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Maternal saturated-fat-rich diet promotes leptin resistance in fetal liver lipid catabolism and programs lipid homeostasis impairments in the liver of rat offspring

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Abstract

We aimed to analyze if an overload of saturated fat in maternal diet induced lipid metabolic impairments in livers from rat fetuses that persist in the offspring and to identify potential mechanisms involving fetal leptin resistance.

Female rats were fed either a diet enriched in 25% of saturated fat (SFD rats) or a regular diet (controls). Fetuses of 21 days of gestation and offspring of 21 and 140 days of age were obtained and plasma and liver were kept for further analysis. Livers from a group of control and SFD fetuses were cultured in the presence or absence of leptin. Leptin or vehicle was administered to control fetuses during the last days of gestation and, on day 21, fetal livers and plasma were obtained. Lipid levels were assessed by thin-layer chromatography and mRNA gene expression of CPT1, ACO and PPARα by RT-PCR.

Liver lipid levels were increased and CPT1 and ACO were down-regulated in fetuses and offspring from SFD rats compared to controls. After the culture with leptin, control fetal livers showed increased ACO and CPT1 expression and decreased lipid levels, while fetal livers from SFD rats showed no changes. Fetal administration of leptin induced a decrease in ACO and no changes in CPT1 expression.

In summary, our results suggest that a saturated fat overload in maternal diet induces fetal leptin resistance in liver lipid catabolism, which might be contributing to liver lipid alterations that are sustained in the offspring.

Keywords: Leptin; Liver lipid catabolism; Saturated-fat-rich diet; Offspring; Fetus

1. Introduction

Currently, the great offer of unhealthy diets, rich in saturated fat, increases the prevalence of metabolic disorders [1–3]. The metabolic syndrome is already established in the worldwide population and is not limited to overweight or obese persons. This disorder refers to a group of metabolic anomalies as dyslipidemias, glucose intolerance, among others, which have a direct relationship with an increase in the risk of developing diabetes, cancer, cardiovascular diseases and other health complications [4–6]. A worrying fact is the increase in the incidence of overweight, metabolic syndrome and related metabolic alterations in young people from neonates to teenagers (www.who.int/features/factfiles/obesity/es) [7–10]. This fact predicts an unhealthy adulthood and an old age with many needs for clinical and pharmacological therapies [6,7,9,11]. It has been well established that the burden of metabolic complications can be perinatally originated [12–16]. The Barker hypothesis states that neonates born small for gestational age are prone to develop cardiovascular diseases later in life. Since then, many investigations have demonstrated that maternal overweight also induces metabolic alterations from fetal life to adulthood in the offspring [9,14,16,17]. We and others have described metabolic abnormal outcomes in fetuses from rats fed with different approaches of diets rich in saturated fat, with increases in caloric input [18–20]. Many metabolic alterations have been described in fetuses and offspring from mothers with metabolic impairments [21–24]. Lipid metabolic alterations harm the liver, which is an important organ for lipid homeostasis and lipid trafficking through the organism. Studies in humans and animal models have shown that perinatal exposure to a lipid enriched environment may develop fatty liver disease later in life [25–29]. Liver fat depots are unlikely to persist and induce fatty liver disease in the adult offspring unless specific alterations in the mechanisms that control liver lipid metabolism are programmed *in utero*. Animal models allow the researcher to control the nutrition for long periods of time, and many works have shown different liver metabolic alterations in healthy nourished offspring born to obese mothers, suggesting that the maternal environment alters the offspring's liver lipid metabolism permanently [25,26,30].

Then, a fat overload in the maternal diet may induce different degrees of fatty liver disease in the offspring. However, there are still some questions to be addressed as whether the liver is affected from

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fetal life and which are the mechanisms involved in such metabolic malprogramming.

Leptin is an adipokine that activates lipid catabolism in peripheral tissue like adipocytes, muscle and liver [31–33]. This adipokine increases fatty acid oxidation in mitochondria and peroxisomes [34–36]. The binding of leptin to its receptor triggers intracellular signaling mechanisms that involve phosphorylation and activation of several proteins, such as Janus kinase 2, AMP-activated protein kinase, the signal transducer and activator of transcription STAT-3 and the peroxisome proliferator-activated receptor PPARα. The transcription factors PPARα and STAT-3 mediate the expression of leptin-induced lipid catabolic enzymes [32,37], such as the acetyl-CoA oxidase ACO and the carnitine palmitoyl transferase CPT1 [38,39]. We have shown that fetuses from rats fed a saturated-fat-rich diet through pregnancy and lactation display hyperleptinemia and liver lipid overaccumulation [24].

This work aimed to investigate whether the increase in liver lipid depots, observed in the fetuses from rats fed a saturated-fat-rich diet, persists later in life in the offspring (a) at 21 days of age immediately after weaning and (b) at 140 days of age, after healthy nourishing. Furthermore, we wanted to investigate if leptin resistance could be involved in the programming of lipid liver alterations and to establish a role for fetal hyperleptinemia in the programming of alterations in liver lipid homeostasis.

2. Materials and methods

2.1. 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 animal protocol was approved by the local institution (Comité Institucional de Cuidado y Uso de Animales de Experimentación, CD N 1497/2013), which follows the suggestions of the "Guide for the Care and Use of Laboratory Animals" of the US National Institutes of Health (NIH Publication, 8th Edition, 2011; http://www.ncbi.nlm.nih.gov/books/NBK54050/?report=reader).

2.2. Diet

At 6 weeks of age, female rats were categorized into two groups: one fed a commercial standard rat chow (Asociación Cooperativa Argentina) (controls) and the other fed a saturated-fat-rich diet (SFD rats). The saturated-fat-rich diet consisted of the standard rat chow (commercially purchased) enriched with 25% of saturated animal fat provided by manual supplementation with butter (Sancor Co., Buenos Aires, Argentina). Both diets were analyzed by the National Food Institute (Instituto Nacional de Alimentos) and the analysis showed that the food composition of the control diet was as follows: 25% of protein, 51% of carbohydrates and 5% of fat (grams per 100 g of total chow); the food composition of saturated-fat-rich diet was 22% of protein, 40% of carbohydrates and 26% of fat (grams per 100 g of total chow). The latter provides 45% calories from fat, 54% calories from carbohydrates and 20% calories from proteins. Control and SFD rats were fed the corresponding diet for 8 weeks before mating and throughout pregnancy and lactation (Fig. 1).

2.3. Experimental design

2.3.1. Fetuses

At 14 weeks of age, control and SFD females 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 euthanized by decapitation and their fetuses were immediately removed. Fetuses were euthanized by decapitation and their fetal livers were carefully removed under a stereomicroscope using microsurgical dissecting instruments, fixed in RNAlater (Ambion, Carlsbad, CA, USA) and kept at −20°C for PCR assays or cultured in the presence or absence of leptin as explained below.

2.3.2. Offspring

We aimed to continue our work through the study of the offspring from both dietary groups. To accomplish this, some controls and SFD rats were allowed to finish their pregnancy. The control and SFD rats were fed the corresponding diet throughout pregnancy and until the end of the lactation period (21 days of age). One group of pups from both control and SFD rats was allowed to grow up until 21 days of age and other group was allowed to grow up until 140 days of age. Offspring from both dietary groups were fed with commercial standard rat chow from weaning to euthanasia at 140 days of age. On these two dates, the offspring were weighed and euthanized by decapitation. Plasma was obtained and kept at −80°C. Livers were removed, weighed and kept at −80°C for lipid analysis or fixed in RNAlater and kept at −20°C for PCR assays (Fig. 1).

2.3.3. Culture of fetal liver explants

Aiming to investigate potential leptin resistance induced by the altered maternal environment, we cultured liver explants of fetuses of 21 days of gestation from controls and SFD rats in the presence or absence of leptin. Immediately after being removed, fetal livers were cultured for 3 h in a metabolic bath under a controlled atmosphere (CO_2: 5%; O_2: 95%) at 37°C, in 1 ml KRB medium in the presence or absence of leptin (100 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA). The concentration was chosen from previous works that showed leptin lipid catabolic effects in the range 20–1000 ng/ml [40,41]. In this experimental design, we obtained four groups of livers: (1) fetal livers from control rats cultured in the absence of leptin, (2) fetal livers from control rats cultured in the presence of leptin, (3) fetal livers from SFD rats cultured in the absence of leptin and (4) fetal livers from SFD rats cultured in the presence of leptin. Immediately after the culture, livers were kept at −80°C for lipid analysis or fixed in RNAlater and kept at −20°C for PCR assays (Fig. 1).

2.3.4. Fetal administration of leptin

Aiming to establish the role of fetal circulating leptin in the modulation of the fetal liver expression of genes involved in lipid catabolism, we administered leptin to fetuses at term gestation, as described previously [24]. Briefly, on day 19 of gestation, a group of control mothers were shortly anesthetized with 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 subcutaneously on their backs through the uterine wall with 50 μl of either saline (control+saline group) or leptin (20 ng, control+leptin group). After the administration, the left uterine horn was

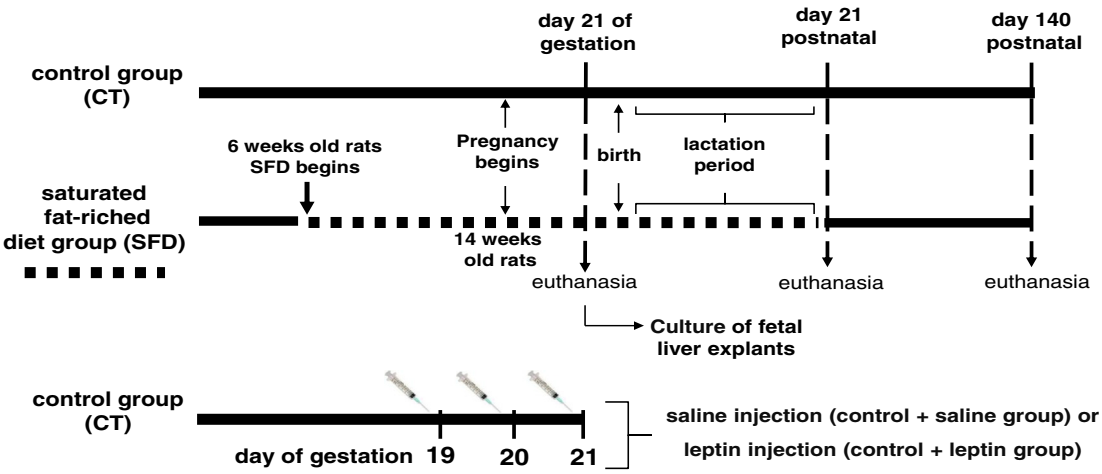

Fig. 1. Schematic representation of the experimental design.

Table 1
Primer sequences and optimum cycle number for each primer pair used for gene expression analysis by semiquantitative PCR in the fetus, 21-day-old and 140-day-old offspring.

Gene	ACO	CPT1	PPARα	L30
Forward primer sequence (5′–3′)	CCAATCACGCAATAGTTCTGG	TATCGTCGCACATTAGACC	TCACACAATGCAATCCGTTT	CCATCTTGGCGTCTGATCTT
Reverse primer sequence (5′–3′)	CGCTGTATCGTATGGCGAT	CATCTATGACCTCCTGGCA	GGCCTTGACCTTGTTCATGT	TGGCGAGGATAACCAATTTC
Fetal liver (optimum cycle number)	25	35	31	23
Fetal liver (injected group) (optimum cycle number)	29	35	25	22
Fetal liver (cultured group) (optimum cycle number)	32	33	31	24
Day 21 postnatal liver (optimum cycle number)	29	35	32	25
Day 140 postnatal liver (optimum cycle number)	23	35	30	24

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 (Fig. 1). After 3 h of the injection on the last day, the mothers were euthanized by decapitation, fetuses were collected and their plasma was obtained. Fetal livers were carefully removed, stored in RNAlater and kept at −20°C for future PCR analysis. The concentration was chosen based on previous *in vivo* studies that showed the effects of 20 ng of leptin on fetal liver gene expression [24]. Plasma leptin levels were assessed in the saline-injected and leptin-injected fetuses, for validation of the method. The leptin-injected fetuses showed higher leptin plasma levels than the saline-injected ones (Supplementary Fig. 1).

2.4. Metabolic assays

2.4.1. Glycemia measurement

Glycemic values of 21- and 140-day-old offspring were measured by the Accu-Chek reagent strips and a glucometer (Accu-Chek; Bayer Diagnostics) in blood obtained from the tail vein.

2.4.2. Plasma leptin, triglycerides and cholesterolemia measurement

Fetal leptinemia was assessed by enzyme immunoassay (Bertin Pharma, Martillac, France). Triglyceridemia and cholesterolemia from 21- and 140-day-old offspring were measured by an enzymatic colorimetric commercial kit (Wiener Lab., Rosario, Argentina).

2.4.3. Liver lipid concentrations

An aliquot of 110±10 mg of each of the livers of 21- and 140-day-old offspring was homogenized in 1 ml saline phosphate buffer and the protein content in homogenates was measured by the Bradford assay [42]. Liver tissue lipids were extracted from 500 ml of each homogenate by three rounds of organic extraction with methanol:chloroform (2:1), following the method of Bligh and Dyer, as performed previously [24,43]. Volumes of lipid extraction equivalent to 400 mg protein were developed by thin-layer chromatography in thin silica gel plates (Merck) using hexane:ether:acetic acid (80:20:2, by volume) as the developing solvent mixture. Lipid species were stained with iodine vapors, identified, quantified by comparison with known amounts of standards on the same plate and analyzed densitometrically with Image J software (www.imagej.software.informer.com). Results are expressed as micrograms per milligram of protein.

2.5. Semiquantitative analysis of PPARα, CPT1 and ACO gene expression

Liver RNA from fetuses, pups (21 days of age) and adult offspring (140 days of age) was extracted as follows: Livers were homogenized in TRI Reagent (Molecular Research Center, Ohio, USA) and RNA extracted according to the manufacturer's instructions. Briefly, samples were subjected to a two-phase separation step and then RNA precipitated and finally resuspended in RNase-free sterile water. cDNA was synthesized by incubating 2 μg of extracted RNA in a first-strand buffer containing 200 U MML-V enzyme (Promega, Madison, WI, USA), 7.5 mM random primer hexamers (Promega) and 0.5 mM of each of all four dNTPs (Invitrogen, Carlsbad, CA, USA). The reaction mixture was incubated for 60 min at 37°C followed by 15 min at 70°C. cDNA (2 μl, selected to work within the linear range) was amplified by PCR in a buffer containing dNTPs, magnesium chloride solution, Taq polymerase (GoTaq Polymerase; Promega) and each specific primer in accordance with the Taq polymerase manufacturer's instructions. Primers (Table 1) were designed using the public Web page Primer3 (http://frodo.wi.mit.edu/primer3) and purchased from Invitrogen. All primer pairs included splicing sites within the amplicon. The mRNA amount of the ribosomal protein L30 (L30) was used as an internal control. The optimum cycle number was determined for each primer pair for livers from fetuses, pups and adult offspring (Table 1). 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 (Biodynamics, Buenos Aires, Argentina) gels. The images were taken with the ImageQuant spectrophotometer and software and quantified with Image J software.

2.6. Statistical analysis

Data are presented as the mean±standard error. The data obtained were checked for normality prior to the statistical analysis with the Shapiro–Wilk test (Statistix 10 software). Groups were compared by Student's *t* test or two-way ANOVA in conjunction with Bonferroni's test as post-test (Prism 5 software). In all cases, differences were considered statistically significant at $P<.05$.

Table 2
Weight and metabolic parameters in offspring of 21 and 140 days of age from control rats and rats fed a saturated-fat-rich diet (SFD rats).

Offspring age		21 Days		140 Days	
		Control	SFD	Control	SFD
Triglyceridemia (mg/dl)	Male	148±13	262±39*	128±9	125±12
	Female	127±11	251±31**	113±9	109±9
Glycemia (mg/dl)	Male	109±6	105±5	100±4	93±3
	Female	106±5	106±6	100±3	104±6
Cholesterolemia (g/dl)	Male	84±6	83±11	93±3	85±5
	Female	88±6	86±11	91±7	80±6
Body weight (g)	Male	43.52±0.78	46.19±0.89*	555±19	606±19
	Female	42.11±0.74	44.88±0.52*	327±11&&	352±12&&
Liver weight (g)	Male	1.68±0.03	1.83±0.03*	15.64±0.43	16.39±0.61
	Female	1.58±0.05	1.88±0.05*	10.14±0.15&&	11.22±0.29&&

Data were obtained from one rat from each litter of six mothers in each gender.
Statistical test: two-way ANOVA, post-test: Bonferroni.
$P<.05$ vs. males of the same age and dietary treatment, statistical differences between genders.
* $P<.05$ vs. controls of the same gender and age, statistical differences between dietary groups.
** $P<.01$ vs. controls of the same gender and age, statistical differences between dietary groups.
&& $P<.01$ vs. males of the same age and dietary treatment, statistical differences between genders.

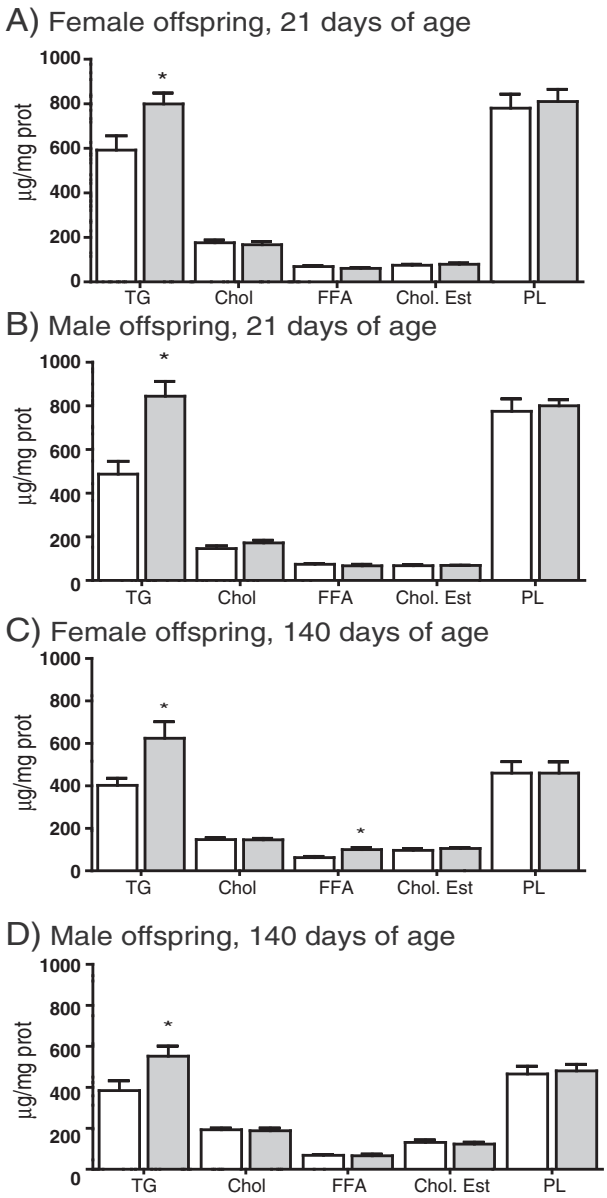

Fig. 2. Tissue liver lipid levels in the offspring from control rats (open bars) and rats fed a saturated-fat-rich diet (gray bars). (A) Female offspring of 21 days of age. (B) Male offspring of 21 days of age. (C) Female offspring of 140 days of age. (D) Male offspring of 140 days of age. TG, triglycerides; Chol, cholesterol; FFA, free fatty acids; Chol. Est., cholesterol esters; PL, phospholipids. Each bar represents the mean±standard error of the values from the analysis of six livers from six individuals from six different mothers. Statistical differences between dietary groups: *P<.05 compared to the control value of rats of the same age, gender and lipid moiety. Statistical test: Student's t test.

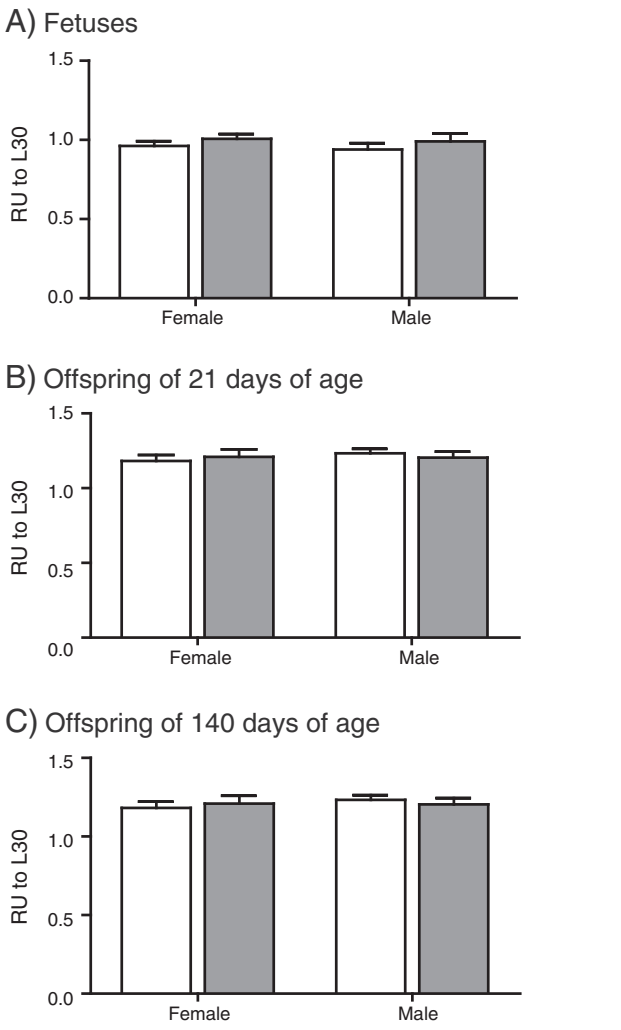

Fig. 3. Liver gene expression of PPARα in the fetuses and the offspring from control rats (open bars) and rats fed a saturated-fat-rich diet (gray bars). (A) Fetuses. (B) Offspring of 21 days of age. (C) Offspring of 140 days of age. Values are expressed as mean± standard error of the optical density units of PPARα relative to the optical density units of L30 as the housekeeping gene. Values were obtained from six different individuals from six different mothers. Statistical test: two-way ANOVA. Post-test: Bonferroni.

3. Results

3.1. Saturated fat in the maternal diet programs metabolic alterations in rat offspring

The pregnant control rats ate more food than the pregnant SFD group (control: 75±2 g/day per kilogram of rat vs. SFD: 58±2 g/day per kilogram of rat, P<.01). Nevertheless, as the saturated-fat-supplemented diet was more caloric, the SFD group consumed more calories than the controls (control: 264±2 kcal/day per kilogram of rat, SFD: 297±2 kcal/day per kilogram of rat, P<.01). We have previously found that fetuses from rats fed a saturated-fat-rich diet show an increase in glucose, triglycerides and cholesterol plasma levels [24]. In this work, we found increased triglyceridemia in pups (21 days old) (P<.01) from SFD rats (Table 2) and no changes in adult (140 days old) offspring from SFD rats when compared to controls (Table 2). Glucose and cholesterol plasma levels showed no differences between the dietary groups in pups and adult offspring. Also, no differences were found between females and males in all the groups studied. Offspring of 21 days of age from SFD rats were heavier than the controls, and also their livers were heavier than the ones from control offspring (P<.05) (Table 2). Offspring of 140 days of age from SFD rats showed no changes in body weight or liver weight compared to controls (Table 2). In the adult offspring, males were heavier than females (P<.01) and livers from males were heavier (P<.01) than those from females in both dietary groups (Table 2).

3.2. Saturated fat in the maternal diet programs lipid overaccumulation in livers from rat offspring

Female and male offspring from saturated-fat-rich diet fed mothers (SFD mothers) showed increased hepatic lipid levels. Female and male

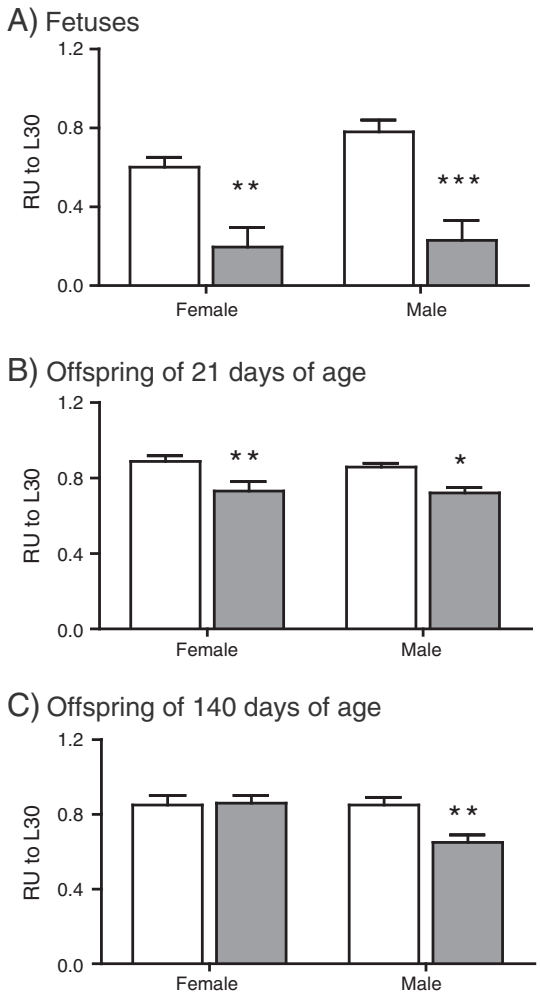

Fig. 4. Liver gene expression of CPT1 in the fetuses and the offspring from control (open bars) and rats fed a saturated-fat-rich diet (gray bars). (A) Fetuses. (B) Offspring of 21 days of age. (C) Offspring of 140 days of age. Values are expressed as mean± standard error of the optical density units of CPT1 relative to the optical density units of L30 as the housekeeping gene. Values were obtained from six different individuals from six different mothers. Statistical differences between the dietary groups: *P<.05, **P<.01 and ***P<.001 compared to the control value of rats of the same age and gender. Statistical test: two-way ANOVA. Post-test: Bonferroni.

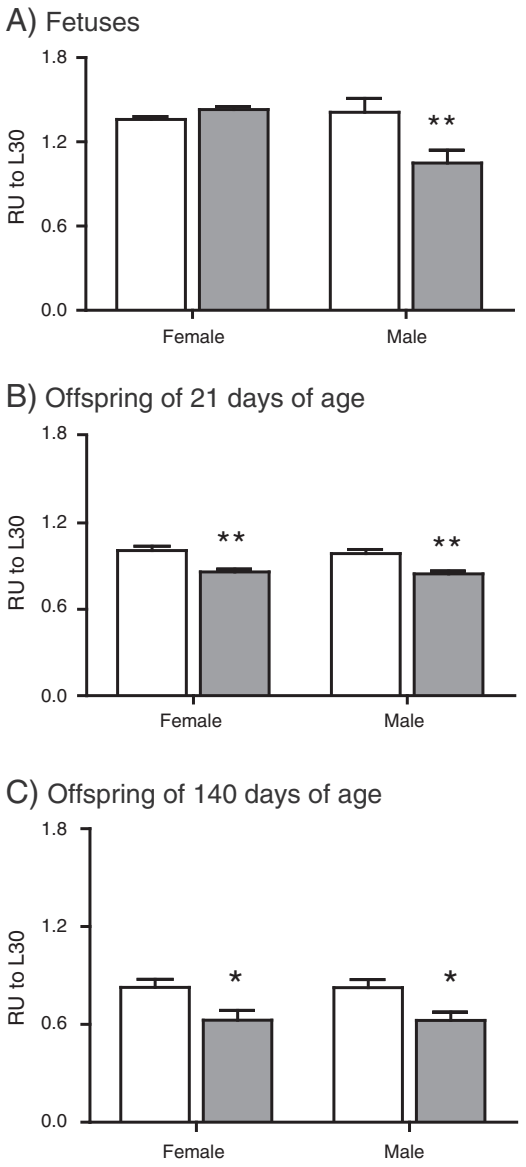

Fig. 5. Liver gene expression of ACO in the fetuses and the offspring from control rats (open bars) and rats fed a saturated-fat-rich diet (gray bars). (A) Fetuses. (B) Offspring of 21 days of age. (C) Offspring of 140 days of age. Values are expressed as mean± standard error of the optical density units of ACO relative to the optical density units of L30 as the housekeeping gene. Values were obtained from six different individuals from six different mothers. Statistical differences between the dietary groups: *P<.05 **P<.01 compared to control value of rats of the same age and gender. Statistical test: two-way ANOVA. Post-test: Bonferroni.

pups of 21 days of age from SFD rats showed an overaccumulation of triglycerides (P<.05) compared to controls (Fig. 2A and B). Aiming to study if this liver lipid accumulation persisted later in life, we assessed liver lipid levels in the 140-day-old offspring of SFD and control rats. Female rats born to SFD mothers showed increased triglycerides and free fatty acid levels (P<.05) in their livers compared to the female offspring of the same age born to control mothers (Fig. 2C). Also, livers from male offspring of SFD rats showed increased triglyceride levels (P<.05) when compared to controls (Fig. 2D).

3.3. Saturated fat in the maternal diet induces alterations in the expression of genes involved in lipid catabolism in livers from rat offspring

We next aimed to study the causes of liver lipid overaccumulation in the offspring from SFD rats. Therefore, we studied the liver gene expression of two enzymes and a transcription factor involved in lipid catabolism: CPT1, ACO and PPARα.

We have previously shown that fetuses from rats fed a saturated-fat-rich diet show liver lipid overaccumulation [24]. In this study, livers from male and female fetuses from SFD rats showed no changes in PPARα expression compared to controls (Fig. 3A). In addition, livers from female and male offspring (both pups and adults) from SFD rats showed no changes in PPARα expression when compared to controls (Fig. 3B and C).

Regarding gene expression of CPT1, livers from female and male fetuses from the SFD rats showed a decrease compared to controls (P<.01) (Fig. 4A). Similarly, livers from female and male offspring of 21 days of age displayed decreased expression of CPT1 (P<.05) compared to controls (Fig. 4B). Regarding offspring of 140 days of age, livers from males from SFD rats displayed a decrease in CPT1 expression (P<.01) while females showed no changes (Fig. 4C).

Regarding gene expression of ACO, livers from female fetuses from SFD rats showed no changes compared to controls, whereas those

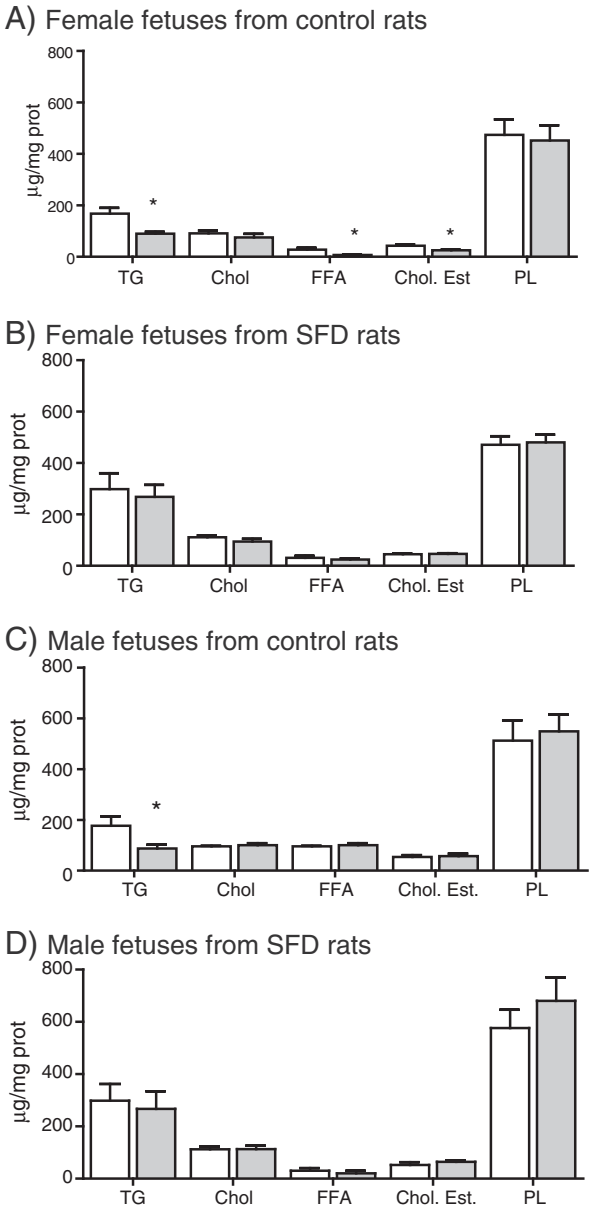

Fig. 6. Lipid levels in liver explants cultured for 3 h in the absence (open bars) or presence of 100 ng/ml of leptin (gray bars). (A) Livers of female fetuses from control rats. (B) Livers of female fetuses from rats fed a saturated-fat-rich diet. (C) Livers of male fetuses from control rats. (D) Livers of male fetuses from rats fed a saturated-fat-rich diet. TG, triglycerides; Chol, cholesterol; FFA, free fatty acids; Chol. Est., cholesterol esters; PL, phospholipids. Each bar represents the mean±standard error of the values from the analysis of six livers from six individuals from six different mothers. Statistical differences between presence and absence of leptin: *P<.05 compared to the value without leptin addition of rats of the same gender and lipid moiety. Statistical test: Student's t test.

from male fetuses from the SFD group showed decreased expression compared to controls (P<.01) (Fig. 5A). Also, ACO expression was down-regulated in livers from 21-day-old (P<.01) and 140-day-old (P<.05) female and male offspring from SFD rats compared to controls (Fig. 5B and C, respectively).

3.4. Leptin exerts different effects on liver lipid metabolism in fetuses from control and SFD rats

We aimed to investigate whether there was a potential *in utero* leptin resistance in liver lipid catabolism induced by the maternal

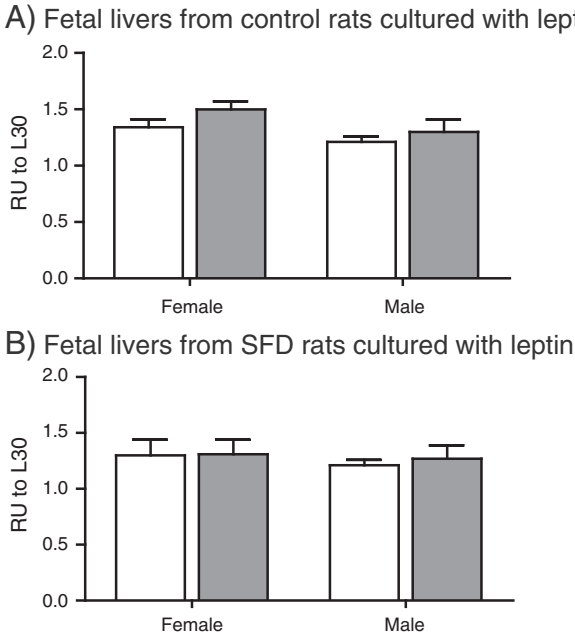

Fig. 7. Analysis of leptin effects on PPARα gene expression of fetal livers. (A) Livers of fetuses from control rats were cultured for 3 h either without additions (open bars) or with 100 ng/ml of leptin (gray bars). (B) Livers of fetuses from rats fed a saturated-fat-rich diet were cultured for 3 h either without additions (open bars) or with 100 ng/ml of leptin (gray bars). Values are expressed as mean±standard error of the optical density units of PPARα relative to the optical density units of L30 as the housekeeping gene. Values were obtained from six different fetuses from six different mothers. Statistical test: two-way ANOVA. Post-test: Bonferroni.

altered environment. To accomplish this, fetal liver explants were cultured in the presence or absence of leptin 100 ng/ml.

3.4.1. Tissue lipid levels

Leptin induced a decrease in triglycerides (47%), free fatty acids (75%) and cholesteryl ester (42%) levels in liver explants from female fetuses from control rats (P<.05) (Fig. 6A). In contrast, leptin displayed no effect on the livers from female fetuses from the SFD group (Fig. 6B). Also, leptin induced a decrease in triglycerides (50%) in liver explants of male fetuses from control rats (P<.05), and it showed no effects on liver explants of male fetuses from SFD rats (Fig. 6C and D).

3.4.2. Expression of genes involved in lipid catabolism

Regarding PPARα gene expression, leptin induced no changes in liver explants from female and male fetuses from control (Fig. 7A) and SFD rats (Fig. 7B).

Regarding liver gene expression of CPT1, female and male fetuses from control rats displayed an increase in the presence of leptin (P<.01) (Fig. 8A). Differently, no effects were observed in livers of female and male fetuses from SFD rats (Fig. 8B).

Regarding liver gene expression of ACO, female and male fetuses from control rats showed an increase after the culture with leptin (P<.01) (Fig. 9A). In contrast, we found no effects after the culture with leptin in the expression of ACO from female and male fetuses from SFD rats (Fig. 9B).

3.5. Effects of leptin administration to control fetuses on the expression of PPARα, CPT1 and ACO

We aimed to analyze whether fetal hyperleptinemia (mimicking a condition observed in fetuses from SFD rats) during the last days of gestation induced changes in the gene expression of liver lipid

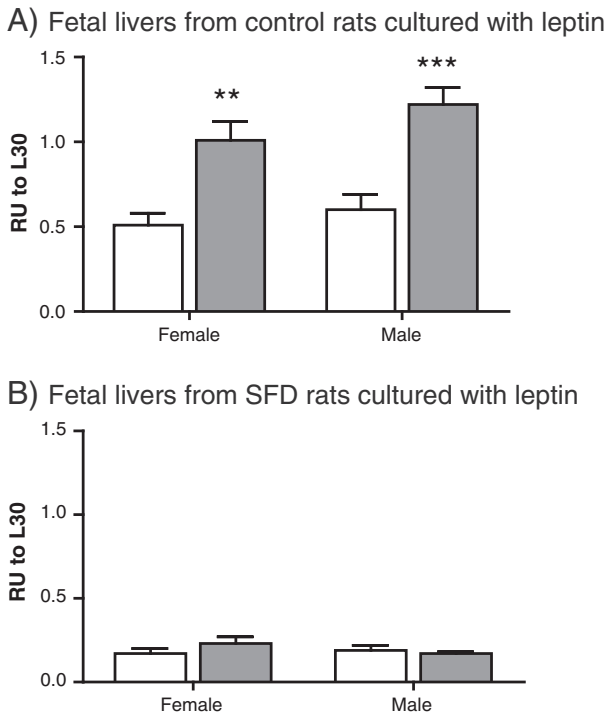

Fig. 8. Analysis of leptin effects on CPT1 gene expression of fetal livers. (A) Livers of fetuses from control rats were cultured for 3 h either without additions (open bars) or with 100 ng/ml of leptin (gray bars). (B) Livers of fetuses from rats fed a saturated-fat-rich diet were cultured for 3 h either without additions (open bars) or with 100 ng/ml of leptin (gray bars). Values are expressed as the mean±standard error of the optical density units of CPT1 relative to the optical density units of L30 as the housekeeping gene. Values were obtained from six different fetuses from six different mothers. Statistical differences between livers that received leptin and those that received no leptin from rats of the same gender: $^{**}P<.01$, $^{***}P<.001$. Statistical test: two-way ANOVA. Post-test: Bonferroni.

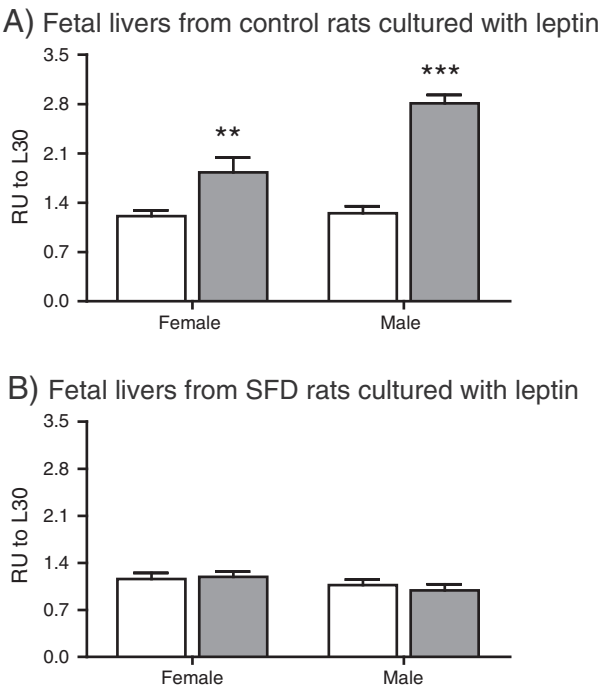

Fig. 9. Analysis of leptin effects on ACO gene expression of fetal livers. (A) Livers of fetuses from control rats were cultured for 3 h either without additions (open bars) or with 100 ng/ml of leptin (gray bars). (B) Livers of fetuses from rats fed a saturated-fat-rich diet were cultured for 3 h either without additions (open bars) or with 100 ng/ml of leptin (gray bars). Values are expressed as mean±standard error of the optical density units of ACO relative to the optical density units of L30 as the housekeeping gene. Values were obtained from six different fetuses from six different mothers. Statistical differences between livers that received leptin and those that received no leptin from rats of the same gender: $^{**}P<.01$, $^{***}P<.001$. Statistical test: two-way ANOVA. Post-test: Bonferroni.

catabolic enzymes. Intrafetal administration of leptin during the last 3 days of gestation induced an increase in leptin circulating levels in both males and females (Supplementary Fig. 1) and no changes in the expression of PPARα (Fig. 10A) and CPT1 (Fig. 10B) in livers from female and male fetuses compared to those from saline-injected fetuses. Differently, ACO expression was decreased in livers from leptin-injected male fetuses ($P<.01$) compared to those from saline-injected male fetuses. In contrast, leptin showed no effects in liver ACO expression when administered to female fetuses compared to the saline-injected ones (Fig. 10C).

4. Discussion

The data presented here show many alterations in lipid metabolism in the livers of the offspring born to rats fed a 26% saturated-fat-rich diet (SFD rats). We evidenced similar lipid metabolism alterations in fetuses and in offspring 21 days of age and 140 days of age. A relevant finding was that livers of fetuses from SFD mothers displayed no changes when exposed to leptin, suggesting *in utero* leptin resistance. We also provide data suggesting that fetal hyperleptinemia could be involved in the development of liver lipid homeostasis impairments.

We have previously shown that SFD rats display an increase in maternal weight gain during pregnancy and that their fetuses were macrosomic [24]. In this work, the offspring of 21 days of age from the SFD group were heavier and exhibited heavier livers than controls. Later in life, offspring of 140 days of age born to SFD rats showed no changes in body weight or liver weight when compared to controls. The young offspring showed increased triglyceridemia and liver lipid overaccumulation, while the elder offspring showed unchanged lipid plasma profile and did show the liver lipid overaccumulation. Other researchers have shown that adult offspring of rats fed high-fat diets (60% calories from fat) that continue or not eating the high-fat diet after weaning display fatty livers together with overweight and hypertriglyceridemia [28,44,45]. The 140-day-old offspring of both groups ate regular rat chow from weaning to sacrifice, and this might be the reason why the adult offspring from SFD rats exhibited no overweight or hypertriglyceridemia as the young offspring. Nevertheless, without the high lipid caloric input, they still maintained the liver lipid metabolism anomalies. Moreover, in our study, maternal saturated-fat-rich diet has 45% calories from fat and adult offspring evidenced liver alterations without overweight or hyperlipidemia. These results highlight the importance of the dietary composition, not only for the final caloric input but also for the source of energy used for maternal nourishing. Our investigation shows that liver damage can be induced in the offspring by the maternal diet despite having healthy nutrition from weaning.

Aiming to find a reason for the increase in liver lipid levels in the offspring from SFD rats, we studied the expression of PPARα, CPT1 and ACO, key genes involved in the lipid catabolism of the liver. We found that livers from fetuses and offspring from SFD rats showed several alterations depending on the age and on the gender. CPT1 expression was decreased in fetuses, young offspring and male adult offspring, while ACO expression was decreased in male fetuses and in young and adult offspring. The decrease in these enzymes could be implicated in the development of liver lipid overaccumulation. The offspring that received high saturated fat input during perinatal life could have adapted to down-regulate the lipid catabolism machinery, reducing

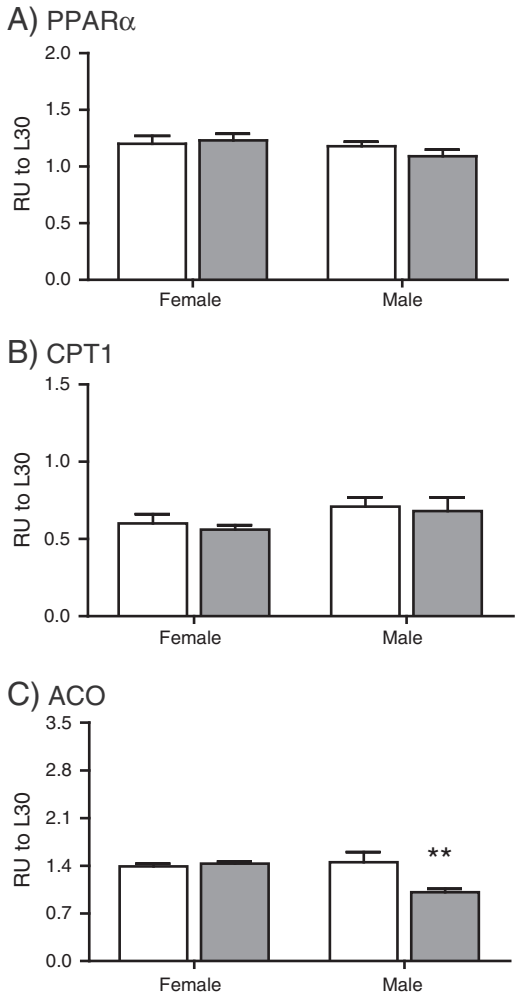

Fig. 10. Gene expression analysis in livers from fetuses administered with leptin (20 ng/day at 19, 20 and 21 days of gestation) (gray bars) vs. saline-administered fetuses (open bars). (A) PPARα expression, (B) CPT1 expression and (C) ACO expression. Values are expressed as the mean±standard error of the optical density units of each gene relative to the optical density units of L30 as the housekeeping gene. Values were obtained from six different fetuses from six different mothers. Statistical differences between the livers from fetuses treated with leptin and those with saline of the same gender: $^{**}P<.01$. Statistical test: two-way ANOVA. Post-test: Bonferroni.

fat utilization as energy source. The development of this adaptation might be the cause of liver lipid overaccumulation that induces lipotoxicity and is harmful for liver function [46,47].

PPARα expression showed no changes in all the developmental stages studied. Several works have shown decreased expression of ACO and CPT1 (targets of PPARα), together with clear markers of fatty liver disease [48–51]. As PPARα activity regulates the expression of both CPT1 and ACO [36,52,53], the fact that ACO and CPT1 are down-regulated independently of PPARα expression might indicate a decrease in PPARα activity in the SFD group.

Liver metabolic programming begins *in utero*. Aiming to find a reason for the decreased lipid catabolism enzyme expression observed in fetuses and offspring from SFD rats, we studied the effect of leptin on liver catabolism in fetuses from control and SFD rats. Livers from control fetuses responded to leptin-activating lipid catabolism, decreasing lipid levels and increasing the expression of genes of catabolic enzymes. In contrast, livers of fetuses from SFD rats showed no changes in lipid catabolism after the culture with leptin. ACO and CPT1 showed no response to leptin in fetal livers and were down-regulated in the subsequent developmental stages studied in the offspring from SFD rats. This suggests that leptin resistance may continue later in life and cause the alterations in lipid levels and enzyme expression observed in young and adult offspring. Other researchers have suggested liver leptin resistance in rats with fatty livers [35]. Here we provide data denoting that liver leptin resistance might be programmed during intrauterine life, conditioning the liver lipid metabolism of the offspring in the future.

We have previously shown that fetuses from SFD rats are hyperleptinemic [24]. Aiming to establish a role for fetal leptinemia, we mimicked the fetal hyperleptinemia observed in fetuses from SFD rats, in fetuses from control rats. Therefore, we assessed the expression of the genes involved in lipid catabolism in livers from control fetuses administered daily with leptin. The expression of ACO was down-regulated by leptin administration to male fetuses. The same down-regulated expression was observed in male fetuses from SFD rats. As hyperleptinemia is a common feature of SFD and control leptin-injected fetuses, we speculate that hyperleptinemia could be inducing impaired leptin receptor signaling, causing the down-regulation of ACO in livers from male SFD fetuses [35,54]. Nevertheless, the other alterations observed in gene expression in livers from SFD fetuses could not be explained by our experiment of fetal leptin administration. Thus, further research is needed to clarify this point.

In summary, we found lipid overaccumulation and abnormal expression of lipid catabolic enzymes in the livers from fetuses and offspring from rats fed a diet enriched in saturated fat. We also found liver leptin resistance in fetuses from the SFD mothers, suggesting that liver lipid homeostasis impairments observed in the offspring from SFD rats might be programmed by *in utero* leptin resistance.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2015.08.019.

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