Saturated fat-rich diet increases fetal lipids and modulates LPL and leptin receptor expression in rat placentas

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Abstract

Metabolic alterations in obese and overweight mothers impact the placenta and the fetus, leading to anomalies in fetal growth and lipid accretion. The primary aim of the study was to examine the effect of a saturated fat-rich diet (FD) on growth, lipid accretion, and lipases, leptin and leptin receptor (ObR) expression in the placenta and fetal liver. We also aimed to find a role for fetal leptin in the modulation of placental and fetal liver lipase and ObR expression. Six-week-old rats were fed with a standard rat chow (control) or a 25% FD for 7 weeks until mating and during pregnancy. Also, in a group of control rats, fetuses were injected with leptin on days 19, 20, and 21 of pregnancy. On day 21, we assessed lipidemia, insulinemia, and leptinemia in mothers and fetuses. In the placenta and fetal liver, lipid concentration was assessed by thin layer chromatography (TLC) and the gene expression of lipoprotein lipase (LPL), endothelial lipase, insulin receptor (*Insr*), leptin, and ObR by RT-PCR. The FD induced hypertriglyceridemia and hyperleptinemia ($P<0.01$) in mothers and fetuses, an increase in maternal ($P<0.05$) and fetal weight ($P<0.01$), overaccumulation of lipids in fetal liver ($P<0.01$), and enhanced leptin expression in the placenta and fetal liver ($P<0.05$). Placental expression of IR and LPL was increased ($P<0.05$), and ObR decreased ($P<0.05$) in the FD group. Fetal administration of leptin induced the placental and fetal liver downregulation of ObR ($P<0.05$) and upregulation of LPL expression ($P<0.05$). The FD led to increased fetal lipid levels, which may result from high maternal lipid availability and fetal leptin effects.

Introduction

Obesity and overweight have been widely studied because they entail serious medical problems such as non-alcoholic fatty liver disease, cardiovascular disease, stroke, and endothelial dysfunction (Lteif *et al.* 2005, Chen *et al.* 2006, Bell 2009, Khoshdel *et al.* 2012). Maternal obesity and overweight affect fetal growth and development. These metabolic impairments will impact the mechanisms that will regulate metabolism in adulthood, programming the development of metabolic and cardiovascular diseases in the following generations (Taylor & Poston 2007, Fernandez-Twinn 2012). Physiological maternal insulin resistance is exacerbated by obesity and overweight and contributes to maternal adiposity and hyperlipidemia, which leads to enhanced lipid transfer through the placenta to the developing fetus.

Table 1 Nutritional composition in 100 g diet

	Standard diet	FD	FD (% from total calories)
Proteins (g)	25	22	19
Carbohydrates (g)	50	40	35
Fat (g)	5	27	47
Energy (kcal)	352	512	

FD, saturated fat-enriched diet.

The placenta acts like a supplier and an exchange organ of oxygen, nutrients, and water. It also provides hormones and growing factors that modulate fetal and placental growth and development (Jones *et al.* 2007, Sibley 2009). Thus, placental expression and the activity of specific transporters will determine the maternal–fetal transport of nutrients. Lipids esterified in triglycerides, phospholipids, and cholesteryl esters are carried as lipoproteins in the maternal circulation. To be transported to the fetal circulation, lipids have to be hydrolyzed to small and simple molecules. Lipases, such as lipoprotein lipase (LPL) and endothelial lipase (EL), are key proteins expressed on the surface of trophoblast cells, where they hydrolyze esterified lipids from lipoproteins, so that they can be acquired by the placenta (Herrera *et al.* 1987, Gauster *et al.* 2007). Expression and activity of these transporters is a limiting step in lipid transport through the placenta to the fetus (Jones *et al.* 2007, Duttaroy 2009). Increased lipid circulating levels both in mothers and fetuses, and fetal tissue accumulation of lipids, are related to fetal overgrowth, the development of macrosomia, and the programming of metabolic alterations in adulthood (Buckley *et al.* 2005, Jansson *et al.* 2006, Shankar *et al.* 2008, Misra *et al.* 2011).

Insulin and leptin are hormones that modulate metabolism and growth. Insulin receptor (INSR) has two isoforms: INSRa, the shortest one, which lacks the 36 bp exon 11, and INSRb, the longer isoform. Both isoforms trigger metabolic and mitogenic effects (Yamaguchi *et al.* 1991), although INSRa has more affinity for IGF2 and has been found in fetal tissues and cancer. This suggests a growth-promoting effect of INSRa, while INSRb has been found to be increased in tissues from insulin resistance and diabetes experimental models (Frasca *et al.* 1999, Sesti *et al.* 2001).

Leptin is produced mainly by adipocytes in response to insulin signaling and lipid accumulation (Maiorana *et al.* 2007). As a result of alternative splicing, leptin receptor (*Lepr*) has six isoforms (a–f) (Tartaglia 1997). The e isoform is the soluble one, the b isoform is the longest splice variant with complete signal transduction capacity, and the others (a, c, d, and f) are short isoforms with a truncated carboxy-terminal cytoplasmic tail. Signaling transduction comprises several mechanisms that are shared by cytokines such as interleukins 2–7 (Fruhbeck 2006). Although *Leprb* is the isoform that displays all the signaling pathways, the *Lepra* isoform has also a signaling ability. Indeed, *Lepra* induces the expression of transcription factors *in vitro* and a reduction in body weight *in vivo* (Murakami *et al.* 1997, Grasso *et al.* 1999). The a and b isoforms are expressed in the rat placenta, showing an increment in their expression at term gestation, suggesting an important role of leptin during gestation and at term pregnancy (Smith & Waddell 2002, Szczepankiewicz *et al.* 2006).

In the human placenta, leptin is expressed in response to insulin, human chorionic gonadotropin, and estradiol and induces placental growth (Coya *et al.* 2001, Maymo *et al.* 2011). Leptin induces lypolysis and fatty acid oxidation in adipocytes, muscle, and placenta (Muoio *et al.* 1997, Fruhbeck & Gomez-Ambrosi 2001, White *et al.* 2006). Also, leptin enhances placental amino acid transport to the fetus (Jansson *et al.* 2003, von Versen-Hoynck *et al.* 2009). These facts indicate an ability of leptin to modulate the maternal-to-fetal transport of nutrients and suggest that leptin is a key molecule in the feto-maternal dialog, responding to fetal nutritional requirements (Forhead & Fowden 2009).

Table 2 Primer sequences and optimum cycle number for each primer pair used for gene expression analysis by semi-quantitative PCR in the placenta and fetal liver

Genes	Forward primer (5′–3′)	Reverse primer (5′–3′)	Placenta	Fetal liver
Insr	CGAGTGCTGCTCATGTCCTA	GTGGAGGAGATGTTGGGAAA	35	36
L30	CCATCTTGGCGTCTGATCTT	TGGCGAGGATAACCAATTTC	24	26
Lep	TTCACACACGCAGTCGGTAT	TGAGCTATCTGCAGCACGTT	30	38
Lepra	GTTCCTGGGCACAAGGACTTAAT	ACTGTTGGGAGGTTGGTAGATTG	34	35
Leprb	GTTCCTGGGCACAAGGACTTAAT	GGTTCCCTGGGTGCTCTGA	35	–
Lpl	CCCTAAGGACCCCTGAAGAC	GGCCCGATACAACCAGTCTA	32	28
El	ACCAGAGTGGTGGGACGTAG	GGACAGCCTCCTGTTGATGT	30	–
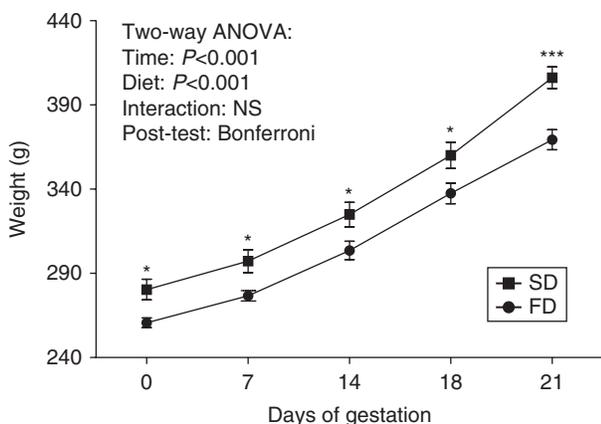
Figure 1
Maternal weight during gestation in the control rats (SD) and the saturated fat-rich diet-fed group (FD). $n=11$ in each experimental group. Statistical differences between the SD and FD groups: $*P<0.05$, $***P<0.001$.

The excess of saturated fat in meals leads the general population to obesity and overweight. Based on this, we aimed to establish an experimental model of rats fed with a diet enriched only with 25% of saturated fat, and a normal protein and carbohydrate content, from puberty to term pregnancy. Further, we aimed to investigate the effects of a saturated fat-rich maternal diet on placental and fetal liver lipid concentrations, LPL, EL leptin, and ObR expression, as well as to investigate the role of leptin in placental lipid transport to the developing fetus.

Materials and methods

Animals

Albino Wistar rats were bred in our laboratory with free access to a commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina) and tap water. The animals were kept in a room at a controlled temperature of 20 °C, with 14 h light:10 h darkness lighting cycles. The guidelines for the care and use of animals approved by the local institution were followed, according to the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985, http://grants1.nih.gov/grants/olaw/references/phspol.htm).

Diet At 6 weeks of age, female rats were categorized into two groups: the control group, fed with a commercial standard rat chow (SD), and the saturated fat-enriched diet (FD), fed with a standard rat chow enriched with 25% of saturated animal fat provided by supplementation with

butter (Sancor Co., Buenos Aires, Argentina). The food composition is detailed in Table 1. The SD and FD rats were fed with the corresponding diet seven weeks before mating and continued the diet throughout pregnancy.

Experimental design

The SD and FD females of 13 weeks of age were caged with control males. The presence of sperm cells in vaginal smears confirmed the pregnant state (day 1 of pregnancy). The animals were allowed to develop pregnancy until day 21 of gestation, when the mothers were shortly anesthetized in a CO_2 chamber. After blood collection from the abdominal aorta in heparinized tubes with heparinized syringes, the animals were killed by cervical dislocation and fetuses and placentas were immediately removed. Fetuses were killed by decapitation and fetal blood was collected in heparinized tubes. Maternal and fetal plasma was obtained by blood centrifugation, and conserved at −80 °C for further determinations. Placentas were weighed and kept at −80 °C for lipid analysis or immediately stored in RNAlater (Ambion, Carlsbad, CA, USA) and kept at −20 °C for gene expression analysis. Fetuses were weighed and fetal organs were carefully removed under a stereomicroscope using microsurgical dissecting instruments. Fetal organs were also weighed and fetal livers kept at −80 °C for lipid analysis or fixed in RNAlater (Ambion) and kept at −20 °C.

Fetal administration of leptin Aiming to establish the role of fetal leptin in the modulation of the placental and fetal liver expression of *Lepr*, *Insr*, *EL*, and *LPL*, we administered leptin to fetuses at term gestation, as described previously (Martinez *et al*. 2011). On day 19 of gestation, a group of control mothers were shortly

Table 3 Metabolic parameters in rats fed with the standard diet (SD) and the saturated fat-enriched diet (FD). $n=11$ in each experimental group. Fetal plasma was pooled from every fetus of each litter

	SD	FD
Maternal glycemia (mg/dl)	82.1±2.9	85.9±2.1
Maternal triglyceridemia (g/l)	2.15±0.31	4.04±0.59[†]
Maternal cholesterolemia (g/l)	1.16±0.07	1.17±0.09
Fetal glycemia (mg/dl)	44.16±4.13	61.59±6.45*
Fetal triglyceridemia (g/l)	0.51±0.08	0.90±0.07[†]
Fetal cholesterolemia (g/l)	0.61±0.04	0.79±0.06*

Statistically differences between the SD and FD groups $*P<0.05$, [†]$P<0.01$.

anesthetized in a CO_2 chamber, followed by slight vapors of ether. An abdominal incision was performed and the left horn of the uterus was exposed. The animals that had five to seven fetuses in their left uterine horn were used. The fetuses were numbered from the ovary and alternatively injected s.c. on their backs through the uterine wall with 50 μl of either saline (SD+S group) or leptin (20 ng, SD+L group) (Sigma–Aldrich). After the administration, the left uterine horn was carefully introduced in the abdominal cavity and the abdominal muscle layer and the skin layer were independently sewn. The entire surgery lasted <10 min. The animals were completely recovered after 15 min. The procedure was repeated on days 20 and 21 of gestation. After 3 h of the injection in the last day, the mothers were killed, fetuses and placentas collected and weighed, and fetal organs carefully removed and weighed. Fetal liver and placentas were stored in RNAlater and kept at −20 °C for future analysis. The concentration was chosen based on previous *in vitro* studies that showed the effects of 20 ng/ml (1 nM) leptin on fetal and placental lipid metabolism in fetuses (White *et al.* 2004, 2007). In addition, similar studies have shown that a single s.c. administration of 80 ng leptin/g to adult rats induces a threefold increase in their leptinemia (de Oliveira *et al.* 2007). Although the clearance rates of adults and fetuses are not the same, we estimate that leptin levels in the fetal circulation after leptin administration could increase up to twofold.

Metabolic assays

Glycemia measurement Glycemic values were measured by the Accu-Chek reagent strips and a glucometer (Accu-Chek; Bayer Diagnostics) in blood obtained from the tail vein of the mothers and fetal decapitation.

Insulinemia Maternal and fetal insulin levels were measured in maternal and fetal plasma by a commercial EIA (Mercodia Ultrasensitive Rat Insulin ELISA kit, Uppsala, Uppland, Sweden), following the manufacturer's instructions. Briefly, samples and calibrators were allowed to interact with an anti-insulin antibody coated on the microplate and with the enzyme conjugate. After 2 h of incubation, the unspecific binding was washed and the substrate added. After 15 min, the reaction was stopped and the plate read at 450 nm.

Leptinemia Maternal and fetal leptin levels were measured in plasma by a commercial EIA (TiterZyme EIA rat leptin; Assay Designs, Ann Arbour, MI, USA) following

the manufacturer's instructions. Briefly, plasma samples or standards were allowed to interact for 1 h at 37 °C with a rabbit anti-leptin antibody coated on a microplate. After gently washing the wells, a secondary HRP-conjugated antibody was added, allowed to interact for 30 min at 37 °C and washed. The substrate was then added and after 30 min, the reaction was stopped and the plate read at 450 nm.

Plasma triglycerides and cholesterolemia measurement

Maternal and fetal triglyceridemia and cholesterolemia were measured by an enzymatic colorimetric commercial kit (Wiener Lab., Rosario, Argentina).

Placental and fetal liver lipid concentrations

Placenta and fetal liver were homogenized in 1000 μl saline phosphate buffer and the protein content in homogenates was measured by the Bradford assay (Bradford 1976). Tissue lipids were extracted from 500 μl of each homogenate by three rounds of organic extraction with methanol:chloroform (2:1), following the method of Bligh & Dyer, as performed previously (White *et al.* 2004). Volumes of lipid extraction equivalent to 400 μg protein were developed by thin layer chromatography (TLC) in thin silica gel plates (Merck) using hexane:ether:acetic acid (80:20:2, by vol.) as the developing solvent mixture. Lipid species were stained with iodine vapors, identified, and quantified by comparison with the known amounts of standards on the same plate, and analyzed densitometrically with Image J software (www.imagej.software.informer.com). Results are expressed as microgram per milligram of protein.

Semi-quantitative analysis of IRA, IRB, ObRa, ObRb, LPL, EL, and leptin gene expression

RNA from the placenta and fetal liver was extracted. The tissues kept in RNAlater from Ambion at −20 °C were

Table 4 Litter size, fetal, placental, and fetal organ weight

	SD	FD
Fetal weight (g)	3.292±0.07	3.834±0.08[†]
No. of fetuses	12.0±0.6	14.0±0.7*
Placental weight (mg)	534.6±9.2	591.7±8.1[‡]
Fetal liver weight (mg)	309.8±10	313.1±13
Fetal pancreas (mg)	21.8±1.1	27.3±0.8[‡]
Fetal intestine (mg)	93±1.63	101.3±1.4[‡]
Fetal stomach (mg)	17±0.6	22.3±0.5[‡]
Fetal lungs (mg)	97.1±1.3	102.0±2.5
Fetal heart (mg)	23.9±1.3	24.5±0.9

Statistically differences between the controls (SD) and saturated-fat-rich diet (FD) rats: *$P<0.05$, [†]$P<0.01$, [‡]$P<0.001$. $n=11$ litters.
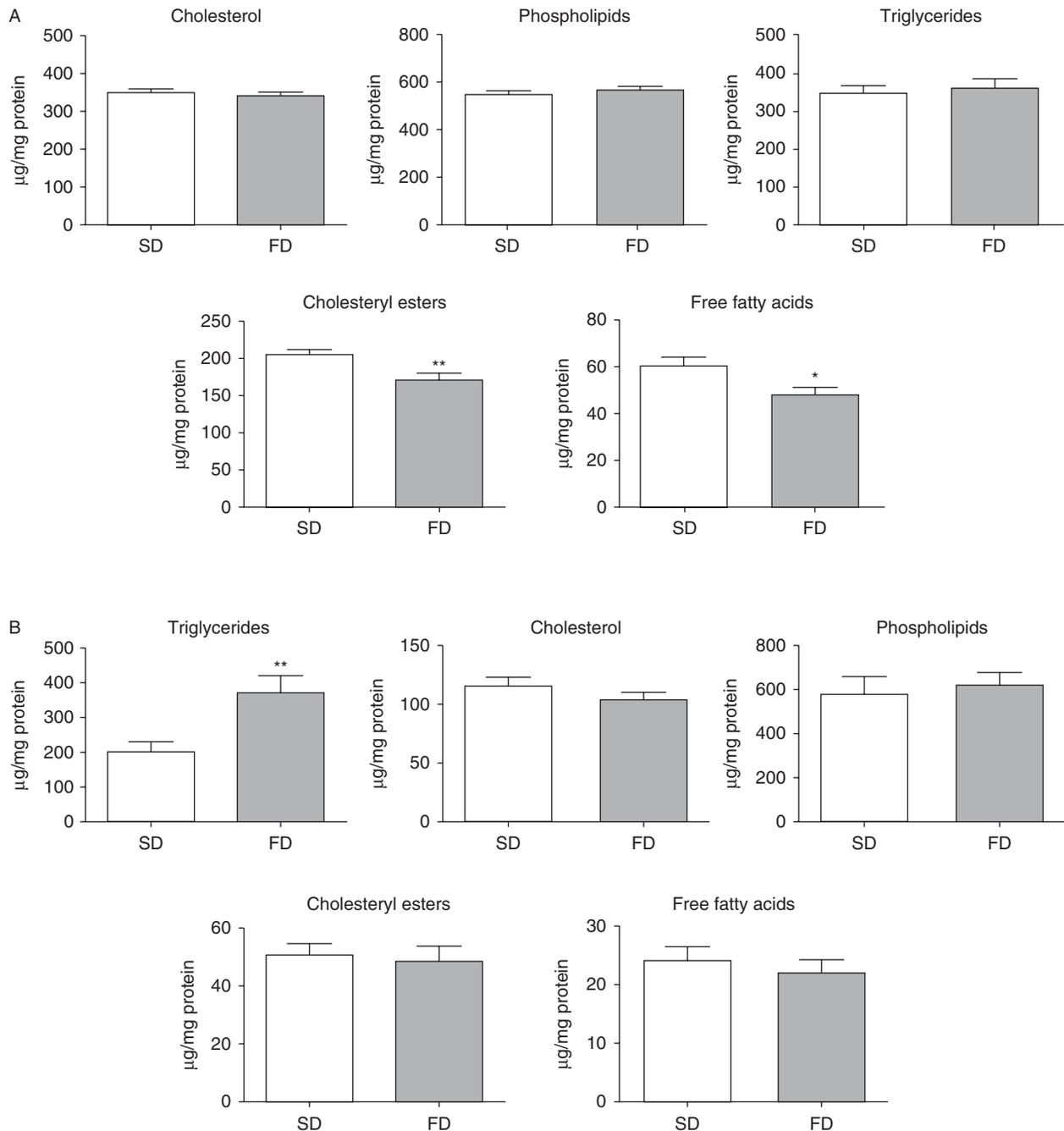
Figure 2

Lipid concentrations in the placenta (A) and fetal liver (B) from the control rats (SD) and rats fed with the saturated fat-rich diet (FD). $n=8$ in each experimental group. Statistical differences between the SD and FD groups: *$P<0.05$, **$P<0.01$.

homogenized in Tri Reagent (Biodynamics, Buenos Aires, Argentina) and RNA extracted accordingly to the manufacturer's instructions. Briefly, samples were subjected to a two-phase separation step, and then RNA was precipitated and finally resuspended in RNase-free sterile water. Primers (Table 2) were designed using the public web-page

Primer3 (http://frodo.wi.mit.edu/primer3) and purchased from Invitrogen. Primers for IR amplification were designed to bind to exons 10 and 12, so they amplified both isoforms. The products of amplification differed in size depending on the inclusion (IRB 237 bp) or exclusion (IRA 201 bp) of exon 11. All primer pairs included splicing
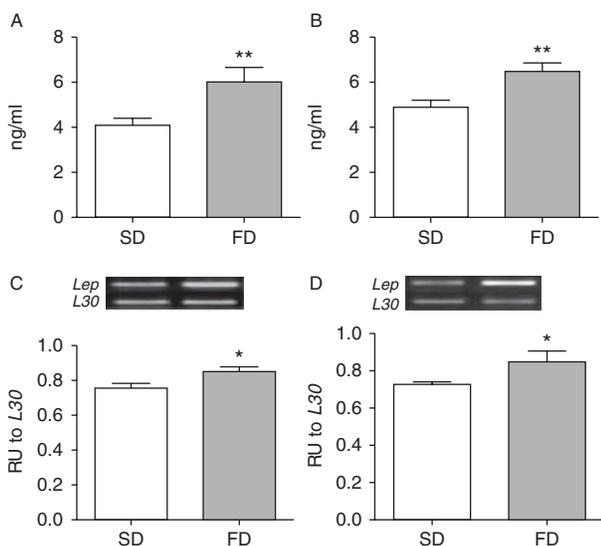
Figure 3

Leptin levels in maternal (A) and fetal (B) plasma from the control rats (SD) and rats fed with the saturated fat-rich diet (FD), $n=11$ in each experimental group. Leptin gene expression in relative units (RU) to the housekeeping gene *L30* in the placenta (C) and fetal liver (D) from the control rats (SD) and rats fed with the saturated fat-rich diet (FD), $n=8$ in each experimental group. Statistical differences between the SD and FD group: *$P<0.05$, **$P<0.01$.

sites within the amplicon. The mRNA amount of the ribosomal protein L30 (L30) was used as an internal control. cDNA was synthesized by incubating 2 μg of extracted RNA in a first-strand buffer containing 200 U MMLV enzyme (Promega), 7.5 mM random primer hexamers (Promega), and 0.5 mM of each of all four dNTPs (Invitrogen). The reaction mixture was incubated for 60 min at 37 °C followed by 15 min at 70 °C. The optimum cycle number was determined for each primer pair for the placenta and fetal liver (Table 2). Each cycle consisted of denaturation at 95 °C for 15 s, primer annealing at 58 °C for 30 s, and extension at 72 °C for 15 s. PCR products were electrophoresed on 2% agarose gels for leptin, ObR, and lipases or on 3% agarose gels for IR, so both isoforms were identified based on migration distances from the loading point. The images were taken with the Image-Quant spectrophotometer and software and quantified with Image J software.

Statistical analysis

Relative differences between samples were analyzed by Student's *t*-test or ANOVA with Bonferroni post-test, when appropriate. A *P* value <0.05 was considered as statistically significant.

Results

Effects of the saturated fat-rich diet on maternal and fetal metabolism

Mothers fed with the saturated fat-rich diet began their pregnancies with a significant increase in body weight ($P<0.05$; Fig. 1). Moreover, they gained more weight than the control group during pregnancy (SD: 108.6 ± 5.8, FD: 126.4 ± 4.9, $P<0.05$). The mothers fed with the saturated fat-rich diet showed the most prominent increase in weight during the last days of pregnancy (Fig. 1). Indeed, the weight of mothers fed the saturated fat-rich diet was markedly increased ($P<0.001$) on day 21 compared with the control group (Fig. 1). Although the control pregnant rats consumed higher amounts of food than the ones fed with the saturated fat-rich diet (SD: 75 ± 2 mg/kg, FD: 57 ± 2 mg/kg food per day, $P<0.001$), energy intake of the control group was lower than that of pregnant rats fed the saturated fat-rich diet (SD: 264 ± 3 kcal/kg per day, FD: 292 ± 8 kcal/kg per day, $P<0.01$).
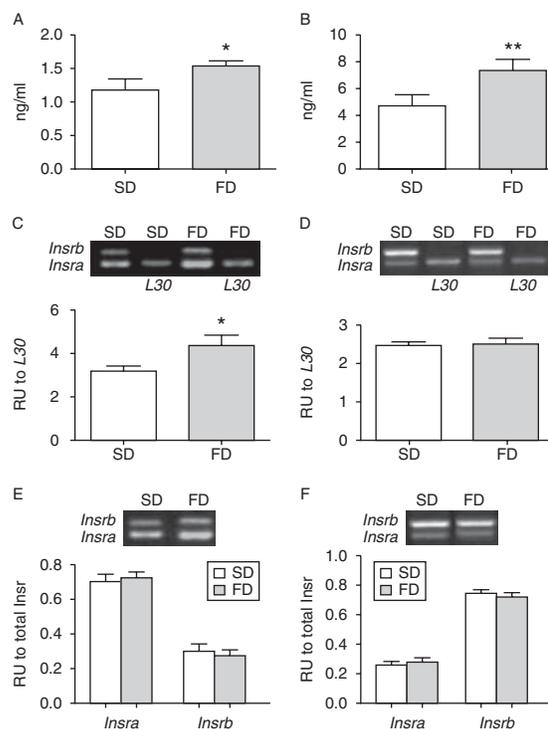
Figure 4

Insulin levels in maternal (A) and fetal (B) plasma obtained from the control rats (SD) and rats fed with the saturated fat-rich diet (FD), $n=11$ in each experimental group. Insulin receptor (*Insr*) gene expression in relative units (RU) to the housekeeping gene *L30* in the placenta (C) and fetal liver (D) from the control rats (SD) and rats fed with the saturated fat-rich diet (FD), $n=8$ in each experimental group. *Insr* isoform expression relative to total *Insr* expression in the placenta (E) and fetal liver (F) from the SD and FD mothers, $n=8$ in each experimental group. Statistical differences between the SD and FD groups: *$P<0.05$, **$P<0.01$.

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On day 21 of gestation, mothers fed with the saturated fat-rich diet exhibited glycemia similar to those of the controls, although plasma triglyceride levels were highly increased ($P<0.01$). Maternal cholesterol levels did not differ between the groups. Conversely, fetuses from the mothers fed with the saturated fat-rich diet showed an increase in glycemia, cholesterolemia ($P<0.05$), and triglyceridemia ($P<0.01$) compared with those from the control group on day 21 of gestation (Table 3).

Fetuses from the mothers that received the saturated fat-rich diet were heavier than those from the control group ($P<0.01$; Table 4). Interestingly, mothers fed with the saturated fat-rich diet had also an increased number of fetuses compared with the control mothers ($P<0.05$; Table 4). Although fetal lung, heart, and liver showed no differences in weight between the two groups, fetal intestine, pancreas, and stomach, as well as placentas were heavier than those from the control group ($P<0.001$; Table 4).

Lipid concentrations in the placenta and fetal liver

We investigated whether the saturated fat-rich diet modifies lipid concentrations in the placenta and fetal liver. We found a decrease in free fatty acid ($P<0.05$) and cholesteryl ester concentrations ($P<0.01$) in the placenta from rats fed the saturated fat-rich diet compared with the controls (Fig. 2A). In contrast, fetal livers from the saturated fat-rich diet-fed group showed a marked increase in triglyceride content ($P<0.01$) compared with those from the controls (Fig. 2B).
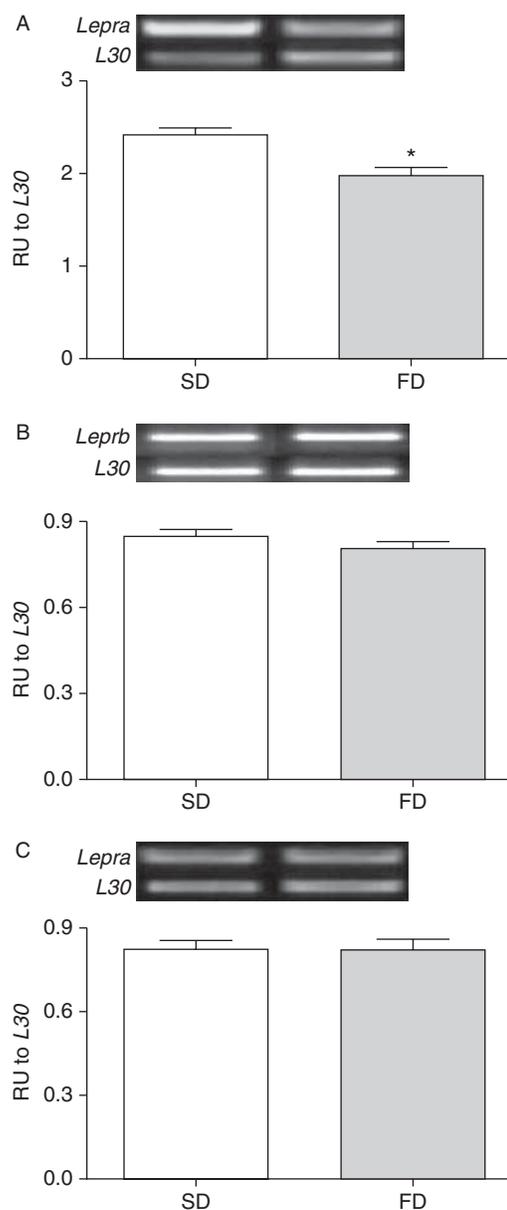
Leptinemia and leptin expression in the placenta and fetal liver

We found increased leptin levels in maternal and fetal plasma in the saturated fat-rich group when compared with the controls ($P<0.01$; Fig. 3A and B). Aiming to investigate the source of the high levels of leptin, we evaluated leptin expression in the placenta and fetal liver. We detected leptin expression in both tissues (Fig. 3C and D) and, interestingly, we found an increase in leptin expression in the placenta and fetal liver in the saturated fat-rich diet group when compared with the controls ($P<0.05$).

Insulinemia and Insr expression in the placenta and fetal liver

Insulin is a hormone that controls many metabolic pathways, vascular function, and cell growth. We found

increased insulin levels in maternal ($P<0.05$) and fetal ($P<0.01$) plasma (Fig. 4A and B). Placentas from the saturated fat-rich diet-fed rats showed an increase in total Insr expression ($P<0.05$) compared with those from the controls (Fig. 4C). We found no changes in total Insr expression in fetal livers from both groups (Fig. 4D). The isoform analysis showed that 30% of the whole placental

Figure 5

Leptin receptor gene expression in relative units (RU) to the housekeeping gene *L30* in the placenta (A. *Lepra* and B. *Leprb*) and fetal liver (C. *Lepra*) from the control rats (SD) and rats fed with the saturated fat-rich diet (FD), $n=8$ in each experimental group. Statistical differences between the SD and FD groups: *$P<0.05$.

Insr expression corresponded to *Insrb* and 70% to *Insra* (Fig. 4E). By contrast, 70% of the total fetal liver Insr expression corresponded to *Insrb* and 30% to *Insra* (Fig. 4F). The saturated fat-rich diet induced no changes in the proportion of isoform expression in the placenta or fetal liver (Fig. 4E and F).

Lepr expression in the placenta and fetal liver

We investigated *Leprb* and *Lepra* expression by semi-quantitative PCR analysis in the placenta and fetal liver. We found that the placenta expressed both isoforms and that the saturated fat-rich diet induced a decrease in *Lepra* ($P<0.05$; Fig. 5A), but no changes in *Leprb* (Fig. 5B) placental expression. On the other hand, we did not detect *Leprb* expression in the fetal liver and although we detected *Lepra*, we found no changes between the control and saturated fat-rich diet groups (Fig. 5C).
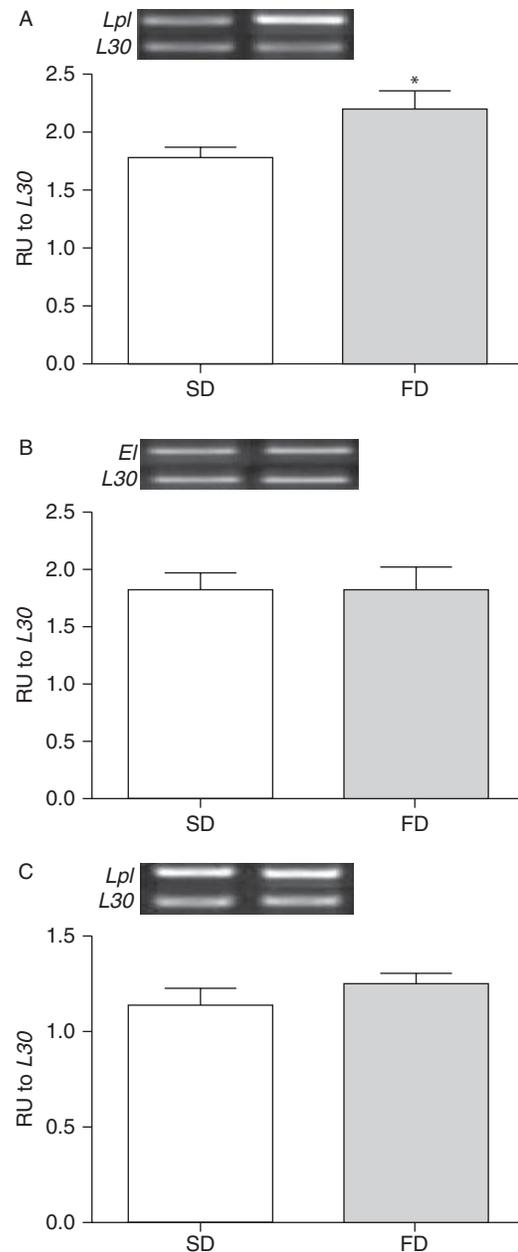
Lipase expression

Lipases are important in placental lipid transfer to the fetus (White *et al.* 2006). We investigated whether a saturated fat-rich diet is able to modify placental and fetal liver *Lpl* and *El* expression. We found an increase ($P<0.05$) in *Lpl* expression (Fig. 6A), while no changes in *El* expression in the placenta from the saturated fat-rich diet group (Fig. 6B). *El* expression was not detected in fetal liver, whereas *Lpl* was expressed in this fetal organ. The saturated fat-rich diet induced no change in the fetal liver expression of *Lpl* (Fig. 6C).

Effects of fetal administration of leptin

To establish whether fetal leptin induces changes in placental and fetal liver expression that could affect feto-placental metabolism and/or growth, fetuses were injected with leptin (20 ng/day) on days 19, 20, and 21 of pregnancy.

Regarding *Insr* expression, we found that total *Insr* expression was unchanged in the placenta and fetal liver from fetuses injected with leptin compared with the saline-treated fetuses (Fig. 7A and B). Also, *Insra* and *Insrb* expression showed no changes after fetal leptin administration in the placenta and fetal liver (data not shown). Regarding *Lepr* expression, we found, similarly to that found in the saturated fat-rich diet treatment, no changes in *Leprb* expression and a decrease in *Lepra* expression in the placenta from leptin-treated fetuses ($P<0.05$) compared with saline-treated ones (Fig. 7C and D). The fetal liver expression of *Lepra* was also decreased ($P<0.05$)
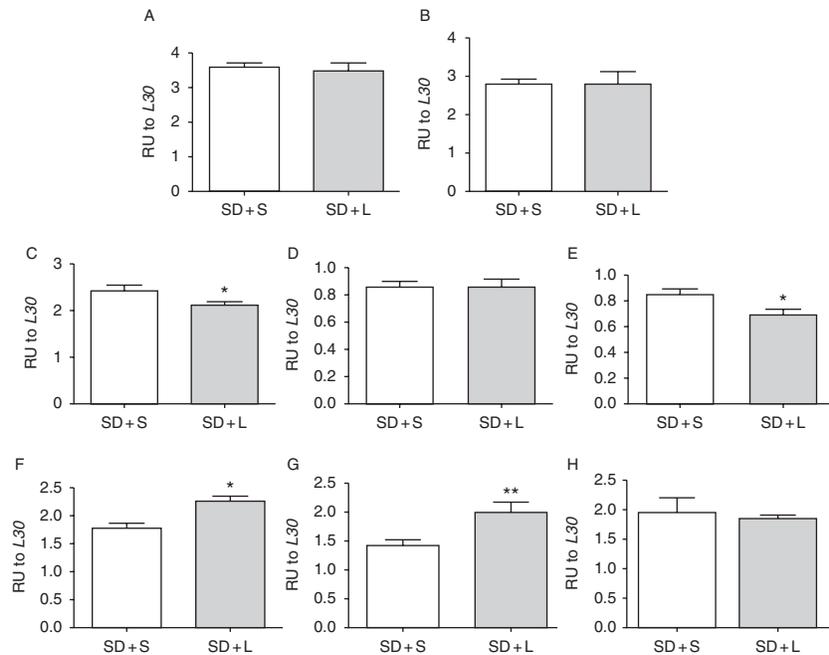

Figure 6
Lipase gene expression in relative units (RU) to the housekeeping gene *L30* in the placenta (A. *Lpl* and B. *El*) and fetal liver (C. *Lpl*) from the control rats (SD) and rats fed with the saturated fat-rich diet (FD), $n=8$ in each experimental group. Statistical differences between the SD and FD groups: *$P<0.05$.

in leptin-treated fetuses (Fig. 7E) compared with saline-treated ones.

As stated before, lipases have an active role in the uptake of lipids and, hence, in the transport of many lipid species. Placentas from fetuses injected with leptin showed an increase in *Lpl* expression ($P<0.05$) compared with the placentas from saline-injected fetuses (Fig. 7F).

Figure 7

Effects of intra-fetal leptin administration on the placental and fetal liver gene expression in relative units (RU) to *L30* compared with saline fetal administration in the control pregnant rats. (A) Total insulin receptor (Insr) expression in the placenta; (B) total Insr in the fetal liver; (C) placental *Lepra* expression; (D) placental *Leprb* expression; (E) fetal liver *Lepra* expression; (F) placental expression of *Lpl*; (G) fetal liver expression of *Lpl*; (H) placental expression of *El* in the placenta and fetal liver from saline-injected fetuses (SD+S) and leptin-injected fetuses (SD+L). $n=11$ fetuses from different mothers in each experimental group. Statistical differences between the SD and FD groups: *$P<0.05$, **$P<0.01$.

This increase was also seen in the liver from leptin-injected fetuses ($P<0.01$) compared with saline-injected ones (Fig. 7G). The fetal administration of leptin induced no changes in placental *El* expression (Fig. 7H).

Discussion

In this work, we developed a model of overweight in pregnant rats by feeding them with a diet rich in saturated fat. Characteristics of the model are that the genetic background is not modified and that the diet has similarities to many fast-food meals, very popular nowadays. Nutritional requirements in rats suggest a dietary intake between 5 and 12% of calories from fat (http://www.nap.edu/openbook.php?record_id=4758&page=11). A report of the WHO and FAO in 2003 suggested a dietary intake between 15 and 30% of calories from fat in humans (www.who.int/mediacentre/news/releases/2003/pr20/en). The saturated fat-rich diet used in this study has 47% of calories from fat, a value higher than the one recommended for both rat and human intakes, but lower than that of other high-fat diets used in rodents in which fat contribution to total calories is 60% (Niculescu & Lupu 2009) and 58% (Softic *et al.* 2012).

Our model showed macrosomia and placentomegaly in rats fed before and during pregnancy with a saturated fat-rich diet. These rats began their pregnancies with an increase in weight and gained more weight during gestation than the control group. Moreover, the most prominent increase in weight gain was during the last 3 days of pregnancy, in which the fetuses are supposed to gain more weight, suggesting that this weight gain corresponds to an increase in feto-placental weight. Additionally, mothers fed with the saturated fat-rich diet showed larger litters than the control ones. In total, more and heavier fetuses and placentas contribute to the increase in maternal weight gain, as seen by others (Jones *et al.* 2009). Conversely, other experimental models fed with high-fat diets have shown a decrease in fetal weight, and a diminished placental efficiency, calculated by a fetal-to-placental weight ratio (Niculescu & Lupu 2009, Hayes *et al.* 2012). In this study, we show an increase in fetal weight, together with an increase in placental weight, and this increase in placental mass might contribute to an enhancement of maternal-to-fetal nutrient transport, inducing an increase in fetal growth.

The increase in lipids provided by the saturated fat-rich diet, together with a decrease in maternal insulin

sensitivity, as evidenced by normal glycemia despite hyperinsulinemia, resulted in high levels of circulating triglycerides. Maternal high circulating levels of triglycerides reach the placenta, where they are hydrolyzed, uptaken as fatty acids, re-esterified within the placenta, and transported in lipoproteins to the fetal circulation. This can result in increased fetal triglyceridemia, which has been linked to fetal overweight, independently of maternal obesity (Merzouk *et al.* 2000). Unexpectedly, placentas from the rats fed with the saturated fat-rich diet showed no lipid accumulation and a relative decrease in some lipid moieties. The saturated fat overload in the maternal diet may induce an increase in the proportion of saturated fatty acid composition of lipid moieties that might lead to decreased iodine staining and an underestimation of the concentrations of lipid moieties. However, decreased concentrations of some lipid moieties in the placenta, together with increased triglyceridemia at both sides of the placenta, could indicate an increase in placental net lipid transport. Further analysis of fatty acid composition and the expression and activity of placental lipid transporters is needed to clarify this point.

On the other hand, we found an overaccumulation of triglycerides in the fetal liver in the saturated fat-rich group, which might be due to the high levels of fetal circulating triglycerides and insulin, as seen by others (Softic *et al.* 2012). Overaccumulation of lipids in the fetal liver has been related to the development of insulin resistance and type 2 diabetes and the programming of metabolic disturbances (McCurdy *et al.* 2009).

The differences observed in the proportion of Insr isoforms between the placenta and fetal liver may cause a preponderance of the growth-promoting effect of *Insr* in the placenta, as the *Insra* isoform is also a target for IGF2. On the other hand, with a preponderance of the *Insrb* isoform, the fetal liver may have both insulin metabolic and mitogenic effects. We found no changes in the fetal liver expression of total Insr and, conversely, an increase in this receptor expression in the placenta from the saturated fat-rich diet-treated rats. The upregulation of INSR expression in the placenta from the saturated fat-rich diet can contribute to the increase in placental mass, although further studies exploring protein expression are needed to clarify this point. Also, this increased placental INSR expression and hyperinsulinemia may enhance glucose and amino acid transport and modulate placental flow, thus improving placental efficiency, discussed above, for the saturated fat-rich diet-fed group (Acevedo *et al.* 2005, Jones *et al.* 2007, 2010, Hiden *et al.* 2009).

Leptin is a hormone produced by adipocytes in response to insulin and lipid storage (Tartaglia 1997, Maiorana *et al.* 2007). Leptin expression in the human placenta and the overexpression of leptin in the placenta from insulin-treated diabetic women have been addressed (Lepercq *et al.* 1998). In previous work, we found decreased concentrations of leptin protein in placental explants from diabetic rats with low insulinemia (White *et al.* 2004). Coya *et al.* (2001) demonstrated the insulin-stimulating effects on leptin expression in human trophoblasts in culture. In our model, with high levels of insulin in mothers and fetuses, we found an increase in leptin expression in the placenta and fetal liver. Although the expression of leptin in the fetal liver has been detected in mouse fetus from day 13.5 of gestation, little is known about the regulation of leptin expression in this organ (Hoggard *et al.* 2000). Fetal liver could be producing leptin in response to insulin, as seen in human trophoblasts, or to insulin-induced storage of lipids, as seen in mature adipocytes (Maiorana *et al.* 2007). Indeed, in rat fetuses, where adipocytes are still immature, placental and fetal liver leptin production could highly contribute to the increase in circulating levels of fetal leptin.

The signaling pathways of *Lepr* have been reviewed (Fruhbeck 2006). We found no changes in *Lepra* expression in the fetal liver from the saturated fat-rich-diet group compared with the controls. We did not detect *Leprb* in the fetal liver, maybe because of low expression, as seen by others in adult mouse livers (Cohen *et al.* 2005). We found *Leprb* expression in the placenta, although we detected no changes in its expression in the saturated fat-rich diet group. It has been shown that *Leprb* is expressed in the basal and labyrinth zones with no variation in gestational day. On the other hand, since labyrinth *Lepra* expression increases at term gestation, this receptor could have a specific role at term and might be under specific gestational regulatory mechanisms (Smith & Waddell 2002). We found that placentas from the saturated-fat rich diet group showed diminished expression of *Lepra*, suggesting nutrient-availability regulatory mechanisms for leptin receptor. Further research is needed to address possible changes in LEPR protein expression and in the activation of downstream pathways.

The fact that placentas showed no lipid overaccumulation is in agreement with the increase in *Lpl* expression observed in the placenta from the saturated fat-rich diet-treated rats. An increase in lipolysis and, consequently, in lipid transport to the fetus would decrease placental lipid content and would increase fetal lipid circulating levels. It has been shown that the gene expression of *Lpl*

in the placenta correlates with fetal triglyceridemia in normal and diabetic pregnancies (Lindegaard *et al.* 2006). These observations may suggest that LPL is promoting maternal lipid transfer to the fetus, although further studies evaluating LPL activity are needed to clarify this point. In this work, we show increased fetal weight, increased levels of triglycerides at both sides of the placenta, and increased *Lpl* expression, together with a decrease in placental relative lipid accumulation, suggesting that there is an increase in maternal-to-fetal lipid outflow.

This study compared *El*, *Lpl*, *Insr*, and *Lepr* gene expression regulation by the maternal saturated fat-rich diet and fetal leptin administration. Fetal *Lepr* is expressed in the human endothelium of umbilical veins and arteries (Akerman *et al.* 2002). Other studies have shown that administration of leptin induces a threefold increase in leptinemia in both fetuses and adult animals (de Oliveira *et al.* 2007, Su *et al.* 2012). We found that fetal leptin administration induced a downregulation of *Lepra* expression in both the placenta and fetal liver from the control rats. Nevertheless, in the fetuses from the saturated fat-rich diet-fed mothers, with high levels of circulating leptin, there was a downregulation of *Lepra* in the placenta but not in the fetal liver. Moreover, *Lpl* expression was upregulated by fetal leptin administration in both the placenta and fetal liver, while the saturated fat-rich diet only induced an upregulation of *Lpl* in the placenta. As fetal circulating levels of leptin are increased in the saturated fat-rich diet group, it seems that placental *Lpl* and *Lepra* expression respond to fetal leptin while liver is not. This suggests that the saturated fat-rich diet induces fetal liver leptin resistance. Leptin is involved in the activation of AMPK and PPARα, both involved in the induction of fatty acid oxidation (Fruhbeck 2006). Further research is needed to address whether a saturated fat-rich diet induces fetal liver leptin resistance, inhibiting the activation of pathways triggered by leptin, which could lead to decreased fatty acid oxidation and thus lipid overaccumulation (Vila *et al.* 2008).

In previous work, we showed a leptin dose-dependent increase in placental lipolysis in the human placenta (White *et al.* 2006). Leptin administration to term gestation fetuses showed an increase in *Lpl* expression in the placenta, suggesting that fetal leptin modulates the transport of lipids to the fetus. Thus, in an intrauterine environment with high levels of leptin, insulin, and growth factors, as in this saturated fat-rich diet model, leptin can be one of the signals increasing the placental transport of lipids, and hence sustaining fetal growth.

In summary, we developed a model of macrosomia without manipulating the genetic background, with a saturated fat-rich diet, which could mimic bad eating habits in humans. We found hyperleptinemia at both sides of the placenta, and identified the placenta and fetal liver as two putative sources of fetal circulating leptin. We also assigned to fetal leptin the ability to induce the expression of the placental lipid transporter *Lpl*. This experimental model would help to understand mechanisms involved in the development of fetal over-growth and organ lipid accumulation that triggers the programming of metabolic disorders in adulthood.

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