPK, and AMPK activity by maternal obesity and ADN treatment. Activation of placental PPARα, p38 MAPK, and AMPK was determined by the phosphorylation and expression of these proteins. Data are mean + SEM [$n = 7$ (C/PBS), $n = 10$ (OB/PBS), and $n = 9$ (OB/ADN)]. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

We recently established a mouse model of maternal diet-induced obesity, which is associated with maternal glucose intolerance and decreased circulating levels of HMW ADN and fetal overgrowth (19). The placentas of obese dams exhibit activation of insulin and mTORC1 signaling and greater nutrient transport capacity, in agreement with obese women giving birth to large babies (6). In addition, this model is associated with fetal hyperglycemia, consistent with reports in pregnant obese women (26). This mouse model is therefore clinically relevant and useful in testing the hypothesis that ADN supplementation prevents the changes in placental function and fetal overgrowth associated with maternal obesity.

Formation of HMW ADN increases the biological activity of ADN in serum and is associated with increased insulin sensitivity in most tissues (27). Consistent with previous reports in mice fed high-fat diets (28), the obese dams had decreased HMW ADN levels, as well as a reduced HMW-to-total ADN ratio in maternal serum. Interestingly, infusion with full-length ADN increased not only total ADN but also the HMW fraction. ADN multimers do not interconvert in circulation, and the formation of HMW complexes is dependent on the adipocyte proper (29). Therefore, it is possible that the increased HMW ADN levels in ADN-supplemented mice were a result of ADN regulating its own multimerization in the adipose tissue. In support of this hypothesis, adipose tissues of ADN-infused dams display increased expression of the ADN assembly chaperone protein DsbA-L (10). The decreased HMW ADN levels, along with elevated fasting insulin and C-peptide levels, in obese dams suggest that maternal obesity is associated with insulin resistance in this mouse model. Importantly, ADN infusion to obese dams normalized circulating levels of HMW ADN, insulin, and C-peptide, consistent with the possibility that ADN plays an important role in regulating maternal insulin sensitivity in pregnancy. Interestingly, ADN supplementation also modestly reduced caloric intake in obese mice, which may be due to the improved leptin resistance, as indicated by reduced fasting leptin levels in these mice.

Changes in placental nutrient transport alter nutrient flux to the fetus and, ultimately, fetal growth, independent of maternal nutrient levels (30, 31). Indeed, large infants of obese nondiabetic women are more likely to have elevated cord serum glucose (26, 32), which may be related to an increased placental glucose transport capacity rather than elevated maternal blood glucose levels (26). Similarly, despite normal maternal serum glucose levels in obese dams, glucose concentrations in the fetal serum were markedly increased, and they returned to control levels by ADN infusion. These data suggest that the effects of maternal obesity and ADN supplementation on fetal glucose levels may be related to the effects on placental transport capacity. In agreement with this hypothesis, maternal obesity in our mouse model was associated with a pronounced increase in in vivo transplacental transfer of glucose. The increase in placental glucose transport is probably due to the marked up-regulation of TPM GLUT1 and GLUT3 expression, given previous reports indicating that these transporters make a significant contribution to glucose transport in mice placentas (33, 34). Fetal hyperglycemia caused by maternal obesity reflects the inability of the fetal pancreas to maintain normoglycemia by increasing insulin secretion, perhaps due to β-cell exhaustion. Hence, the fetal hyperglycemia in response to maternal obesity may represent one of the early events in programming of diabetes. The normalization of fetal hyperglycemia by maternal ADN supplementation therefore suggests that restoration of maternal ADN levels may be sufficient to reverse the in utero fetal programming of diabetes associated with maternal obesity.

Maternal obesity was also associated with enhanced placental transfer of MeAIB, indicating increased placental system A activity, which can be explained by the markedly increased protein expression of the SNAT2 isoform in TPMs in obese animals. The increase in TPM SNAT2 expression is consistent with reports that

SNAT2 represents a highly regulated system A isoform in the placenta (25, 35, 36) and in other tissues (37). In addition, we have previously shown that mTORC1 signaling is a positive regulator of system A transport in human primary trophoblast cells by influencing the plasma membrane trafficking of SNAT2, but not SNAT1 or SNAT4 (25). Importantly, ADN infusion reversed the increase in placental transport of glucose and neutral amino acids associated with maternal obesity, consistent with the possibility that ADN supplementation in maternal obesity prevents fetal overgrowth by normalizing placental function.

Unlike classical insulin-sensitive tissues, such as adipose, muscle, and liver, where insulin function is impaired by metabolic disease, placental insulin signaling has been reported to be activated in pregnancies associated with obesity or diabetes (6, 38). Consistent with these reports in humans, the obese dams displayed increased placental expression of IR-β and increased phosphorylation of IRS-1 and Akt. Collectively, these findings indicate that maternal obesity in mice is associated with activation of placental insulin signaling, which was completely reversed by ADN infusion. Because ADN supplementation also reduced maternal insulin levels, it is likely that the effects of ADN on placental insulin signaling were a result of both the direct effects of ADN in attenuating placental insulin signaling as well as the indirect effects through reduced circulating insulin levels. Insulin signaling regulates critical functions of the placenta, including nutrient transport, and therefore is believed to be essential in determining fetal growth (24, 39). Thus, we propose that the normalization of placental insulin signaling by maternal ADN infusion constitutes the critical event that ultimately prevents fetal overgrowth caused by maternal obesity.

mTORC1 is a central regulator of cellular metabolism and is activated by insulin signaling through Akt phosphorylation. mTORC1 is a key positive regulator of placental amino acid transport (24, 25), and placental mTORC1 signaling has been reported to be activated in obese women (6) and rodents fed a high-fat diet (40). Consistent with these reports, placental mTORC1 activity was elevated in obese dams and mTORC1 signaling returned to control levels by ADN infusion. Taken together, these findings suggest that maternal ADN infusion prevents fetal overgrowth in obese dams by normalizing placental insulin and mTORC1 signaling, which prevents the increased fetal nutrient supply associated with maternal obesity.

Activation of the cellular energy sensor AMPK mediates the effects of ADN on glucose transport and fatty acid oxidation in the liver and muscle (41). In contrast to these tissues, ADN inhibits AMPK activity in PHTs (17), and placental AMPK phosphorylation was reduced by ADN infusion in obese dams. On the other hand, ADN activation of PPARα inhibits insulin signaling in PHTs (17). In line with these in vitro findings, PPARα signaling was reduced in the placentas of obese dams, and this effect was reversed by ADN.

In our previous study, we demonstrated that ADN infusion in insulin-sensitive, control diet-fed pregnant mice with normal ADN levels resulted in an ∼18% reduction in fetal weight (16). Furthermore, the placentas of the ADN-infused dams exhibited decreased expression and activity of system A transporters in isolated TPMs and attenuated placental insulin signaling. Given that fetal growth restriction is associated with neonatal complications, as well as cardiometabolic disorders in later life (42), the potential use of ADN as a treatment in pregnant women with normal ADN levels may be cautioned.

One potential limitation of this study is that sex-specific effects of maternal obesity and ADN supplementation on placental function were not examined. Due to our experimental design, placentas from each litter were pooled to obtain sufficient material for TPM isolations, precluding the possibility of studying male and female placentas separately. However, ADN supplementation completely reversed or normalized most of the placental and fetal outcomes, including placental glucose and amino acid transport, TPM GLUT3 and SNAT2 protein expression, placental insulin and mTORC1 signaling, and fetal growth. If there were significant sexual dimorphism, the expected result would be either no effect of the intervention (female and males responding in opposite directions) or partial effects (one of the sexes not responding). Thus, it is likely that female and male placentas/fetuses respond similarly in our study.

In conclusion, our study provides evidence, for the first time to our knowledge, that maternal ADN supplementation prevents fetal overgrowth caused by maternal obesity mediated by inhibition of placental insulin and mTORC1 signaling, resulting in normalization of placental nutrient transport. These findings suggest that interventions aimed at increasing low maternal ADN may improve the fetal metabolic phenotype associated with maternal obesity and/or GDM. Furthermore the use of synthetic ADN receptor agonists (18) may provide a pharmacological strategy to increase ADN function in maternal and placental tissues to alleviate the adverse metabolic effects of maternal obesity and GDM.

Materials and Methods

Animals and Diets. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center, San Antonio. Complete details of the animal diets and protocols are provided in *SI Materials and Methods*.

ADN Infusion. At E14.5, fasted dams were anesthetized with isoflurane, and a miniosmotic pump (Alzet 1003D; Alza Corporation) was implanted s.c. as previously described (16). Animals in the OB group received either sterile PBS or mouse recombinant full-length ADN (ALX-522-059; Enzo Life Sciences) reconstituted in PBS. These animals were designated as OB/PBS and OB/ADN, respectively. The ADN infusion rate was 0.62 μg·g of body weight^{-1}·d^{-1}. All animals in the control group were implanted with miniosmotic pumps filled with PBS only (C/PBS).

In Vivo Placental Transport. At E18.5, a subgroup of fasted dams was used for measurements of maternal-fetal transfer of the radiolabeled isotopes MeAIB (PerkinElmer) and MeG (PerkinElmer), as previously reported (21). The time point (3 min) for ending the experiments was chosen based on pilot experiments terminating at 1.5, 3.0, and 4.5 min to ensure maternal-to-fetal transport of radiolabeled substrates was in the linear phase (Fig. S4). Complete details of the in vivo placental transport procedure are provided in *SI Materials and Methods*.

Isolation of Layer II TPM. Maternal-facing layer II TPM of the mouse placenta, which is functionally analogous to the human microvillus membrane, was isolated using differential ultracentrifugation and Mg^{2+} precipitation, as previously described (19, 22). TPM enrichment, as determined by alkaline phosphatase activity of TPM/homogenate, was similar across the different groups (Fig. S5). A detailed description of this procedure is provided in *SI Materials and Methods*.

Data Presentation and Statistical Analyses. Data are presented as mean + SEM or mean ± SEM. For fetal and placental data, means of each litter were calculated and used in the statistical analysis. Therefore, *n* represents the number of litters. Because the placental tissues were pooled for TPM isolations, we were not able to separate our findings according to fetal sex. Statistical significance between the C/PBS, OB/PBS, and OB/ADN groups was examined by one-way ANOVA, with Tukey's post hoc test. $P < 0.05$ was considered significant.

Additional information on methods used can be found in *SI Materials and Methods*.

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1. Rhéaume C, Leblanc ME, Poirier P (2011) Adiposity assessment: Explaining the association between obesity, hypertension and stroke. *Expert Rev Cardiovasc Ther* 9(12): 1557–1564.
2. Wolin KY, Carson K, Colditz GA (2010) Obesity and cancer. *Oncologist* 15(6):556–565.
3. Gluckman PD, Hanson MA (2004) Living with the past: Evolution, development, and patterns of disease. *Science* 305(5691):1733–1736.

4. Ogden CL, Carroll MD, Kit BK, Flegal KM (2012) Prevalence of obesity in the United States, 2009-2010. NCHS Data Brief 82, pp 1–8.
5. Catalano PM (2003) Obesity and pregnancy–the propagation of a viscous cycle? *J Clin Endocrinol Metab* 88(8):3505–3506.
6. Jansson N, et al. (2013) Activation of placental mTOR signaling and amino acid transporters in obese women giving birth to large babies. *J Clin Endocrinol Metab* 98(1):105–113.
7. Ehrenberg HM, Mercer BM, Catalano PM (2004) The influence of obesity and diabetes on the prevalence of macrosomia. *Am J Obstet Gynecol* 191(3):964–968.
8. Hendler I, et al. (2005) The levels of leptin, adiponectin, and resistin in normal weight, overweight, and obese pregnant women with and without preeclampsia. *Am J Obstet Gynecol* 193(3 Pt 2):979–983.
9. Jansson N, et al. (2008) Maternal hormones linking maternal body mass index and dietary intake to birth weight. *Am J Clin Nutr* 87(6):1743–1749.
10. Liu M, et al. (2008) A disulfide-bond A oxidoreductase-like protein (DsbA-L) regulates adiponectin multimerization. *Proc Natl Acad Sci USA* 105(47):18302–18307.
11. Halberg N, et al. (2009) Systemic fate of the adipocyte-derived factor adiponectin. *Diabetes* 58(9):1961–1970.
12. Pajvani UB, et al. (2004) Complex distribution, not absolute amount of adiponectin, correlates with thiazolidinedione-mediated improvement in insulin sensitivity. *J Biol Chem* 279(13):12152–12162.
13. Retnakaran R, et al. (2010) Low adiponectin concentration during pregnancy predicts postpartum insulin resistance, beta cell dysfunction and fasting glycaemia. *Diabetologia* 53(2):268–276.
14. Williams MA, et al. (2004) Plasma adiponectin concentrations in early pregnancy and subsequent risk of gestational diabetes mellitus. *J Clin Endocrinol Metab* 89(5): 2306–2311.
15. Jones HN, Jansson T, Powell TL (2010) Full-length adiponectin attenuates insulin signaling and inhibits insulin-stimulated amino Acid transport in human primary trophoblast cells. *Diabetes* 59(5):1161–1170.
16. Rosario FJ, et al. (2012) Chronic maternal infusion of full-length adiponectin in pregnant mice down-regulates placental amino acid transporter activity and expression and decreases fetal growth. *J Physiol* 590(Pt 6):1495–1509.
17. Aye IL, Gao X, Weintraub ST, Jansson T, Powell TL (2014) Adiponectin inhibits insulin function in primary trophoblasts by PPARα-mediated ceramide synthesis. *Mol Endocrinol* 28(4):512–524.
18. Okada-Iwabu M, et al. (2013) A small-molecule AdipoR agonist for type 2 diabetes and short life in obesity. *Nature* 503(7477):493–499.
19. Rosario FJ, Kanai Y, Powell TL, Jansson T (2015) Increased placental nutrient transport in a novel mouse model of maternal obesity with fetal overgrowth. *Obesity (Silver Spring)* 23(8):1663–1670.
20. Kulandavelu S, et al. (2006) Embryonic and neonatal phenotyping of genetically engineered mice. *ILAR J* 47(2):103–117.
21. Jones HN, et al. (2009) High-fat diet before and during pregnancy causes marked up-regulation of placental nutrient transport and fetal overgrowth in C57/BL6 mice. *FASEB J* 23(1):271–278.
22. Kusinski LC, Jones CJ, Baker PN, Sibley CP, Glazier JD (2010) Isolation of plasma membrane vesicles from mouse placenta at term and measurement of system A and system beta amino acid transporter activity. *Placenta* 31(1):53–59.
23. Boura-Halfon S, Zick Y (2009) Phosphorylation of IRS proteins, insulin action, and insulin resistance. *Am J Physiol Endocrinol Metab* 296(4):E581–E591.
24. Jansson T, Aye IL, Goberdhan DC (2012) The emerging role of mTORC1 signaling in placental nutrient-sensing. *Placenta* 33(Suppl 2):e23–e29.
25. Rosario FJ, Kanai Y, Powell TL, Jansson T (2013) Mammalian target of rapamycin signalling modulates amino acid uptake by regulating transporter cell surface abundance in primary human trophoblast cells. *J Physiol* 591(Pt 3):609–625.
26. Acosta O, et al. (2015) Increased glucose and placental GLUT-1 in large infants of obese nondiabetic mothers. *Am J Obstet Gynecol* 212(2):227.e1–227.e7.
27. Liu M, Liu F (2012) Up- and down-regulation of adiponectin expression and multimerization: Mechanisms and therapeutic implication. *Biochimie* 94(10):2126–2130.
28. Liu M, et al. (2012) Fat-specific DsbA-L overexpression promotes adiponectin multimerization and protects mice from diet-induced obesity and insulin resistance. *Diabetes* 61(11):2776–2786.
29. Schraw T, Wang ZV, Halberg N, Hawkins M, Scherer PE (2008) Plasma adiponectin complexes have distinct biochemical characteristics. *Endocrinology* 149(5):2270–2282.
30. Gaccioli F, Lager S, Powell TL, Jansson T (2013) Placental transport in response to altered maternal nutrition. *J Dev Orig Health Dis* 4(2):101–115.
31. Díaz P, Powell TL, Jansson T (2014) The role of placental nutrient sensing in maternal-fetal resource allocation. *Biol Reprod* 91(4):82.
32. Langer O, Yogev Y, Xenakis EM, Brustman L (2005) Overweight and obese in gestational diabetes: The impact on pregnancy outcome. *Am J Obstet Gynecol* 192(6): 1768–1776.
33. Ganguly A, Collis L, Devaskar SU (2012) Placental glucose and amino acid transport in calorie-restricted wild-type and Glut3 null heterozygous mice. *Endocrinology* 153(8): 3995–4007.
34. Ganguly A, et al. (2007) Glucose transporter isoform-3 mutations cause early pregnancy loss and fetal growth restriction. *Am J Physiol Endocrinol Metab* 292(5): E1241–E1255.
35. Jones HN, Jansson T, Powell TL (2009) IL-6 stimulates system A amino acid transporter activity in trophoblast cells through STAT3 and increased expression of SNAT2. *Am J Physiol Cell Physiol* 297(5):C1228–C1235.
36. Thongsong B, Subramanian RK, Ganapathy V, Prasad PD (2005) Inhibition of amino acid transport system a by interleukin-1beta in trophoblasts. *J Soc Gynecol Investig* 12(7):495–503.
37. Hyde R, Hajduch E, Powell DJ, Taylor PM, Hundal HS (2005) Ceramide down-regulates System A amino acid transport and protein synthesis in rat skeletal muscle cells. *FASEB J* 19(3):461–463.
38. Colomiere M, Permezel M, Riley C, Desoye G, Lappas M (2009) Defective insulin signaling in placenta from pregnancies complicated by gestational diabetes mellitus. *Eur J Endocrinol* 160(4):567–578.
39. Larqué E, Ruiz-Palacios M, Koletzko B (2013) Placental regulation of fetal nutrient supply. *Curr Opin Clin Nutr Metab Care* 16(3):292–297.
40. Gaccioli F, et al. (2013) Maternal overweight induced by a diet with high content of saturated fat activates placental mTOR and eIF2alpha signaling and increases fetal growth in rats. *Biol Reprod* 89(4):96.
41. Yamauchi T, et al. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. *Nat Med* 8(11):1288–1295.
42. Barker DJ (2005) The developmental origins of insulin resistance. *Horm Res* 64(Suppl 3):2–7.
43. Aye IL, et al. (2014) Increasing maternal body mass index is associated with systemic inflammation in the mother and the activation of distinct placental inflammatory pathways. *Biol Reprod* 90(6):129.
44. Lanoix D, et al. (2012) Stability of reference proteins in human placenta: general protein stains are the benchmark. *Placenta* 33(3):151–156.