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Maternal Overweight Induced by a Diet with High Content of Saturated Fat Activates Placental mTOR and eIF2alpha Signaling and Increases Fetal Growth in Rats[1]

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ABSTRACT

The mammalian target of rapamycin (mTOR) and the eukaryotic initiation factor 2 (eIF2) signaling pathways control protein synthesis in response to nutrient availability. Moreover, mTOR is a positive regulator of placental nutrient transport and is involved in the regulation of fetal growth. We hypothesized that maternal overweight, induced by a diet with high saturated fat content, i) up-regulates placental mTOR activity and nutrient transport, resulting in fetal overgrowth; ii) inhibits phosphorylation of eIF2 at its alpha subunit (eIF2alpha); and iii) leads to placental inflammation. Albino Wistar female rats were fed a control or high-saturated-fat (HF) diet for 7 wk before mating and during pregnancy. At Gestational Day 21, the HF diet significantly increased maternal and fetal triglyceride, leptin, and insulin (but not glucose) levels and maternal and fetal weights, and placental weights trended to increase. Phosphorylated 4EBP1 (T37/46 and S65) was significantly higher, and phosphorylated rpS6 (S235/236) tended to increase, in the placentas of dams fed an HF diet, indicating an activation of mTOR Complex 1 (mTORC1). Phosphorylation of AMPK and eIF2alpha was reduced in the HF diet group compared to the control. The expression and activity of placental nutrient transporters and lipoprotein lipase (LPL), as well as the activation of inflammatory pathways, were not altered by the maternal diet. We conclude that maternal overweight induced by an HF diet stimulates mTORC1 activity and decreases eIF2alpha phosphorylation in rat placentas. We speculate that these changes may up-regulate protein synthesis and contribute to placental and fetal overgrowth.

fetal overgrowth, maternal obesity, placental inflammation, placental transport, pregnancy

INTRODUCTION

Nearly two thirds of women of reproductive age in the United States are currently overweight or obese (body mass index [BMI] $\geq$ 25) [1], and similar trends have been observed worldwide. Maternal overweight/obesity is associated with pregnancy complications, such as stillbirth and birth injury, and increased risk of delivering both a growth-restricted and a large-for-gestational-age (LGA; >90th percentile) or macrosomic (>4000 g) baby [2–5]. Fetal overgrowth is associated with increased incidence of perinatal complications and development of obesity, diabetes, and cardiovascular disease in childhood and later in life [6]. The mechanisms linking maternal overweight/obesity to fetal overgrowth are not well established, but altered placental metabolism and enhanced placental nutrient transport capacity may contribute [7, 8].

Mammalian target of rapamycin (mTOR) is a central integrator of various signals, such as growth factors, nutrients, energy, and stress, and it is present in the cytoplasm in two complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2) [9]. Activation of mTORC1 increases the phosphorylation of its downstream targets, eIF4E-binding protein 1 (4EBP1), p70 S6 kinase (S6K), and ribosomal protein S6 (rpS6), thus leading to an up-regulation of cap-dependent protein translation [10, 11]. Mammalian TORC2 has been reported to be involved in the regulation of actin cytoskeleton and cell survival through Akt activation [9]. Upstream regulators of mTOR activity include the insulin/PI3K/Akt signal transduction pathway [12], the AMP-activated protein kinase (AMPK) [13], and the extracellular signal-regulated kinase (ERK) 1/2 pathway [14]. The mechanisms by which AMPK regulates mTORC1 activity are complex [15–17] and include the phosphorylation of Tuberin, product of the TSC2 (tuberous sclerosis 2) gene [13], and Raptor [18].

Another master regulator of protein synthesis is eukaryotic initiation factor 2 (eIF2), which plays a major role in protein translation initiation. Phosphorylation of eIF2 at its alpha-subunit (eIF2α) is induced by several stress conditions, such as amino acid deprivation and endoplasmic reticulum (ER) stress, and results in inhibition of global protein synthesis [19].

Placental activity of mTORC1 and eIF2α has been linked to the nutritional, metabolic, and physiological state of the mother. Both these proteins have been shown to be involved in regulating placental development and function (such as transport capacity) and are, therefore, ultimately associated with fetal growth. For example, placental mTORC1 activity increases in association with maternal obesity and fetal overgrowth [20, 21] and decreases in intrauterine growth restriction (IUGR) [22] in human pregnancies. Moreover, mTORC1 is a positive regulator of System A and L amino acid transporters in cultured primary trophoblast cells [23–25] and villous explants [22] from human placenta. In pregnancies at high altitude, which are associated with chronic hypobaric hypoxia and low birth weights, phosphorylation of placental eIF2α and ER stress were increased, and placental protein synthesis and cell proliferation decreased [26].

The placenta plays a critical role in providing an adequate supply of nutrients necessary for optimal fetal growth and

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development. System A is a sodium-dependent transporter that mediates the uptake of small, nonessential neutral amino acids [27]. The three System A isoforms, sodium-coupled neutral amino acid transporter (SNAT)1, SNAT2, and SNAT4 are all expressed in the placenta [28]. System A activity is critical also for placental transport of essential amino acids because it establishes the high intracellular concentration of nonessential amino acids that are then used to drive the exchange for extracellular essential amino acids via System L [29]. System L consists of a light chain (L-type amino acid transporter [LAT] 1 or LAT2) and a heavy chain (4F2hc/CD98). The activity of placental amino acid transporters System L and System A has been associated with maternal nutritional status [7, 8], with maternal overnutrition and undernutrition being linked to increased and decreased placental amino acid transport, respectively [30, 31]. Moreover, placental System L and System A activities are decreased in IUGR pregnancies [32–35] and increased with fetal overgrowth [20, 30, 36] in humans and relevant animal models. Placental transport of glucose occurs via facilitated diffusion mediated by glucose transporters (GLUTs), which transport glucose down its concentration gradient. Whereas GLUT1 is believed to be the predominant transporter mediating transplacental glucose transfer in humans, both GLUT1 and GLUT3 are involved in rodents [37]. We previously demonstrated that GLUT1 expression was increased in trophoblast plasma membranes (TPMs) isolated from mice fed a high-fat diet [30]. Fatty acids are released from maternal lipoproteins by placental lipases, including lipoprotein lipase (LPL), and transferred across the placenta by facilitated transport mediated by fatty acid transport proteins (FATPs) [38, 39]. Zhu et al. [40] recently reported increased expression of placental fatty acid transporters in diet-induced obesity in sheep.

Inflammatory responses to pregnancy have been reported to be exaggerated in maternal obesity [41]. In the placenta, inflammatory pathways may be activated in response to inflammatory stimuli or through infiltration of maternal immune cells with subsequent increased cytokine production [42]. The key intracellular mediators of the inflammatory response include NF-κB (nuclear factor-κB), JNK (c-Jun N-terminal kinase), and p38-MAPK (mitogen-activated protein kinase), which are activated by Toll-like receptor (TLR) 4 signaling cascade [43], and STAT3 (signal transducer and activator of transcription 3). An increasing body of evidence suggests that dietary factors, such as elevated circulating levels of lipids, may also be involved in the induction of placental inflammation by maternal obesity [44]. Interestingly, Lager et al. [45] recently demonstrated that the activation of TLR4 by free fatty acids increases System A transport activity in cultured trophoblast cells, providing a possible link between maternal hyperlipidemia and increased placental nutrient transport capacity.

We have developed a model of maternal overweight in which albino Wistar rats are fed a diet rich in saturated fat [46]. In the current study, we used this model to test the hypotheses that maternal overweight i) up-regulates placental mTOR activity and nutrient transport, resulting in fetal overgrowth; ii) inhibits eIF2α phosphorylation; and iii) activates placental inflammatory pathways.

MATERIALS AND METHODS

Animals and Diets

Albino Wistar rats were housed in the animal facility of CEFYBO-CONICET Institute (School of Medicine, University of Buenos Aires, Argentina) at a controlled temperature of 20°C with a 14L:10D cycle and free access to commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina) and tap water. The rats were provided by the Central Animal Facilities of the Faculty of Science (University of Buenos Aires), and the guidelines for animal care approved by the local institution were followed, according to the *Principles of Laboratory Animal Care* (NIH publication number 85-23, revised 1985, http://grants1.nih.gov/grants/olaw/references/phspol.htm). At 6 wk of age, female rats were divided into two groups and fed either the standard rat chow (control diet group [CON]; nutritional composition in 100 g of diet: 25 g protein, 50 g carbohydrate, and 5 g fat; 352 Tkcal/100 g; n = 9) or standard rat chow supplemented with 25% of saturated animal fat (high-saturated-fat [HF] diet group; butter supplementation; SanCor Co.; nutritional composition in 100 g of diet: 21 g protein, 38 g carbohydrate, and 28 g fat; 505 kcal/100 g; n = 10) for 7 wk before mating with standard diet-fed males and throughout pregnancy. Pregnancy was confirmed by the presence of sperm cells in vaginal smears, which was defined as Gestational Day (GD) 1.

Metabolic Assays

Glucose concentrations in maternal and fetal blood were measured with Accu-Chek reagent strips and glucometer (Bayer Diagnostics). Plasma maternal and fetal triglyceride levels were determined by the enzymatic TG Color kit (Wiener Lab Group). Insulin and leptin levels were measured in maternal and fetal plasma by Mercodia Ultrasensitive Rat Insulin ELISA Kit (Mercodia) and TiterZyme EIA Rat Leptin (Assay Designs), respectively, following the manufacturer's instructions.

Collection of Tissue Samples and Preparation of Placental Homogenates and TPM Vesicles

At GD21 (term: GD22), dams were fasted for 6 h and euthanized using CO_2 inhalation, followed by cervical dislocation. Maternal blood was collected in heparinized tubes from the abdominal aorta. After laparotomy, fetuses and placentas were collected and weighed. In each litter, all the pup and placental weights were used to calculate a mean weight for that litter, which then was used to further determine the mean ± SEM in each experimental group. Fetuses were killed by decapitation, and fetal blood was collected. Maternal and fetal plasma were obtained by centrifugation, and plasma was stored at −80°C for subsequent analyses. For the preparation of placental homogenates and TPM samples, all the placentas from each litter were pooled together; the n number in each group represents the number of litters, i.e., nine litters in the CON group and 10 litters in the HF diet group. The CON group had a total of 123 pups/placentas, and the HF diet group had a total of 132 pups/placentas (litter size was not significantly different between groups, as shown in Table 1). Placentas were homogenized in ice-cold buffer D (250 mM sucrose and 10 mM Hepes-Tris [pH 7.4], with protease and phosphatase inhibitors), frozen in liquid nitrogen, and stored at −80°C until use. Subsequently, homogenates for Western blot were thawed on ice and centrifuged at 13 400 × *g* for 15 min at 4°C to remove large cellular debris, and the supernatant was collected for further analysis.

TPMs were isolated from rat placental homogenates as previously described [31]. Homogenates were centrifuged at 10 000 × *g* for 15 min; pellets were resuspended, homogenized in 12 ml of buffer D, and centrifuged at 10 000 × *g* for 15 min. The resulting supernatants were combined and spun at 125 000 × *g* for 30 min. The pelleted, crude membrane fraction was resuspended in 3 ml of buffer D, and 12 mM $MgCl_2$ was added. The suspension was stirred for 20 min on ice. After centrifugation at 2500 × *g* for 10 min, the supernatant was centrifuged at 125 000 × *g* for 30 min. The final pellet was resuspended in buffer D to give a final protein concentration of 10–20 mg/ml. The protein concentration in total placental homogenates and TPM samples was determined using the Pierce BCA Protein Assay kit (Thermo Scientific). TPM enrichments were assessed using standard activity assays for alkaline phosphatase [47]. Only TPM preparations with an alkaline phosphatase enrichment of more than 10-fold compared to placental homogenates were included in the study. Enrichment of alkaline phosphatase activity was not significantly different in TPM samples obtained from CON (22.4 ± 6.4, n = 9) or HF diet (22.4 ± 5.9, n = 8) dams.

Western Blot Analysis

Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (35-V constant, overnight at 4°C). The membranes were then stained with the Amido Black staining solution for total proteins (Sigma-Aldrich) [48] to evaluate the quality of the transfer and for further normalization. Blocking was carried out for 1 h at room temperature in 5% nonfat milk in TBS-Tween, and membranes were incubated in primary antibody (diluted in 1% bovine serum albumin in TBS-Tween) overnight at

TABLE 1. Maternal, fetal, and placental characteristics at GD21.

Characteristic	Control diet (n = 9)*	High saturated fat diet (n = 10)*	$P^{\dagger}$
Maternal weight (g)	373.5 ± 17.6	448.6 ± 18.2	<0.01
Maternal blood glucose (mg/dl)	85.6 ± 5.2	88.9 ± 4.9	0.65
Maternal plasma triglycerides (g/L)	2.2 ± 0.2	4.8 ± 0.9	<0.05
Maternal plasma insulin (ng/ml)	1.3 ± 0.3	2.3 ± 0.2	<0.05
Maternal plasma leptin (ng/ml)	5 ± 0.6	7.3 ± 0.6	<0.05
Fetal weight (g)	3.48 ± 0.07	3.72 ± 0.06	<0.05
Fetal blood glucose (mg/dl)	45 ± 4.1	63.2 ± 9.2	0.10
Fetal plasma triglycerides (g/L)	0.48 ± 0.02	0.65 ± 0.02	<0.01
Fetal plasma insulin (ng/ml)	3.2 ± 0.5	6.8 ± 0.9	<0.01
Fetal plasma leptin (ng/ml)	3.1 ± 0.5	4.8 ± 0.4	<0.05
Placental weight (mg)	562.3 ± 12.6	592.1 ± 14.3	0.14
Litter size	13.7 ± 0.4	13.2 ± 1.3	0.75

* Data are expressed as mean ± SEM.
† Statistical differences were evaluated by *t*-test; $P < 0.05$ was considered statistically significant.

4°C. The expression of the following proteins in placental homogenate was determined using antibodies purchased from Cell Signaling Technology: total and phosphorylated 4EBP1 (T37/46, S65, and T70; catalog nos. 9452, 9459, 9451, and 9455), total and phosphorylated ribosomal protein S6 (rpS6, S235/236; nos. 2217 and 4858), total and phosphorylated Akt (T308 and S473; nos. 9272, 4056, and 4060), total and phosphorylated AMPKα (T172; nos. 2532 and 2535), phosphorylated TSC2 (S1387; no. 5584), total and phosphorylated ERK1/2 (T202; nos. 4695 and 4370), total and phosphorylated eIF2α (S51; nos. 5324 and 3398), phosphorylated JNK (T183/Y185; no. 4668), phosphorylated STAT3 (Y705; no. 9145), total IκBα (no. 4812), and caspase-1 (no. 2225). Protein expression of FATPs, LPL, SNATs, and GLUTs was analyzed in TPM preparations. The antibodies for FATP6 (no. ab84183), LPL (no. ab21356), and GLUT9 (no. ab82910) were purchased from Abcam. The antibody for FATP4 was obtained from Santa Cruz Biotechnology (no. sc-101271). The antibodies for GLUT1 (no. 07–1401) and GLUT3 (no. AB1344) were from Millipore. A polyclonal SNAT2 antibody generated in rabbit [49] was received as a generous gift from Dr. P. Prasad at the University of Georgia (Augusta, GA). Affinity-purified polyclonal anti-SNAT1 and -SNAT4 antibodies were generated by Eurogentec. After washing, the membranes were incubated with the appropriate peroxidase-conjugated secondary antibody and visualized using enhanced chemiluminescence detection solution (Thermo Scientific) and a G:BOX gel imaging system (Syngene). Densitometry analysis was performed with ImageJ software (http://rsbweb.nih.gov/ij/; National Institutes of Health). The expression of the target protein in each individual lane was normalized for the total protein staining to adjust for unequal loading. The mean of all the samples within a single gel was calculated and set to 100%; the expression of the target protein in each sample was then calculated as a percentage of that mean.

TPM Amino Acid Transporter Activity Measurements

The activity of the System A and System L amino acid transporters in TPM vesicles was determined as described previously [33], with minor modifications. TPM vesicles were preloaded by incubation in 300 mM mannitol and 10 mM Hepes-Tris (pH 7.4) overnight at 4°C. Subsequently, vesicles were pelleted and resuspended in a small volume of the same buffer to a final protein concentration of 5–10 mg/ml. After warming the samples to 37°C, 30 μl of vesicles were rapidly mixed (1:2), with the appropriate incubation buffer including ^{14}C-methyl-aminoisobutyric acid (MeAIB; 150 μM) or ^{3}H-L-leucine (0.375 μM). After 15 sec, the uptake of radio-labeled amino acids was terminated by addition of 2 ml ice-cold PBS. Subsequently, vesicles were rapidly separated from the substrate medium by filtration on mixed ester filters (0.45-μm pore size; Millipore) and washed with 4 × 2-ml ice-cold PBS. Filters were dissolved in 2 ml liquid scintillation fluid and counted. Protein content of the vesicles was determined by the Pierce BCA Protein Assay kit (Thermo Scientific), and uptakes were expressed as picomoles per milligram of protein per 15 sec. In all uptake experiments, each condition was studied in triplicate. In studies of MeAIB transport, 150 mM NaCl or 150 mM KCl was used in incubation buffers to assess total and sodium-independent uptake, respectively: Na^{+}-dependent uptake of MeAIB (corresponding to System A activity) was then calculated by subtracting Na^{+}-independent uptake from total uptake. In leucine transport experiments, the nonmediated flux was determined in the presence of 30 mM unlabeled L-leucine, and mediated uptake was calculated by subtracting nonmediated transport from total uptake.

LPL Activity Assay

LPL activity was measured in TPM samples by Roar LPL Activity Assay Kit (Roar Biomedical, Inc.) following the manufacturer's instructions. After performing a titration curve with increasing quantities of TPM and incubation times (data not shown), we used 8 μg of sample per well and an incubation time of 30 min for the remaining experiments. Each sample was analyzed in duplicate, and the results are expressed as nanomoles of substrate hydrolyzed per milligram of protein.

Data Presentation and Statistical Analysis

Data are presented as mean and SEM. GraphPad Prism 5 was used for the statistical analysis (GraphPad Software). Statistical differences between two groups were evaluated by unpaired, two-tailed *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Maternal, Fetal, and Placental Characteristics

Maternal, fetal, and placental characteristics are presented in Table 1. Maternal (+20%, $P < 0.01$) and fetal (+6%, $P < 0.05$) weights were significantly higher in the HF diet group than in the control group, though the increase in placental weight failed to reach statistical significance (+5%, $P = 0.14$). Litter size was not different between groups. Plasma triglyceride, insulin, and leptin levels were elevated in the HF diet dams and in their pups. Maternal and fetal blood glucose concentrations were not significantly altered by the HF diet, indicating that the dams were not diabetic.

Placental mTORC1 Signaling Is Activated by Maternal HF Diet

To test the hypothesis that placental mTOR signaling is increased by feeding the dams an HF diet, we determined the phosphorylation of rpS6 and 4EBP1, two well-established functional readouts for mTORC1 signaling. Phosphorylation of 4EBP1 at T37/46 and S65 was significantly higher (+39% and +43%, respectively, $P < 0.05$), and phosphorylation of rpS6 at S235/236 was trending toward an increase (+48%, $P = 0.07$), in the HF diet group compared to in the control group. Levels of phosphorylated 4EBP1 at T70, total 4EBP1, and total rpS6 were not different between groups (Fig. 1).

The Activity of Placental AMPK, but Not Akt or ERK1/2, Is Inhibited in Response to HF Diet

To investigate which upstream signaling pathway could influence mTORC1 activity in the placenta of dams fed an HF

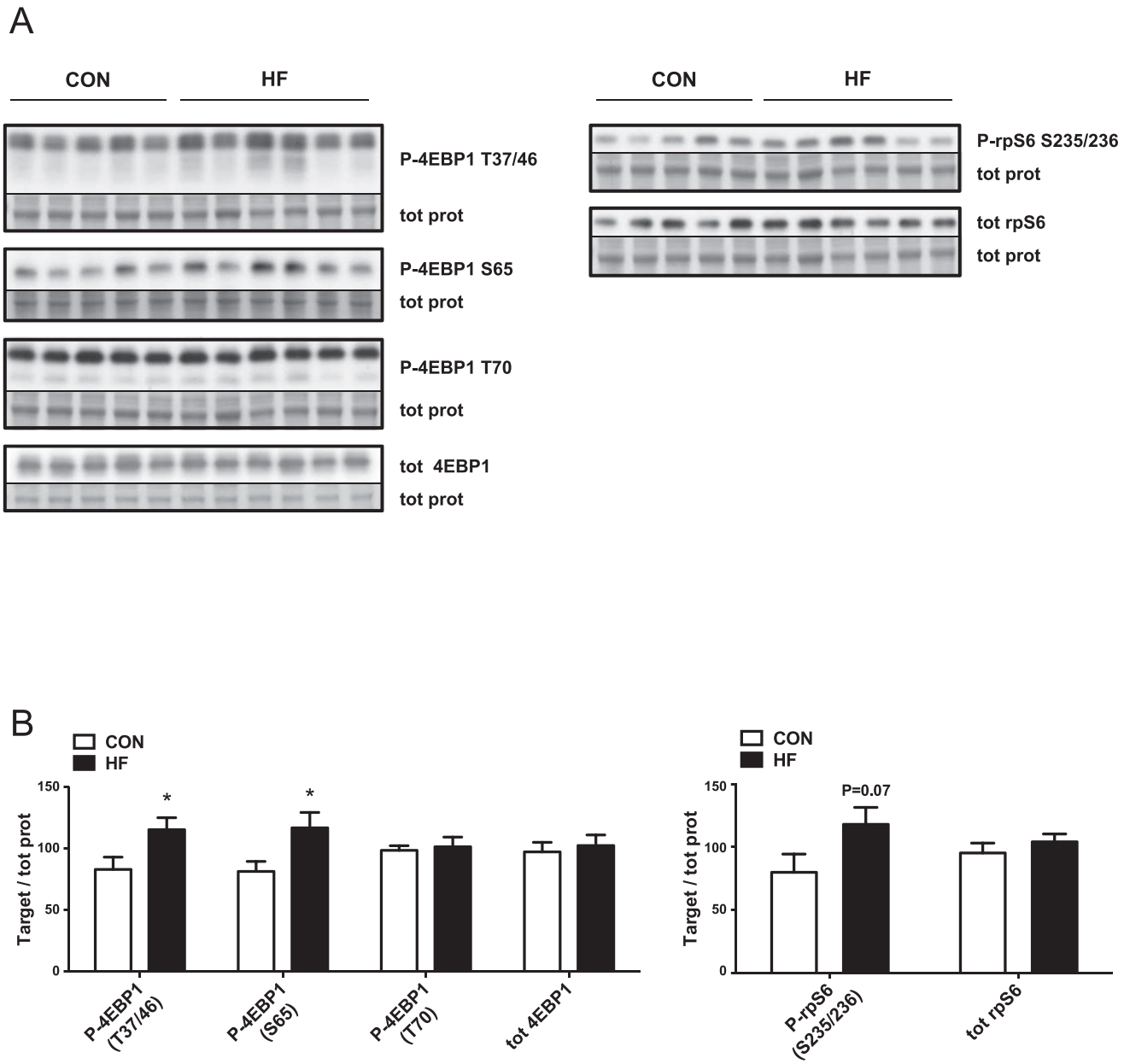

FIG. 1. Placental protein expression of total and phosphorylated 4EBP1 and rpS6 in response to an HF diet. Expression of total and phosphorylated 4EBP1 (T37/46, S65, and T70) and rpS6 (S235/236) was analyzed by Western blot in the placenta of dams fed a CON or HF diet. **A**) Representative Western blots. **B**) Summary of the data, mean + SEM. CON, n = 9; HF, n = 10. *$P < 0.05$.

diet, we studied the phosphorylation of AMPKα at T172 (stimulated by increased intracellular AMP levels), Akt at T308 (indicator of insulin/IGF-I signaling activity), and S473 (readout for mTORC2 activity) and ERK1/2 at T202 (member of the MAPK signal transduction cascade). Phosphorylation of AMPKα at T172 was significantly lower in the placenta of dams fed an HF diet (−28%, $P < 0.01$). AMPK is known to be a negative regulator of mTORC1 mediated by numerous mechanisms, including phosphorylation of TSC2 at S1345; however, placental TSC2 phosphorylation (S1345) did not differ between the two groups (data not shown). Phosphorylation of placental Akt and ERK1/2 as well as the expression of total AMPK, Akt, and ERK1/2 was not influenced by maternal diet (Fig. 2).

Maternal HF Diet Decreases Placental eIF2α Phosphorylation

We evaluated the total expression and phosphorylation of placental eIF2α, which is regulated by various cellular stresses such as viral infection, heat shock, nutrient deprivation, and accumulation of unfolded or denatured proteins. We observed a significant decrease (−35%, $P = 0.01$) of phosphorylated eIF2α in placentas of dams fed an HF diet compared to those of controls, whereas the levels of total eIF2α remained unchanged between the two groups (Fig. 3).

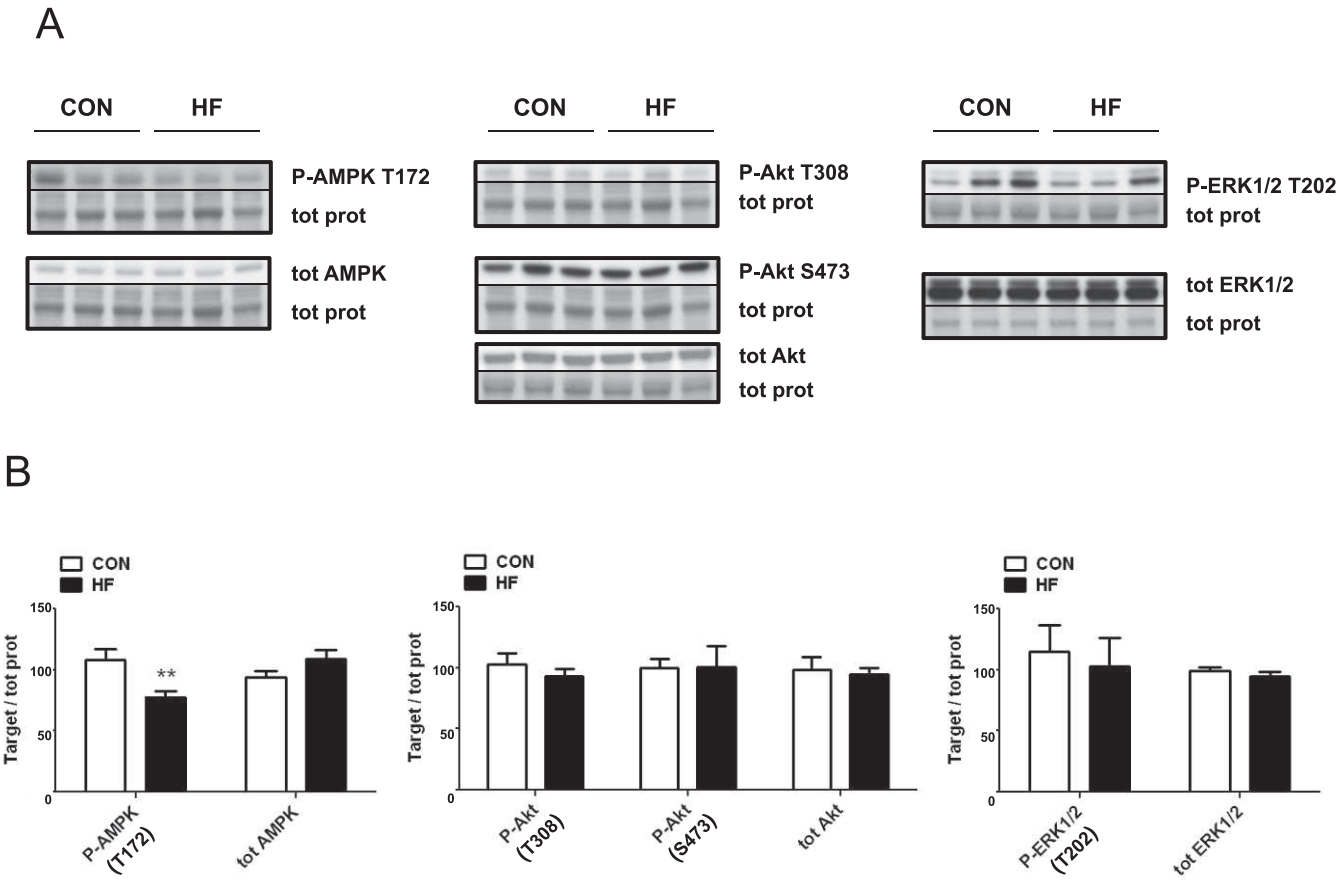

FIG. 2. Phosphorylation and total levels of upstream regulators of mTORC1 in the placenta in response to an HF diet. **A**) Representative Western blots showing phosphorylation and total levels of placental AMPK, Akt, and ERK1/2 in the CON and HF groups. **B**) Summary of data, mean + SEM. CON, n = 9; HF, n = 10. $^{**}P < 0.01$.

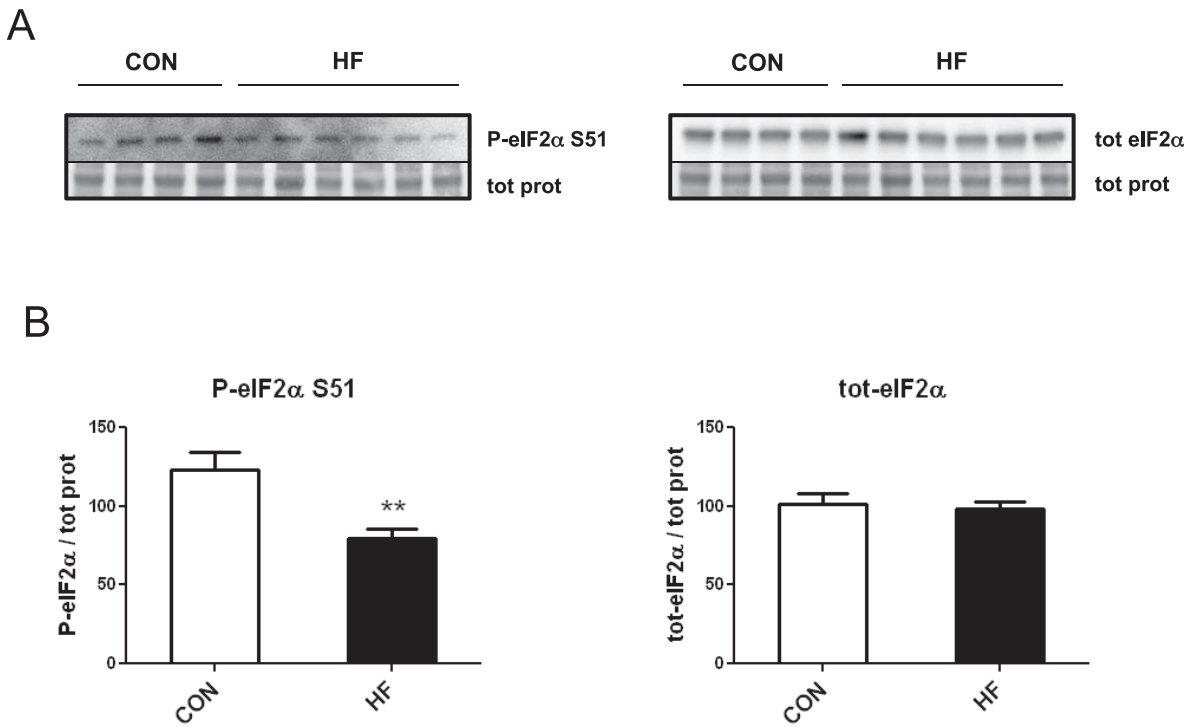

FIG. 3. Expression and phosphorylation of placental eIF2α in response to an HF diet. **A**) Representative Western blots showing phosphorylation and total levels of placental eIF2α in the CON and HF groups. **B**) Summary of data, mean + SEM. CON, n = 9; HF, n = 10. $^{**}P < 0.01$.

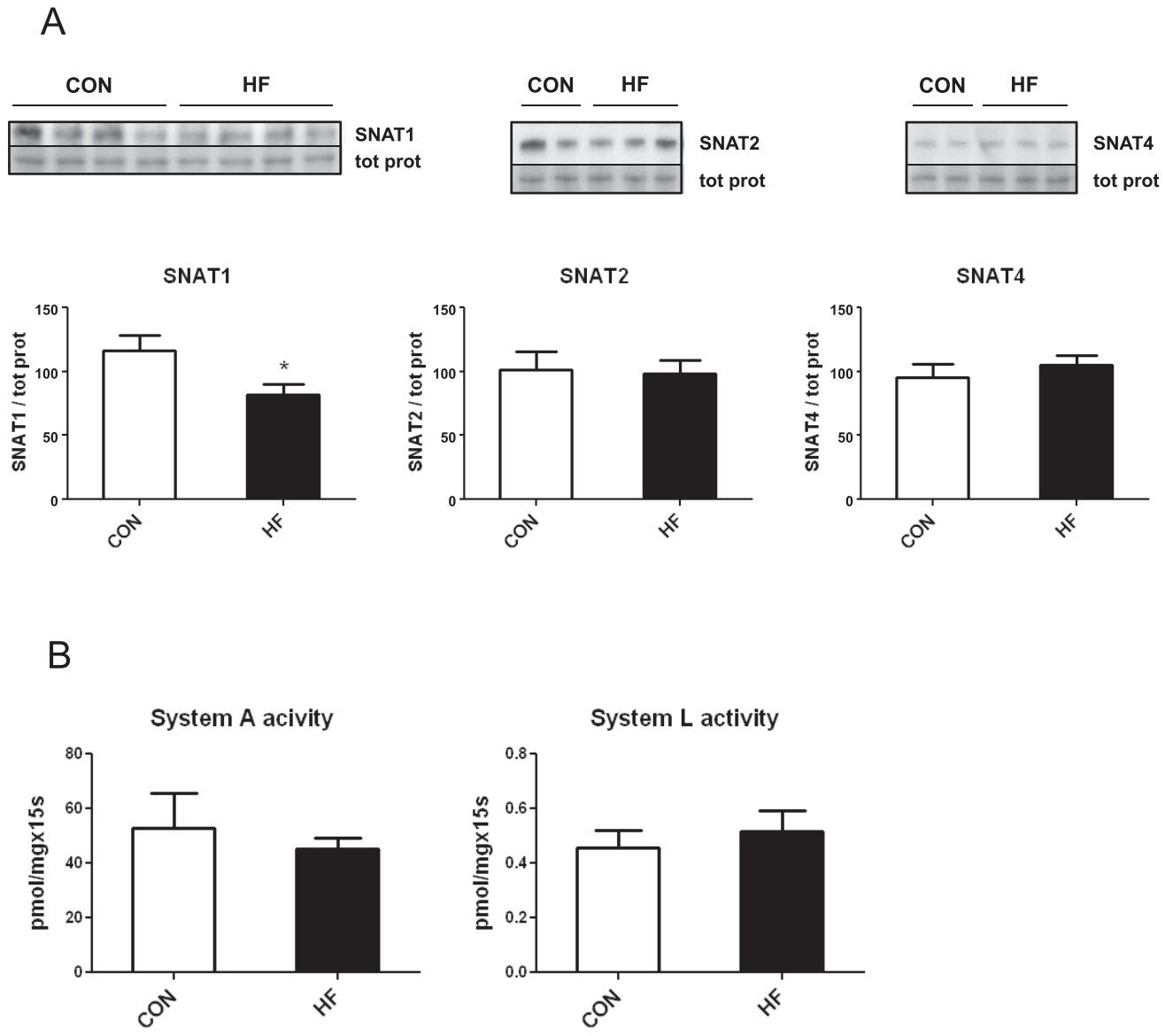

FIG. 4. Protein expression of System A isoforms and amino acid transport activity in TPM in response to an HF diet. **A**) Representative Western blots showing TPM expression of SNAT1, SNAT2, and SNAT4 in response to maternal diet. Bar graphs summarize the Western blot data. **B**) System A transport activity was determined as sodium-dependent [^{14}C]-MeAIB uptake, and System L transport was measured as [^{3}H]-L-leucine uptake in isolated TPM vescicles. MeAIB, 2-methylaminoisobutyric acid; Leu, leucine. CON, n = 9; HF, n = 10. Results are shown as mean + SEM. *$P < 0.05$.

Effect of Maternal HF Diet on the Activity and/or Expression of Proteins Involved in Placental Amino Acid, Glucose, and Fatty Acid Transport

We examined the effect of maternal HF diet on the expression and/or activity of proteins involved in the maternal-fetal nutrient exchange of amino acids, glucose, and fatty acids. We determined the expression of System A amino acid transporters SNAT1, SNAT2, and SNAT4 in TPM isolated from placentas of dams fed the CON or the HF diet, and we detected a significant decrease in SNAT1 expression in the HF diet group, whereas the expression of SNAT2 and SNAT4 was not influenced by maternal diet (Fig. 4A). System A and System L amino acid transport activity was not different between groups (Fig. 4B). The TPM expression of glucose transporters GLUT1, GLUT3, and GLUT9 and of LPL and of fatty acid transporters FATP4 and FATP6 did not change in response to maternal HF diet (Figs. 5 and 6). Consistent with the results of the protein expression levels, LPL activity in the TPM was not different between groups (Fig. 6B).

Placental JNK, IκB, STAT3, and Caspase-1 Inflammatory Pathways Are Not Regulated by Maternal HF Diet

We tested the hypothesis that feeding the dams an HF diet leads to placental inflammation. We studied the phosphorylation of JNK and STAT3 and the expression of IκB and cleaved caspase-1. The phosphorylation or expression of these targets did not differ between the two groups (Fig. 7).

DISCUSSION

The novel finding in this report is that maternal overweight in the rat, induced by a diet with high saturated fat content, inhibits AMPK and activates mTORC1 signaling in the placenta. In addition, maternal overweight is associated with

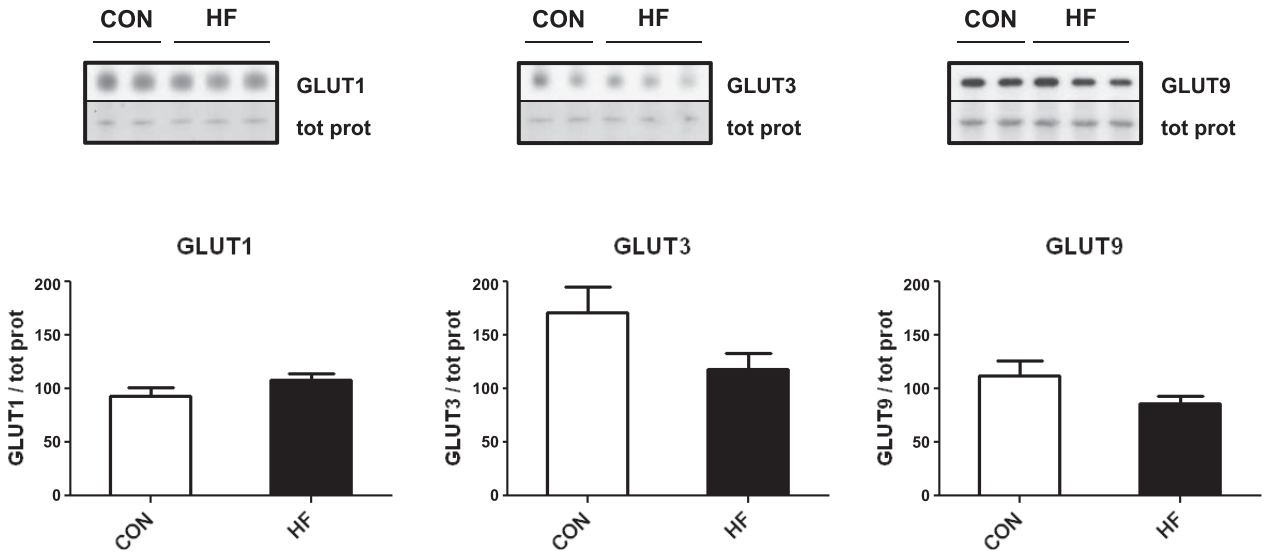

FIG. 5. TPM protein expression of glucose transporters in response to an HF diet. Western blots showing expression of GLUT1, GLUT3, and GLUT9 in TPM samples from dams fed a CON or HF diet. Bar graphs summarize the Western blot data, mean + SEM. CON, n = 9; HF, n = 10.

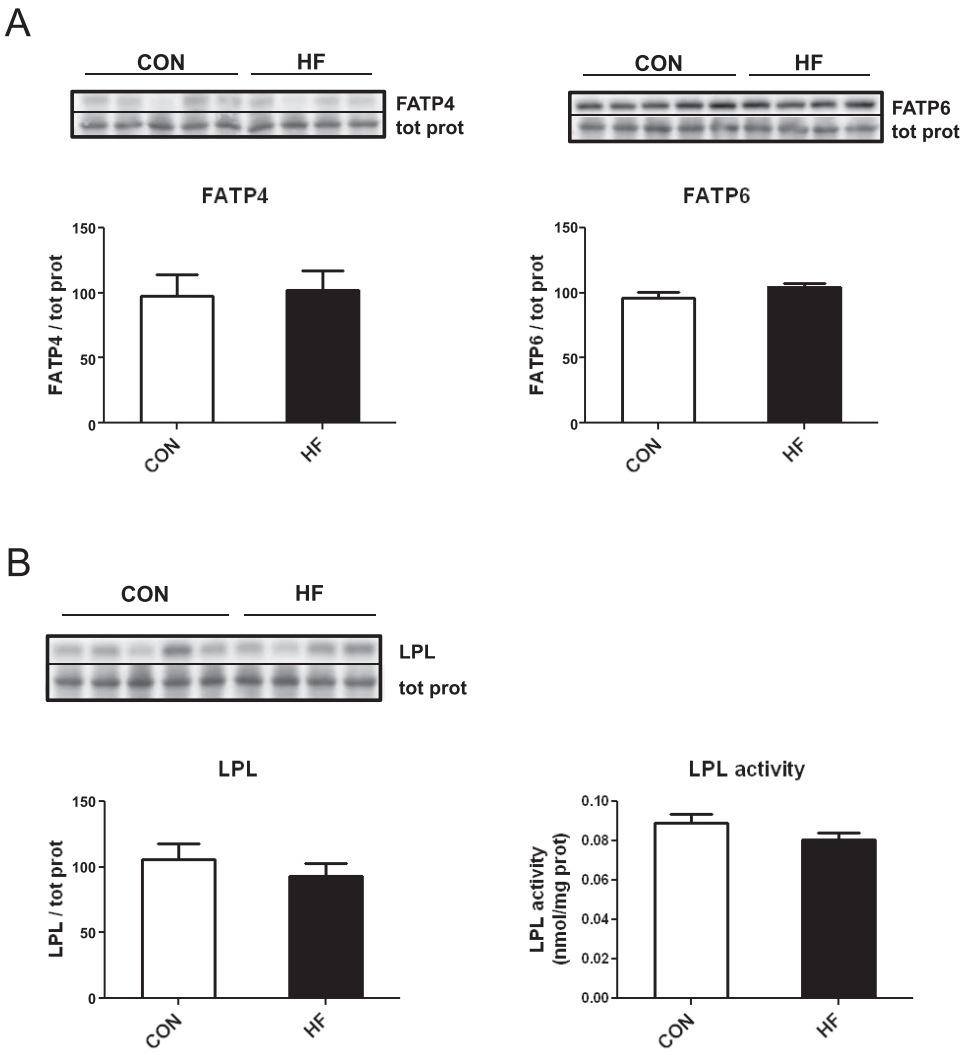

FIG. 6. Protein levels of FATPs and expression/activity of LPL in TPM in response to an HF diet. **A**) Western blots showing TPM expression of FATP4 and FATP6 in response to maternal diet. **B**) LPL expression and activity in TPM samples isolated from dams fed a CON or HF diet. Bar graphs summarize the data. CON, n = 9; HF, n = 10. Results are shown as mean + SEM.

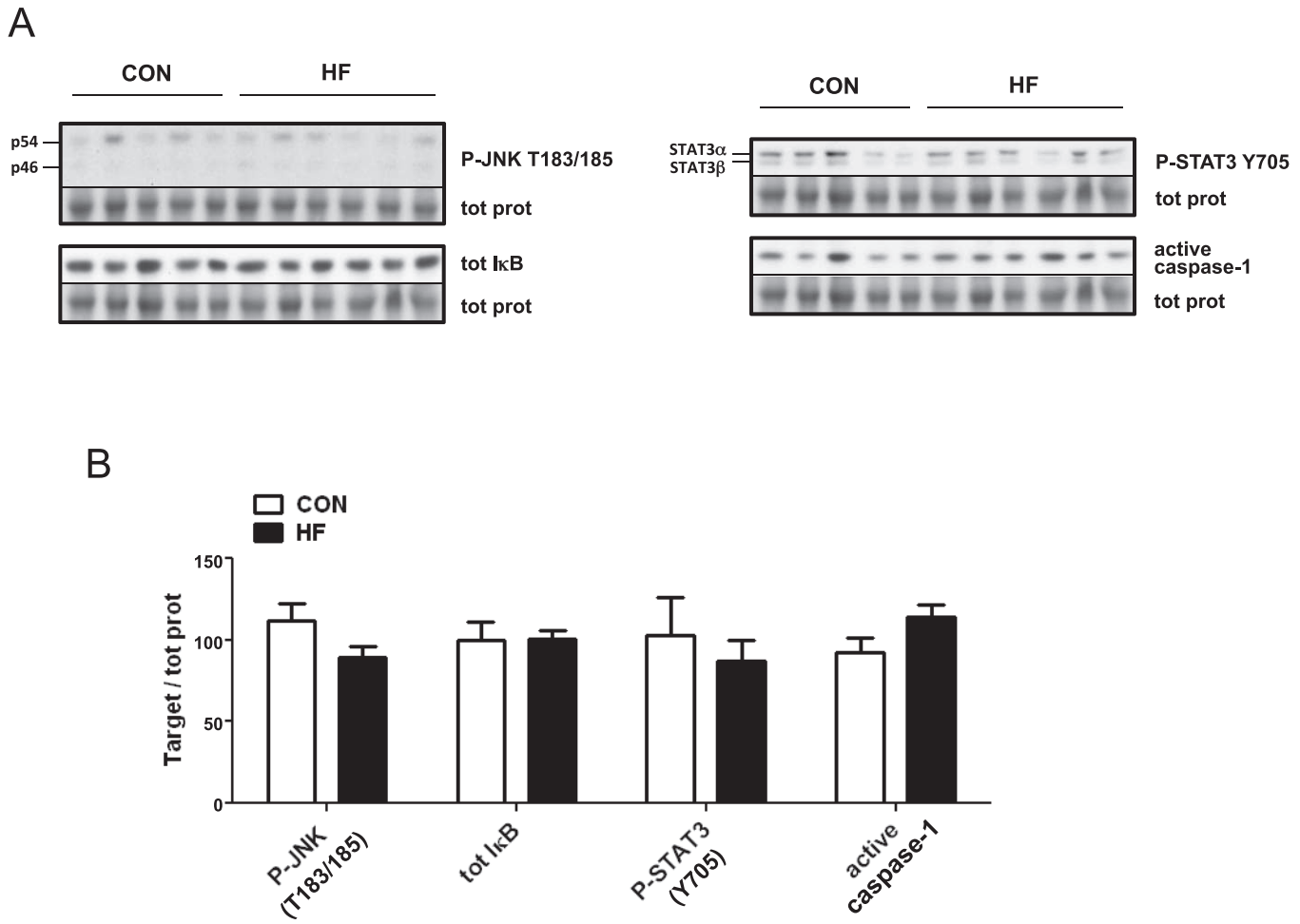

FIG. 7. Phosphorylation or expression of placental inflammatory markers in response to an HF diet. **A**) Representative Western blots showing placental expression of phosphorylated JNK (p54 and p46) and STAT3 (STAT3α and STAT3β) and total levels of IκB and active caspase-1 in response to maternal diet. **B**) Summary of data, mean + SEM. CON, n = 9; HF, n = 10.

a decreased phosphorylation of placental eIF2α. Because mTORC1 stimulates and eIF2α phosphorylation inhibits protein synthesis, these findings are consistent with an increased placental protein synthesis in response to maternal overweight, which could contribute to placental and fetal overgrowth.

Maternal overweight was induced by feeding rats a standard chow supplemented with saturated fat. The relevance of this model is underlined by the fact that overweight and obese individuals typically have a high dietary fat intake [50], and increasing numbers of women enter pregnancy overweight or obese. Most of the high-fat diets used to model human obesity in animals have a percentage of calories from fat ranging between 55% and 60%. We developed a butter-supplemented diet with a more modest (47%) proportion of fat to total calories [46]. Importantly, this HF diet is associated with fetal overgrowth, a common outcome in pregnancies of overweight and obese women [2, 3, 51], providing further support for the clinical relevance of this model. Moreover, dams fed the HF diet had a marked increased in maternal circulating levels of triglycerides, whereas glucose levels were not different in the two experimental groups. Thus, our model resembles overweight/obese pregnant women without diabetes. Interestingly, the HF diet induced fetal hypertriglyceridemia, which may lead to increased fetal adiposity and contribute to the increased fetal weights in this group.

We previously proposed that the placenta functions as a nutrient sensor, regulating the flux of nutrients to the fetus by integrating signals on maternal nutrient availability and fetal demand [7, 52]. At the molecular level, this function could be exerted by mTOR and/or eIF2α, two key regulators of cellular metabolism controlled by nutrient availability [53–55]. We demonstrated that in the placenta of dams fed the HF diet, the expression of phosphorylated 4EBP1 (at T37/46 and S65) is significantly higher and phosphorylation of rpS6 (at S235/236) trended toward an increase. These data are in agreement with previous findings [20, 31, 54] suggesting that placental mTORC1 signaling is involved in sensing the nutritional/metabolic/endocrine status of the mother. Moreover, it has been demonstrated that the hierarchical phosphorylation of 4EBP1, with the first phosphorylation step (at T37/46) priming the protein for the second phosphorylation event (at S65 or T70), results in the release of eIF4E and stimulation of translation [56]. In contrast, maternal HF diet did not affect mTORC2 signaling, as assessed by the phosphorylation of Akt at S473 [57]. Furthermore, phosphorylation of placental eIF2α at S51 was significantly lower in the HF group compared to that in the control group. Phosphorylated eIF2α acts as an inhibitor of its guanine nucleotide exchange factor eIF2B, leading to translation inhibition. Thus, both the activation of mTOR, a well-established stimulator of protein synthesis, and

decreased eIF2α phosphorylation in response to HF diet are expected to activate protein synthesis.

While the role of eIF2α as an amino acid sensor has been established in other tissues, very little is known about the role of eIF2α in the adaptation of placental function to nutrient availability. Yung et al. [26, 58] demonstrated an increased phosphorylation of placental eIF2α in pregnancies of nonnative women residing at high altitude and in pregnancies complicated by IUGR with or without preeclampsia. Their data suggest that both of these complications are characterized by placental ER stress, which increases phosphorylation of eIF2α and down-regulates protein synthesis, thus contributing to the altered placental structure and function often observed in these pregnancies. Moreover, in Sprague-Dawley rats, eIF2α phosphorylation is significantly increased in the placentas of dams fed a low-protein diet compared to their control counterparts [59]. Our data are in general agreement with these previous findings, suggesting a positive association between stress/undernutrition and eIF2α phosphorylation. The lower placental eIF2α phosphorylation in the HF group may be due to lower levels of ER stress in the placentas of mothers fed an HF diet.

In order to identify upstream regulators of mTORC1 activity, we analyzed AMPK, Akt, and ERK1/2 pathways. High intracellular AMP levels activate the energy-sensing kinase AMPK [60], which is a well-known inhibitor of mTOR signaling [13]. We found that AMPK phosphorylation was significantly lower in the placentas from dams fed the HF diet, thus providing a potential mechanism for placental mTORC1 activation by diet-induced maternal overweight. Although the lack of change in TSC2 phosphorylation (S1345) does not support a role of AMPK inhibition in activation of placental mTOR signaling in response to high-fat diet, there are other possible mechanisms linking the AMPK and the mTORC1 signaling pathways, such as mTOR phosphorylation (T2446) [15], p53 [61], and Raptor phosphorylation (S722/792) [18]. Thus, the potential effect of AMPK inhibition on the mTOR signaling pathway in placentas of dams fed an HF diet requires further investigation. Our data are in agreement with a recent report showing a negative correlation between placental AMPK activity and maternal BMI and birth weight [20]. In the same study, placental mTOR activity was increased in obese mothers giving birth to large babies. The observation that placental AMPK is inhibited in response to a high-fat diet is also in line with previous animal studies that reported decreased AMPK activity in the kidney of C57BL/6J mice fed a high-fat diet [62]. Similarly, in rats on an HF diet, decreased AMPK activity was reported in white adipose tissue, heart, and liver in the absence of systemic inflammation, hyperglycemia, or elevated free fatty acids [63]. Consistent with this model, we found that an HF diet was associated with maternal hyperleptinemia, hyperinsulinemia, and euglycemia. We did not examine the mechanisms involved in the down-regulation of placental AMPK activity by the HF diet, but changes in upstream modulators of AMPK activity, such as LKB-1 (liver kinase B-1), which is activated by low AMP/ATP ratios, and CaMKK (calcium calmodulin-dependent protein kinase kinase), could be involved [60]. Mammalian TOR activity is also regulated by the PI3K/Akt and MAPK/ERK signal transduction pathways, which are regulated by insulin/IGF-I levels and other growth factors [12, 64]. We did not detect any differences in the phosphorylation of placental Akt (T308), functional readout for insulin/IGF-1 signaling, or ERK1/2 (T202) in the two groups. These results indicate that the insulin/IGF-1 and MAPK/ERK signaling pathways are not up-regulated by a maternal HF diet in our rat model and do not appear to be involved in the activation of mTORC1 by the HF diet. Moreover, our data suggest that diet-induced maternal overweight is associated with placental insulin resistance, because the HF diet led to a significant increase in maternal and fetal insulin circulating levels but no activation of insulin signaling pathways in the placenta.

Recent data demonstrated that placental mTOR constitutes a positive regulator of trophoblast amino acid transporters in vitro [23, 24]. Moreover, studies in animals [31] and humans [20, 22] suggest that placental mTOR functions as a nutrient sensor, matching fetal growth with maternal nutritional status by regulating placental nutrient transport [53, 54]. Our results indicate that maternal overweight induced by HF diet does not significantly alter the TPM expression or activity of amino acid transporters (although we detected a significantly lower expression of SNAT1 isoform) or the expression of glucose or fatty acid transporters. Despite these findings, fetal plasma triglycerides were significantly elevated in the HF diet group. We speculate that, in this group, the high levels of maternal plasma triglycerides result in an increased facilitated transport of fatty acids across the placenta mediated by FATPs [65, 66], which contributes to fetal hypertriglyceridemia.

Maternal obesity has been associated with placental inflammation resulting in an elevated production of pro-inflammatory cytokines by trophoblast cells and expanded infiltration of maternal immune cells, such as natural killer cells, dendritic cells, and macrophages [43, 67]. The elevated circulating levels of lipids observed in pregnancies complicated by maternal obesity could contribute to placental inflammation through the activation of TLR4 [44]. Together, the high cytokine and lipid levels may induce inflammatory signaling pathways, including JNK, NFκB, p38-MAPK, caspase-1, and STAT3, leading to the transcription of inflammatory related genes. However, in this study we did not detect any difference in the activation/status of any of these pathways between the two groups, suggesting that the HF diet alone does not induce placental inflammation in our model of maternal overweight. Because macrophage accumulation and inflammation in the human placenta has only been observed in morbidly obese women (BMI $>$ 40) [42], it is possible that more significant maternal adiposity than in our animal model is required to activate placental inflammatory pathways.

In conclusion, our findings indicate that HF diet-induced maternal overweight activates mTORC1, possibly through the inhibition of AMPK, and down-regulates eIF2α phosphorylation in rat placentas. We speculate that these changes may up-regulate protein synthesis and contribute to the modest placental and fetal overgrowth. This rat model of maternal overweight induced by an HF diet shares many characteristics with human pregnancies complicated by overweight and obesity, including high dietary fat intake, maternal hypertriglyceridemia, hyperleptinemia and hyperinsulinemia, activation of placental mTOR signaling, inhibition of placental AMPK, and fetal overgrowth. We expect that this model will help us better understand the impact of maternal overweight/obesity on placental function and pregnancy outcomes.

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