Activation of placental insulin and mTOR signaling in a mouse model of maternal obesity associated with fetal overgrowth

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Rosario FJ, Powell TL, Jansson T. Activation of placental insulin/IGF-I/mTOR and leptin signaling pathways in obese mice. Am J Physiol Regul Integr Comp Physiol 310: R87–R93, 2016. First published October 21, 2015; doi:10.1152/ajpregu.00356.2015.—Fetal overgrowth is common in obese women and is associated with perinatal complications and increased risk for the child to develop metabolic syndrome later in life. Placental nutrient transport capacity has been reported to be increased in obese women giving birth to large infants; however, the underlying mechanisms are not well established. Obesity in pregnancy is characterized by elevated maternal serum insulin and leptin, hormones that stimulate placental amino acid transporters in vitro. We hypothesized that maternal obesity activates placental insulin/IGF-I/mTOR and leptin signaling pathways. We tested this hypothesis in a mouse model of obesity in pregnancy that is associated with fetal overgrowth. C57BL/6J female mice were fed a control (C) or a high-fat/high-sugar (HF/HS) pelleted diet supplemented by ad libitum access to sucrose (20%) solution. Placentas were collected at embryonic day 18.5. Using Western blot analysis, placental mTOR activity was determined along with energy, inflammatory, leptin, and insulin signaling pathways (upstream modulators of mTOR). Phosphorylation of 4E ribosomal protein (S-235/236), 4E-BP1 (T-37/46), Insulin receptor substrate 1 (Y-608), Akt (T-308), and STAT-3 (Y-705) was increased in obese dams. In contrast, expression of placental caspase-1, IκBα, IL-1β, and phosphorylated-JNK (K46/S-T183/Y185) was unaltered. Fetal amino acid availability is a key determinant of fetal growth. We propose that activation of placental insulin/IGF-I/mTOR and leptin signaling pathways in obese mice stimulates placental amino acid transport and contributes to increased fetal growth.

fetal growth; intracellular signaling proteins; fetal programming; pregnancy; maternal-fetal exchange

MATERNAL OBESITY DURING PREGNANCY is associated with a higher risk of pregnancy complications, as well as increased birth weight (55). Furthermore, children born to obese mothers are more likely to become obese themselves (58). Infants of obese mothers have increased visceral adiposity (10) and elevated risk for metabolic syndrome later in life (9, 22). Large-for-gestational age babies of overweight and obese mothers show increased lipolysis and a propensity for decreased insulin sensitivity already at birth (1) and are at particular risk to establish a metabolic trajectory leading to obesity, Type 2 diabetes, and cardiovascular disease in childhood and beyond (2, 45).

The mechanisms linking obesity in pregnancy to altered fetal growth and programming of adult disease are not well established, but there is an increasing awareness that changes in placental metabolism and nutrient transport capacity may contribute to altered fetal growth in maternal obesity (21). We recently generated a novel mouse model of maternal obesity by feeding a diet high in saturated fat, cholesterol, and simple sugars, resembling a diet common in Western societies (52). This resulted in fetal overgrowth associated with maternal metabolic alterations similar to that observed in pregnant women with high body mass index (BMI). Furthermore, the protein expression of specific glucose and amino acid transporter isoforms and amino acid transport activity were markedly elevated in placentas of obese dams (52), consistent with the possibility that fetal overgrowth in response to maternal obesity may be caused by increased placental nutrient transport. However, the placental signaling events linking maternal obesity to upregulation of placental nutrient transport remain to be established.

Mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that integrates growth factor signaling with information on cellular levels of nutrients, oxygen, stress, and energy to regulate cell growth, mediated by effects on gene expression and protein translation. The mTOR protein is present in the cytoplasm in two complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2) (37). mTORC1, the canonical target of rapamycin and it is analogs, is responsive to nutrient abundance, energy sufficiency, and growth factor signaling. mTORC1 signaling activity is commonly assessed by phosphorylation of downstream targets S6 kinase 1 (S6K1), ribosomal protein S6 (rpS6), and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Phosphorylation of 4E-BP1 results in its dissociation from eukaryotic translation initiation factor 4E (eIF4E), allowing eIF4E to associate with the scaffold for the cap-binding complex, eukaryotic translation initiation factor 4G (or eIF4G). The fully assembled cap-binding complex, eIF4E, has the binding and helicase activities required to recruit mRNA to the ribosomes and initiate translation (14). mTORC2 has been reported to be involved in the regulation of actin cytoskeleton and cell survival through RAC-α serine/threonine-protein kinase (Akt) activation (60). Upstream regulators of mTOR activity include the insulin/phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway (24), the AMPK, leptin (46) and the ERK 1/2 pathway (33).

The placenta has been proposed to function as a nutrient sensor matching fetal growth to maternal nutrient availability by altering placental growth and nutrient transport capacity (28). Placental mTOR signaling in the placenta is believed to

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constitute a key component of the placental nutrient sensor (32, 51). Using the phosphorylation of well-established downstream targets as functional readouts, placental mTOR activity is positively correlated to fetal growth in animal models (3, 18, 32, 57) and in humans (27, 48). Moreover, mTOR is a positive regulator of System A and System L amino acid transporter in the placenta (53).

Obesity in nonpregnant individuals is associated with a low-grade chronic inflammation, which is believed to contribute to insulin resistance (41). Obese pregnant women also show signs of inflammation, including elevation of circulating cytokines such as TNF-α, monocyte chemotactant protein-1 (MCP-1), IL-1, and IL-6 (5, 7, 11, 47), compared with pregnant women with normal BMI. In the placenta, inflammatory pathways may be activated in obese women in response to inflammatory stimuli or through infiltration of maternal immune cells with subsequent increased cytokine production (11). The key intracellular mediators of the inflammatory response include NF-κB, JNK, caspase-1, and p38-MAPK, which are activated by Toll-like receptor 4 signaling cascade and STAT3. The cellular effects of leptin are largely mediated by the JAK/STAT signaling pathway, which includes the phosphorylation of STAT3 at Tyr-705, resulting in STAT3 dimerization and migration to the nucleus, where transcription initiation is affected (17). However, Tyr-705-STAT3 phosphorylation can also be induced by other cytokines and hormones (36).

Maternal obesity is associated with elevated circulating levels of insulin and leptin (9, 11). These hormones have been shown to activate mTOR in nonplacental cells (17, 37). In cultured primary human trophoblast cells, insulin stimulates amino acid uptake mediated by a mTOR-dependent mechanism (50). Furthermore, maternal circulating levels of adiponectin are markedly decreased in obese pregnant women (26) and mice (52) compared with lean controls. Because maternal adiponectin inhibits insulin signaling in the placenta (4, 29, 54), the low maternal adiponectin associated with maternal obesity may contribute to activation of placental insulin signaling. In the current study, we used a novel mouse model of maternal obesity with fetal overgrowth and a maternal endocrine and metabolic profile similar to that of obese pregnant women (52) to test the hypothesis that maternal obesity activates placental insulin/IGF-1/mTOR and leptin signaling pathways.

MATERIALS AND METHODS

Animals and diets. The Institutional Animal Care and Use Committee at the University of Texas Health Science Center San Antonio approved all of the study’s protocols. Female ~12-wk-old C57BL/6J mice (n = 24), which were proven breeders (one previous litter; The Jackson Laboratory, Bar Harbor, ME), were housed five per cage under controlled conditions (25°C, 12:12-h light-dark cycle). Starting at 13 wk of age, animals were fed ad libitum with a control (D12450B, 10.6 kcal/100 kcal) or high-fat pellet diet (Western diet D12951B, 41 kcal/100 kcal) supplemented with ad libitum access to sucrose (20%) solution (high fat/high sugar, HF/HS) (52). The sucrose solution was supplemented with vitamins (Vitamin Mix V10001, 10 g/4,000 kcal) and minerals (Mineral Mix S10001, 35 g/4,000 kcal). Diets and vitamin and mineral mixes were purchased from Research Diets (New Brunswick, NJ). All animals had free access to water. When females on the HF/HS diet had increased their body weight by 25%, which occurred after 4–6 wk on the diet, they, and age-matched females on the control diet, were mated by overnight housing with a male on a control diet. On the next morning, mating was confirmed by the presence of a vaginal plug [defined as embryonic day (E) 0.5], and dams were maintained on their respective diets throughout gestation. At E18.5, dams were euthanized for collection of tissue samples.

Collection of placental tissue. Dams were fasted (4 h) and then euthanized at E18.5 by carbon dioxide inhalation. After laparotomy, fetuses and placentas were collected and quickly dried on blotting paper; any remaining fetal membranes were removed and weighed. All placentas in each litter were pooled and washed in PBS and transferred to 3 ml of buffer D [250 mM sucrose, 10 mM HEPES-Tris, and 1 mM EDTA (pH 7.4) at 4°C], protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) was added at a dilution of 1:1,000, and the mixture was homogenized using a Polytron (Kinetica, Bohemia, NY), frozen in liquid nitrogen, and stored at −80°C until analysis.

Western blot analysis. Western blot analysis was performed as previously described (32). In brief, 10 μg of total protein were loaded onto a SDS-PAGE, and electrophoresis was performed at a constant 100 V for 2 h. Proteins were transferred onto nitrocellulose membranes overnight at a constant 30 V. After the transfer, membranes were blocked in 5% blotting grade blocker nonfat dry milk (Bio-Rad, Hercules, CA) in TBS (wt/vol) plus 0.1% Tween 20 (vol/vol) for 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4°C. Subsequently, membranes were incubated with the appropriate secondary peroxidase-labeled antibodies for 1 h. After washing, bands were visualized using enhanced chemiluminescence detection reagents (GE Healthcare, Chalfont, St. Giles, Buckinghamshire, UK). Blots were stripped as described previously (51) and reprobed for β-actin as a loading control. Analysis of the blots was performed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). For each protein target, the mean density of the control sample bands was assigned an arbitrary value of 1, and data are presented relative to control. The relative density of the target protein in each lane was divided by the density of the corresponding β-actin band as a loading control. Furthermore, β-actin did not differ between control and HF/HS group (data not shown).

Data presentation and statistics. Data are presented as means ± SE. For fetal and placental data, means of each litter were calculated and used in the statistical analysis. Therefore, n represents the number of litters. Statistical significance of differences between control and HF/HS groups was assessed using Student’s unpaired t-test. A P value <0.05 was considered significant.

RESULTS

Fetal and placental weights. In the present study, placental samples were used from dams fed a control and HF/HS diet in which maternal metabolism and placental amino acid transport have been reported previously (52). Fetal weights were increased by 18% (P > 0.01; n = 12 in each group) at E18.5 in the HF/HS group compared with control (52). This was not due to a difference in litter size, which was essentially the same in the control (6.9 ± 0.14; n = 12) and HF/HS group (6.8 ± 0.32; n = 12). Placental weights were not different between groups.

Placental mTORC1 signaling is activated by maternal HF/HS diet. To test the hypothesis that placental mTOR signaling is increased by feeding the dams a HF/HS diet, we determined the phosphorylation of rpS6 and 4E-BP1, two well-established functional readouts for mTORC1 signaling. The phosphorylation of ribosomal protein S6 (S-235/236), a component of the 40S ribosome and a physiologically relevant S6K1 substrate, was increased by more than 150% (P < 0.001, Fig. 1, A and B) in the HF/HS diet group compared with the control group. Similarly, feeding of HF/HS diet significantly
Increased the phosphorylation of 4E-BP1 at T-37/46 (+ 89%, \( P < 0.001 \), Fig. 2, A and B) compared with controls. There was no significant difference in the total S6 and 4E-BP1 expression level between control and HF/HS group placenta (Figs. 1 and 2, A and B).

**Maternal HF/HS diet inhibits placental AMPK phosphorylation.** To investigate which upstream signaling pathways could influence mTORC1 activity in the placenta of dams fed an HF/HS diet, we measured AMPK activation. Phosphorylation of AMPK\( \alpha \) at T-172, a functional readout of AMPK activity, was significantly lower in the placenta of dams fed a HF/HS diet (−75%, \( P < 0.0001 \)). There was no significant difference in the total placental AMPK expression between control and HF/HS groups (Fig. 3, A and B).

**Maternal HF/HS diet activates placental insulin/IGF-I signaling.** Placental insulin/IGF-I signaling activity was assessed by determining phosphorylation of insulin receptor substrate 1 (IRS-1) and Akt (Fig. 4, A and B). Phosphorylation of IRS-1 at Y-608 (+ 50%, \( P < 0.001 \)) and Akt at T-308 (+ 90%, \( P < 0.001 \)) was significantly higher in the placenta of dams fed a HF/HS diet compared with controls. There was no significant difference in the total IRS-1 or Akt expression between the two groups.

**Placental JNK, IkB, and caspase-1 inflammatory pathways are not regulated by maternal HF/HS diet.** We tested the hypothesis that feeding the dams a HF/HS diet leads to placental inflammation. We studied the expression of caspase-1 (analyzing 50- and 20-kDa bands together), IkB\( \alpha \), JNK\(^{46/54-T183/Y185}\), and IL-1\( \beta \) in the placental homogenates of control and HF/HS groups. The phosphorylation or total expression of these targets did not differ between the two groups (Fig. 5, A and B).

**Maternal HF/HS diet activates placental STAT3 signaling.** We have previously reported increased circulating leptin levels in dams fed a HF/HS diet (52), and STAT3 is a key mediator of intracellular signaling downstream of the leptin receptor. Phosphorylation of STAT3 at Y-705 was significantly higher in the placenta of dams fed an HF diet (+ 60%, \( P < 0.001 \)) compared with controls. Total STAT3 expression was not significantly different between the control and HF/HS groups (Fig. 6, A and B).

**DISCUSSION**

In this study, we demonstrate that placental insulin, mTOR, and STAT3 signaling pathways, all known to be positive regulators of placental amino acid transporters, are activated in a mouse model of obesity in pregnancy, which is associated with fetal overgrowth. We propose that these pathways stimulate placental amino acid transport and contribute to increased fetal growth.

We developed the mouse model of obesity in pregnancy used in the current study to resemble maternal obesity in women, and a number of previously published observations suggest that it is relevant for the human condition. First, maternal obesity was induced by feeding mice a high-fat diet supplemented with high sugar (52), which is comparable with the diet in overweight/obese women (reviewed in Refs. 19, 23, 43). Second, in this model, maternal obesity is associated with fetal overgrowth (52), which is a common clinical outcome in obese women (6, 16, 20, 39, 55). Third, maternal endocrine changes (increased fasting serum insulin and leptin, decreased
adiponectin) and changes in placental nutrient transport (increased amino acid and glucose transport capacity) (52) replicate findings in obese women giving birth to large babies (27). Moreover, the activation of placental insulin and mTOR signaling and inhibition of AMPK signaling reported here are strikingly similar to changes in placentas of large infants of obese mothers (27). Therefore, this model addresses a critical need for an animal model of obesity in pregnancy that is clinically relevant.

Placental signaling pathways linking maternal nutrition and metabolism to changes in nutrient transport may include mTOR, which is regulated by a wide range of factors, including amino acids, glucose, oxygen, energy status, and insulin/IGF-I, leptin, and TNF-α signaling (59). In the current study, we show that phosphorylation of ribosomal protein S6 and 4E-BP1, representing well-established functional readouts of the mTORC1 signaling pathway, was increased in placentas of HF/HS-fed mice, consistent with the elevated circulating levels of maternal insulin in obese dams that we reported previously (52). Maternal serum leptin levels are also increased in obese dams (52), which may contribute to the increased phosphorylation of STAT3 in placentas of obese dams observed in the present study. These signaling changes may contribute to the increased placental amino acid transport in maternal obesity (27, 52) because insulin stimulates placental system A and L amino acid transport in cultured primary human trophoblast cells (31, 49), and insulin and leptin have been reported to stimulate system A amino acid transport in placental villous fragments (25). Moreover, we have previously reported that activation of STAT3 by IL-6 stimulates system A amino acid transport in cultured trophoblast cells (30).

Placental AMPK phosphorylation in mice fed a control (C) or HF/HS diet. A: representative Western blots of AMPK (T-172) and total AMPK in homogenates of mice placenta at E18.5. B: summary of the Western blot data. After normalization to β-actin, the mean density of control samples was assigned an arbitrary value of 1. Subsequently, individual control and HF/HS density values were expressed relative to this mean. Values are given as means ± SE; n = 12/each group, *P < 0.05 vs. control, using unpaired Student’s t-test.

Placental IRS-1 and Akt phosphorylation in mice fed a control (C) or HF/HS diet. A: representative Western blots of IRS-1 (Y-608), Akt (T-308), and total IRS-1/Akt in homogenates of mice placenta at E18.5. B: summary of the Western blot data. After normalization to β-actin, the mean density of control samples was assigned an arbitrary value of 1. Subsequently, individual control and HF/HS density values were expressed relative to this mean. Values are given as means ± SE; n = 12/each group, *P < 0.05 vs. control, using unpaired Student’s t-test.
Sex differences in the rate of fetal growth have long been recognized (40) and the sex of the fetus may affect the ability of the placenta to respond to adverse stimuli (12, 42, 44, 56). Because placentas were pooled for isolation of trophoblast plasma membranes for subsequent analysis of amino acid transport activity (52), we were not able to separate our findings, according to fetal sex or study placental morphology. One potential limitation of this study is, therefore, that sex-specific effects of maternal obesity on placental function and morphology were not examined. Furthermore, the current study does not address the question of whether the marked effects on placental signaling are caused by the high-fat diet, the obesogenic metabolic environment, or a combination of these factors. In addition, it remains to be demonstrated that fetuses of HF/HS dams have increased adiposity. As with all animal studies, extrapolation of findings in this mouse model to pregnant women with obesity has to be done with caution. The mouse has a large litter and differences in maturity at birth (13), and placental structure (8) between humans and mice introduce some limitations in using the mouse as a model for human pregnancy. However, the extensive functional similarities between the mouse and human placentas (15) suggest that this animal model is relevant for the human.

**Perspectives and Significance**

Our findings indicate that HF/HS diet-induced maternal obesity in mice activates placental insulin/mTOR and STAT3 signaling. Given the well-established role of mTOR as a positive regulator of placental amino acid transport (53), we...
propose that the activation of placental mTOR in our HF/HS model increases placental amino acid transport capacity (52). We have previously reported that this mouse model of maternal obesity induced by an HF/HS diet shares many characteristics with human pregnancies complicated by obesity, including high dietary fat and sugar intake, maternal hyperleptinemia, hyperinsulinemia, and increased placental amino acid nutrient transfer and fetal overgrowth (52). In this study, we demonstrate that our mouse model of maternal obesity and fetal overgrowth is associated with changes in placental signaling that closely resembles the signaling changes in placentas of obese women giving birth to large babies (27). Obesity and metabolic syndrome may, in part, originate in fetal life. In particular, babies of mothers with obesity are often large at birth and have increased adiposity, which predisposes them for the development of metabolic disease later in life (9, 22). This model will help us better understand the impact of maternal overweight/obesity on placental function and pregnancy outcomes.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
Author contributions: F.J.R., T.L.P., and T.J. conception and design of research; F.J.R. performed experiments; F.J.R. analyzed data; F.J.R., T.L.P., and T.J. interpreted results of experiments; F.J.R., T.L.P., and T.J. drafted manuscript; F.J.R., T.L.P., and T.J. edited and revised manuscript; F.J.R., T.L.P., and T.J. approved final version of manuscript.

REFERENCES


