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

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Abstract

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 \textit{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 \textit{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 S6 ribosomal protein (S-235/236), 4E-BP1 (T-37/46), IRS-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 \textsuperscript{p46/54 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.

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Introduction

Maternal obesity during pregnancy is associated with an 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). LGA 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 the pregnant woman with high 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 its 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 (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-alpha serine/threonine-protein kinase (Akt) activation (60). Upstream regulators of mTOR activity include the insulin/phosphatidylinositide 3-kinases (PI3K)/Akt signal transduction pathway (24), the AMP-activated protein kinase (AMPK), leptin (46) and the extracellular signal-regulated kinase (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 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 non-pregnant 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 tumor necrosis factor α (TNFα), monocyte chemoattractant protein-1 (MCP-1), interleukin-1 (IL-1) and IL-6 (5, 7, 11, 47), as compared to 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 (nuclear factor-κB), JNK (c-Jun N-terminal kinase), caspase-1 and p38-MAPK (mitogen-activated protein kinase), which are activated by Toll-like receptor (TLR) 4 signaling cascade, and STAT3 (signal transducer and activator of transcription 3). The cellular effects of leptin are largely mediated by the JAK/STAT signaling pathway, which includes the phosphorylation of signal transducer and activator of transcription 3 (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 non-placental cells (17, 37). In cultured primary human trophoblast cells (PHTs) 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) as compared to 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-I/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 protocols. Female C57BL/6J mice (n=24), proven breeders (one previous litter) and approximately 12 weeks old (The Jackson Laboratory, Bar Harbor, ME, USA) were housed 5 per cage under controlled conditions (25°C, 12-hour light/dark cycle). Starting at 13 weeks of age, animals were fed *ad libitum* with a control (D12489B, 10.6 kcal% fat) or high fat pellet diet (Western Diet D12079B, 41 kcal% fat) 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 gm/ 4000 kcal) and minerals (Mineral Mix S10001, 35 gm/ 4000 kcal). Diets, vitamin and mineral mixes were purchased from Research Diets (New Brunswick, NJ, USA). 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 weeks on the diet, they, and age-matched females on control diet, were mated by overnight housing with a male on 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 hr) 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 phosphate buffer saline 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 Corp., St. Louis, MO, USA) was added at a dilution of 1:1000, and the mixture was homogenized using a Polytron (Kinematica, Bohemia, NY, USA), 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 (Biorad, CA) in Tris-buffered saline (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, 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, USA). 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 beta-actin band as a loading control. Furthermore, beta-actin did not differ between control and HF/HS group (data not shown).

**Data presentation and statistics**

Data are presented as means ± 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. 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 as compared to 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
+150% (p<0.001, Figure 1 a & b) in the HF/HS diet group compared to the control group.

Similarly, feeding of HF/HS diet significantly increased the phosphorylation of 4E-BP1 at T-37/46 (+ 89 %, P<0.001, Figure 2 a & b) as compared to controls. There was no significant difference in the total S6 and 4E-BP1 expression level between control and HF/HS group placentas (Figure 1-2 a & 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 AMPKα 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 (Figure 3 a & b).

**Maternal HF/HS diet activates placental insulin/IGF-I signaling**

Placental insulin/IGF-I signaling activity was assessed by determining phosphorylation of IRS-1 and Akt (Figure 4 a & 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 placentas of dams fed a HF/HS diet as compared to controls. There was no significant difference in the total IRS-1 or Akt expression between the two groups.

**Placental JNK, IκB 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), IκBα, JNKp46/54 T183/Y185 and IL-1β 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 (Figure 5a & b).

Maternal HF/HS diet activates placental STAT-3 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 STAT-3 at Y-705 was significantly higher in the placenta of dams fed an HF diet (+60%, P < 0.001) as compared to controls. Total STAT3 expression was not significantly different between the control and HF/HS groups (Figure 6a & 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 reminiscent of the diet in overweight/obese women (reviewed in 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). This model therefore 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 and energy status, insulin/IGF-I, leptin, and tumor necrosis factor (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 an activation of placental mTOR signaling in obese women giving birth to larger babies (27). These findings are also in agreement with placental mTOR activation and increased fetal growth in a model of maternal overweight in the rat (18). We recently demonstrated that mTOR regulates system A and L amino acid transport activity by modulating cell surface abundance of SNAT-2 and LAT-1 isoforms in cultured primary human trophoblast cells (53), providing one possible mechanism underlying the increase in expression of system A and L isoforms in the trophoblast plasma membrane in obese dams (52). To identify upstream signals activating mTOR signaling in placentas of obese dams, we studied AMPK, which is the primary cellular energy sensor and is phosphorylated at Thr-172 in response to increased AMP/ATP ratio. Activation of AMPK is a known negative regulator of mTOR signaling (37) and we have shown previously that placental AMPK is down-regulated in a rat model of maternal overweight and increased fetal growth (18). In this study we demonstrated that the activity of placental AMPK was markedly decreased in maternal obesity induced by HF/HS diet, in agreement with findings in obese women (27). It is
therefore possible that the activation of placental mTORC1 signaling in placentas of obese mice is caused, in part, by AMPK inhibition.

The increased phosphorylation of placental IRS-1 and Akt, well established functional readouts of insulin/IGF-I signaling, in response to maternal obesity is 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 has 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).

Activation of inflammatory pathways by TNFα increases mTOR signaling activity in cell lines of non-placental origin (38). Furthermore, we have reported previously that physiological concentrations of IL-6 and TNF-α stimulate amino acid transport in cultured primary human trophoblast cells (30). Some studies suggest that maternal obesity is associated with chronic low-grade inflammation (10). A variety of cytokines, including, interleukin 1 beta, interleukin 6 and 10, monocyte chemoattractant protein 1 (MCP1), interferon gamma, and TNFα have been observed to be elevated in the maternal circulation in association with obesity in pregnancy in both humans and rodents (34, 35). We found only modest changes in circulating inflammatory markers in obese pregnant women (5). In addition, although maternal obesity in women was associated with increased placental STAT3 and p38MAPK activity, classical inflammatory
pathways such as JNK and NFκB were not activated in the placenta (5). In our mouse model of obesity, we found little evidence of placental inflammation, suggesting that inflammatory signals are unlikely to cause activation of mTOR signaling or placental amino acid transport.

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 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.
References


Figure Legends

Figure 1. Placental S6 phosphorylation in mice fed a control (C) or HF/HS diet. Representative Western blots of S6 (S-235/236) and total S6 in homogenates of mice placenta at E18.5 (a). Summary of the western blot data (b). 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 ± SEM; n=12/each group, *P < 0.05 vs. control; unpaired Student's t test.

Figure 2. Placental 4E-BP1 phosphorylation in mice fed a control (C) or HF/HS diet. Representative Western blots of 4E-BP1 (T-37/46) and total 4E-BP1 in homogenates of mice placenta at E18.5 (a). Summary of the western blot data (b). 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 ± SEM; n=12/each group, *P < 0.05 vs. control; unpaired Student's t test.

Figure 3. Placental AMPK phosphorylation in mice fed a control (C) or HF/HS diet. Representative Western blots of AMPK (T-172) and total AMPK in homogenates of mice placenta at E18.5 (a). Summary of the western blot data (b). 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 ± SEM; n=12/each group, *P < 0.05 vs. control; unpaired Student's t test.
Figure 4. Placental IRS-1 and Akt phosphorylation in mice fed a control (C) or HF/HS diet. Representative Western blots of IRS-1 (Y-608), Akt (T-308) and total IRS-1/Akt in homogenates of mice placenta at E18.5 (a). Summary of the western blot data (b). 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 ± SEM; n=12/each group, *P < 0.05 vs. control; unpaired Student's t test.

Figure 5. Placental of caspase-1, IκBα, JNK\(^{p46/54 \text{T183/Y185}}\) and IL-1β expression mice fed a control (C) or HF/HS diet. Representative Western blots of caspase-1, IκBα, JNK\(^{p46/54 \text{T183/Y185}}\) and IL-1β in homogenates of mice placenta at E18.5 (a). Summary of the western blot data (b). 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 ± SEM; n=12/each group, *P < 0.05 vs. control; unpaired Student's t test.

Figure 6. Placental phosphorylated STAT-3 expression mice fed a control (C) or HF/HS diet. Representative Western blots of STAT-3 (Y-705) and total STAT-3 in homogenates of mice placenta at E18.5 (a). Summary of the western blot data (b). 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 ± SEM; n=12/each group, *P < 0.05 vs. control; unpaired Student's t test.
Figure 1
Figure 2

**a)**

- **C**
- **HF/HS**
- **C**
- **HF/HS**

- **4E-BP1**
- **T-37/46**
- **4E-BP1**
- **β-actin**

**b)**

- **C**
- **HF/HS**

**Relative density**

- **4E-BP1**
- **T-37/46**
- **4E-BP1**

*Statistical significance*
Figure 3
Figure 4
Figure 5
Figure 6