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PPAR ligands improve impaired metabolic pathways in fetal hearts of diabetic rats

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Abstract

In maternal diabetes, the fetal heart can be structurally and functionally affected. Maternal diets enriched in certain unsaturated fatty acids can activate the nuclear receptors peroxisome proliferator-activated receptors (PPARs) and regulate metabolic and anti-inflammatory pathways during development. Our aim was to investigate whether PPARα expression, lipid metabolism, lipoperoxidation, and nitric oxide (NO) production are altered in the fetal hearts of diabetic rats, and to analyze the putative effects of *in vivo* PPAR activation on these parameters. We found decreased PPARα expression in the hearts of male but not female fetuses of diabetic rats when compared with controls. Fetal treatments with the PPARα ligand leukotriene B_4 upregulated the expression of PPARα and target genes involved in fatty acid oxidation in the fetal hearts. Increased concentrations of triglycerides, cholesterol, and phospholipids were found in the hearts of fetuses of diabetic rats. Maternal treatments with diets supplemented with 6% olive oil or 6% safflower oil, enriched in unsaturated fatty acids that can activate PPARs, led to few changes in lipid concentrations, but up-regulated PPARα expression in fetal hearts. NO production, which was increased in the hearts of male and female fetuses in the diabetic group, and lipoperoxidation, which was increased in the hearts of male fetuses in the diabetic group, was reduced by the maternal treatments supplemented with safflower oil. In conclusion, impaired PPARα expression, altered lipid metabolism, and increased oxidative and nitridergic pathways were evidenced in hearts of fetuses of diabetic rats and were regulated in a gender-dependent manner by treatments enriched with PPAR ligands.

Key Words
- diabetes in pregnancy
- fetus
- heart
- nitric oxide
- PPAR

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Introduction

Maternal diabetes can impair the development of the fetus and lead to adverse consequences evident in the offspring both during the perinatal period and in later life (Michael Weindling 2009, Simeoni & Barker 2009, Ali & Dornhorst 2011). The fetal heart is a target organ that can be structurally and functionally affected by maternal diabetes (Molin *et al*. 2004, Corrigan *et al*. 2009). In the fetal period, the heart uses glucose and lactate as main oxidative substrate sources and then switches to fatty acids in the neonate, to assure proper energy metabolism according to dietary and physiological conditions (Finck 2007).

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Peroxisome proliferator-activated receptor alpha (PPARα), the first of the three PPAR isotypes identified, is an important regulator of myocardial lipid metabolism that also contributes to the control of inflammation and oxidative stress (Wahli & Michalik 2012, Lee *et al*. 2013, Palomer *et al*. 2013). PPARα is expressed in the fetal heart and its expression increases after birth (Abbott 2009). In mouse models of diabetes, the fetal heart shows decreased PPARα expression (Lindegaard & Nielsen 2008). In experimental models, both PPARα inactivation and overexpression can lead to metabolic alterations in the heart (Finck 2007, Lindegaard & Nielsen 2008). In adult diabetic patients, chronic inflammation and alterations in energy and lipid homeostasis are involved in cardiac dysfunction (Palomer *et al*. 2013). In the cardiovascular system, several metabolic and anti-inflammatory pathways are regulated by ligands of the nuclear receptor PPARα both through transactivation and transrepression mechanisms (Lefebvre *et al*. 2006, Wahli & Michalik 2012). Fibrates are pharmacological ligands of PPARα used to regulate dyslipidemia, which ameiorate cardiovascular diseases and reduce pro-inflammatory markers in the heart of diabetic patients (Lefebvre *et al*. 2006, Lee *et al*. 2013). The natural ligands of PPARα include leukotriene (LT) B_4, and certain unsaturated fatty acids such as oleic acid and linoleic acid, which can be efficiently transferred through the placenta (Hihi *et al*. 2002, Herrera *et al*. 2006, Jawerbaum & Capobianco 2011).

Diabetes induces a pro-oxidant and pro-inflammatory state in both the non-pregnant and the pregnant states (Calkin & Thomas 2008, Lappas *et al*. 2011). In previous studies, in a mild diabetic rat model during pregnancy we have evaluated and found alterations in PPARα expression and signaling in different fetal organs such as the liver and the lungs (Jawerbaum & White 2010, Martinez *et al*. 2011*a*, Kurtz *et al*. 2012). In the current study, we used the same diabetic rat model to address whether the fetal heart has altered expression of PPARα and lipid-oxidizing enzymes, lipid content, nitric oxide (NO) production, and lipid peroxidation. We also carried out fetal treatments with LTB_4 and evaluated maternal diets enriched with 6% olive oil or 6% safflower oil to identify putative changes in PPARα expression, lipid metabolism, and oxidative and nitridergic pathways. Considering the known sexual differences in PPAR signaling and in metabolic and heart diseases, the hearts from both female and male fetuses were separately analyzed (Kautzky-Willer & Handisurya 2009, Yoon 2009, Garcia-Patterson *et al*. 2011, Mosca *et al*. 2011).

Materials and methods

Animals

Albino Wistar rats bred in our animal facility were fed *ad libitum* with commercial rat chow (Asociación Cooperativa Argentina, Buenos Aires, Argentina). To induce diabetes, at 2 days of age, neonates were injected with streptozotocin (90 mg/kg, s.c., Sigma–Aldrich) diluted in citrate buffer (0.05 M, pH 4.5, Sigma–Aldrich), as previously described (Jawerbaum & White 2010, Martinez *et al*. 2011*a*). The control animals were injected with citrate buffer alone. The diabetic state was confirmed in 2-month-old rats before mating. The rats were considered diabetic when they presented fasting glycemia values higher than 130 mg/dl. The guidelines for the care and use of animals approved by the local institution were followed (EXP-UBA 0011821), according to the Principles of Laboratory Animal Care (NIH publication number 85-23, revised 1985, http://grants1.nih.gov/grants/olaw/references/phspol.htm).

Control and diabetic female rats were mated with control males. The first day of pregnancy was confirmed by the presence of sperm cells in vaginal smears. On this day, both control and diabetic animals were randomized into two groups: group 1, animals whose fetuses were injected with the PPARα ligand LTB_4 or vehicle on days 19, 20, and 21 of pregnancy and group 2, animals fed with diets supplemented with 6% olive oil or 6% safflower oil, enriched in natural PPAR ligands, from days 1 to 21 of pregnancy.

In group 1, to inject the fetuses, the animals ($n=9$ control and $n=9$ diabetic rats) were anesthetized in a CO_2 chamber on days 19, 20, and 21 of pregnancy and a slight anesthesia maintained with ether vapors. An abdominal incision was performed and the left horn of the uterus was exposed. The fetuses were numbered from the ovary and alternatively injected with LTB_4 (0.1 nmol/fetus dissolved in vehicle; Cayman Chemical Co., Ann Arbor, MI, USA) or vehicle (0.3 μl ethanol/fetus, dissolved in saline solution) subcutaneously on their backs through the uterine wall, as described previously (Kurtz *et al*. 2012). The entire surgery lasted <10 min and the animals were completely recovered after 15 min. On day 21 of pregnancy and after 3 h of the last injection, the rats were killed and the hearts from female and male fetuses were explanted and preserved as described below.

In group 2, control and diabetic mothers were fed from days 1 to 21 of pregnancy either with a standard diet or with diets enriched in unsaturated fatty acids that activate PPARs: 6% olive oil (354% enriched in oleic acid) and 6% safflower oil (226% enriched in linoleic acid)

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($n=9$ in each experimental group), as described previously (Martinez *et al*. 2012). The compositions of the diets were as follows: standard diet (g/100 g): carbohydrates (50); proteins (25); fat (5), major fatty acids 16:0 (0.58), 18:0 (0.16), 18:1 (1.27), 18:2 (1.99), 18:3 (0.73); olive oil-supplemented diet (g/100 g): carbohydrates (48); proteins (24); fat (11), major fatty acids 16:0 (1.55), 18:0 (0.26), 18:1 (5.77), 18:2 (2.41), 18:3 (0.57); and safflower oil-supplemented diet (g/100 g): carbohydrates (47); proteins (23); fat (11), major fatty acids 16:0 (0.97), 18:0 (0.25), 18:1 (1.81), 18:2 (6.49), 18:3 (0.55).

All animals from groups 1 and 2 were killed by decapitation on day 21 of pregnancy. Maternal and fetal blood samples were collected in heparinized tubes and plasma was preserved at −80 °C. Under a stereomicroscope, fetuses were sexed and the fetal hearts explanted. The hearts from male and female fetuses were randomly selected and either preserved in RNA stabilization solution (RNAlater, Invitrogen) for further evaluation of gene expression of PPARα and lipid-oxidizing enzymes or preserved at −80 °C for further analysis of lipid content, NO production, and lipoperoxidation.

Metabolic assays

Glycemia values were measured by the Accu-Chek reagent strips and a glucometer Accu-Chek (Bayer Diagnostics) in blood samples obtained from the tail vein of the mothers. The maternal and fetal glycemia and triglyceridemia were measured in plasma by an enzymatic colorimetric commercial kit (Wiener Lab, Rosario, Argentina).

Expression of PPARα and rate-limiting enzymes in lipid oxidation

The gene expression of PPARα and the rate-limiting enzymes in lipid oxidation of acyl CoA oxidase (ACO) and carnitine palmitoyltransferase 1 (CPT1) were evaluated by RT-PCR, a semi-quantitative method, as described previously (Kurtz *et al*. 2012). Briefly, RNA was extracted from the hearts of one female and one male fetus from each rat ($n=9$ rats in each experimental group) with TRI reagent (Genbiotech, Buenos Aires, Argentina) in accordance with the manufacturer's instructions. cDNA was synthesized by incubating 1 μg of extracted RNA in a first-strand buffer containing MMLV enzyme (Promega), random primer hexamers, and each of all four dNTPs (Invitrogen), in accordance with the MMLV manufacturer's instructions. 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 for PPARα were as follows: forward, 5′-TCACACAATGCAATCCGTTT-3′ and reverse: 5′-GGCCTTGACCTTGTTCATGT-3′, whose amplification product is a 177-bp fragment (Kurtz *et al*. 2012). The primers for *Aco* were as follows: forward: 5′-CCAATCACGCAATAGTTCTGG-3′ and reverse, 5′-CGCTGTATCGTATGGCGAT-3′, whose amplification product is a 363-bp fragment (Lillycrop *et al*. 2005). The primers for *Cpt1* were as follows: forward, 5′-TATCGTCGCACATTAGACCGT-3′ and reverse, 5′-CATCTATGACCTCCTGGCACT-3′, whose amplification product is a 715-bp fragment (Cheng *et al*. 2004). The primers for the ribosomal protein L30, used as an internal control, were as follows: forward, 5′-CCATCTTGGCGTCTGATCTT-3′ and reverse, 5′-GGCGAGGATAACCAATTTC-3′, whose amplification product is a 201-bp fragment (Primer 3 Software, Cambridge, MA, USA). The initial step in the reaction was 95 °C for 5 min, followed by 33 cycles for PPARα, 33 cycles for ACO, 34 cycles for CPT1, and 26 cycles for L30, as selected to work within the linear range. 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. The resulting products were separated on a 2% agarose gel and stained with SYBR safe (Invitrogen). The images were taken with an ImageQuant spectrophotometer (GE Healthcare, Buckinghamshire, UK) and the density of the bands was quantified with the Image J Software (NIH, Bethesda, MD, USA) and normalized to L30.

Analysis of lipid content

Lipid content was determined by thin layer chromatography, as described previously (Martinez *et al*. 2011*a*). Briefly, hearts from three female or three male fetuses from each rat were pooled and homogenized ($n=9$ rats in each experimental group) in 500 μl PBS and protein content in the homogenates was measured by the Bradford assay. The tissue lipids were extracted from 470 μl of each homogenate by three rounds of organic extraction in methanol:chloroform (2:1). The lipids were developed by thin layer chromatography in 0.2 mm silica gel plates (Merck), using hexane:ether:acetic acid (80:20:2, v:v:v) as the developing solvent mixture. The lipid species were stained with iodine vapors, identified, and quantified by comparison with known the amounts of standards on the same plate, and densitometric analysis was performed using the Image J Software.

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Evaluation of NO production

NO production was evaluated by measuring the concentration of its stable metabolite nitrates/nitrites, as previously determined (Kurtz *et al.* 2012), by using a commercial assay kit (Cayman Chemical Co.). For this, hearts of two female or two male fetuses from each rat were pooled and homogenized ($n=9$ rats in each experimental group) in 1 ml Tris–HCl buffer of pH 7.6, and an aliquot was separated for protein analysis. The nitrates in the supernatant were reduced to nitrites by nitrate reductase, and the total nitrites were measured by the Griess method (Green *et al.* 1982). The optical densities were measured at 540 nm in a microtiter plate using nitrates as standard.

Analysis of lipoperoxidation

Lipoperoxidation was assessed as described previously (Martinez *et al.* 2011*a*), by measuring the concentrations of thiobarbituric acid reactive substances (TBARS), a method widely used to assess peroxidation of fatty acids (Ohkawa *et al.* 1979). Briefly, hearts from two female or two male fetuses from each rat were pooled and homogenized ($n=9$ rats in each experimental group) in 100 mM Tris–HCl buffer (0.1 mM, pH 7.4). The homogenate was added with 40% trichloroacetic acid (Merck). After centrifugation, the supernatant was added with an equal volume of thiobarbituric acid (46 mM; Sigma–Aldrich), the solution was heated at 95 °C and, after cooling, quantified spectrophotometrically at 540 nm. Different concentrations of malondialdehyde (Sigma–Aldrich) subjected to the same conditions as the tissue homogenates were used as standards.

Statistical analysis

Data are presented as means ± S.E.M. Groups were compared by two-way ANOVA in conjunction with Bonferroni's test. A P value <0.05 was considered statistically significant.

Results

Decreased expression of PPARα and enzymes involved in lipid oxidation in the hearts of fetuses from diabetic rats: effects of fetal treatments with a PPARα activator

We first analyzed the metabolic parameters of experimental group 1 and found that glycemia and triglyceridemia were increased in both mothers and fetuses from diabetic rats compared with controls on day 21 of pregnancy ($P<0.01$), with no gender differences and no effects of injecting with the PPARα activator LTB_4 on fetus in the groups evaluated (Table 1).

When we evaluated the expression of PPARα, a regulator of lipid metabolism and anti-inflammatory processes in the heart of adults and in different fetal tissues (Martinez *et al.* 2011*a*, Kurtz *et al.* 2012, Lee *et al.* 2013), we found a decrease in PPARα expression in the hearts of male but not of female fetuses in the diabetic group compared with controls ($P<0.01$, Fig. 1A). We also evidenced the ability of the PPARα ligand LTB_4 (0.1 nmol, injected into the fetuses through the uterine wall on days 19, 20, and 21 of pregnancy) to upregulate PPARα expression in the hearts of female and male fetuses in the diabetic group and of male fetuses in the control group ($P<0.01$, Fig. 1A). We next evaluated the expression of *Aco* and *Cpt1*, relevant PPARα-target genes that code for rate-limiting enzymes in lipid oxidation. We found a decrease in *Aco* expression in the hearts of male fetuses but not of female fetuses in the diabetic group compared with controls ($P<0.05$, Fig. 1B), and a decrease in *Cpt1* expression in the hearts of male and female fetuses in the diabetic group compared with controls ($P<0.01$, Fig. 1C). The fetal injections with the PPARα ligand LTB_4 (0.1 nmol) led to an increase in *Aco* expression in the hearts of female and male fetuses in the diabetic group as well as in the hearts of female fetuses in the control group

Table 1 Maternal and fetal metabolic parameters in control and diabetic rats from experimental group 1 whose fetuses were injected with LTB_4 or vehicle on days 19, 20, and 21 of pregnancy. Values represent mean ± S.E.M., obtained from mothers and female or male fetuses from $n=9$ rats in experimental group 1. Two-way ANOVA in conjunction with Bonferroni's test was performed

	Glycemia (mg/dl)	**Triglyceridemia** (g/l)
Maternal data		
Control	92 ± 8	1.9 ± 0.3
Diabetic	214 ± 24[a]	4.0 ± 0.3[a]
Fetal data (combined males/females)		
Control vehicle	47 ± 5	0.54 ± 0.06
Control LTB_4	49 ± 10	0.60 ± 0.05
Diabetic vehicle	146 ± 11[b]	0.89 ± 0.07[c]
Diabetic LTB_4	135 ± 9	0.88 ± 0.04

[a]$P<0.001$ diabetic vs control; [b]$P<0.01$, [c]$P<0.001$ diabetic with vehicle vs control with vehicle.

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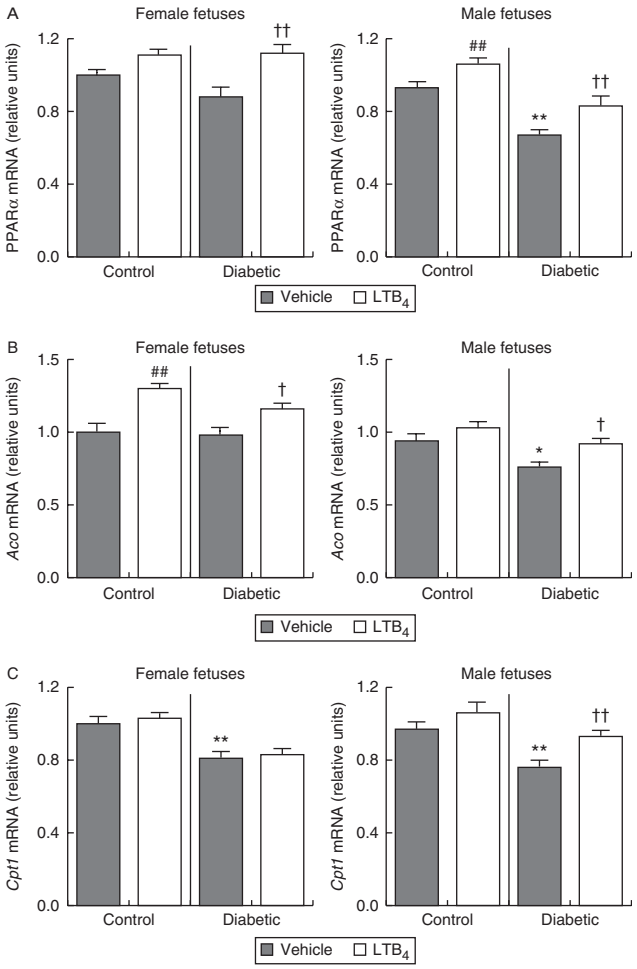

Figure 1
(A) PPARα expression, (B) *Aco* expression, and (C) *Cpt1* expression in the hearts of fetuses from control and diabetic rats injected with the PPARα agonist LTB_4 or with vehicle on days 19, 20, and 21 of pregnancy. Values represent mean ± S.E.M., obtained from female or male fetuses from $n=9$ rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's test was performed. $^{*}P<0.05$, $^{**}P<0.01$ diabetic with vehicle vs control with vehicle; $^{\#\#}P<0.01$ control with LTB_4 vs control with vehicle; and $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ diabetic with LTB_4 vs diabetic with vehicle.

($P<0.05$, Fig. 1B). The fetal treatments with LTB_4 only led to an increase in *Cpt1* expression in the hearts of male fetuses in the diabetic group ($P<0.01$, Fig. 1C).

Effect of maternal diets enriched with olive oil or safflower oil on lipid concentrations

Considering the sex-dependent changes observed in the expression of PPARα and its target genes involved in lipid oxidation in the fetal hearts of diabetic rats, we evaluated lipid concentrations in the hearts of female and male fetuses from control and diabetic rats. We found increased concentrations of triglycerides ($P<0.001$), cholesterol ($P<0.05$), and phospholipids ($P<0.05$) and no changes in cholesteryl esters in the heart of female and male fetuses in the diabetic group compared with controls (Fig. 2). As we have previously shown that dietary supplementation with 6% olive oil and 6% safflower oil can regulate lipid content in different fetal tissues and through developmental stages (Capobianco *et al.* 2008*a*,*b*, Kurtz *et al.* 2014), in this study we analyzed the effect of these dietary supplementations, administered from days 1 to 21 of pregnancy. The metabolic parameters evaluated in these animals (experimental group 2) are shown in Table 2. On day 21 of pregnancy, increased glycemia and triglyceridemia were observed in both diabetic mother and fetus rats fed the standard diet compared with controls fed the standard diet ($P<0.001$), with no gender differences, and no effects of the diets supplemented with 6% olive oil or 6% safflower oil in the groups evaluated (Table 2).

When we analyzed the effect of the maternal diets supplemented with 6% olive and 6% safflower oils on lipid content in the fetal heart, we observed only a few changes, all of them evidenced in the hearts of female fetuses: an increase in triglycerides in the diabetic group fed the olive oil-supplemented diet, a decrease in cholesterol in the control group fed the olive oil-supplemented diet, and an increase in cholesteryl esters in the control group fed the safflower oil-supplemented diet ($P<0.05$, Fig. 2).

The absence of marked effects on lipid content was evidenced even when the olive oil- and the safflower oil-supplemented diets induced relevant changes in PPARα expression in the hearts of both female and male fetuses (Fig. 3A). Indeed, PPARα expression was negatively regulated by the olive oil-supplemented diet in the hearts of female fetuses in the diabetic group and positively regulated by both the olive oil- and the safflower oil-supplemented diets in the hearts of male fetuses in control and diabetic groups ($P<0.05$, Fig. 3A). No effects were observed when expression of *Aco* and *Cpt1* was evaluated in the control and the diabetic groups fed olive oil- and safflower oil-supplemented diets compared with the respective groups fed the standard diet (Fig. 3B and C).

Effect of maternal diets enriched with olive oil or safflower oil on NO production

Our previous studies suggest that PPARα is a relevant regulator of NO production in different fetal organs (Martinez *et al.* 2011*b*, Kurtz *et al.* 2012). Excessive

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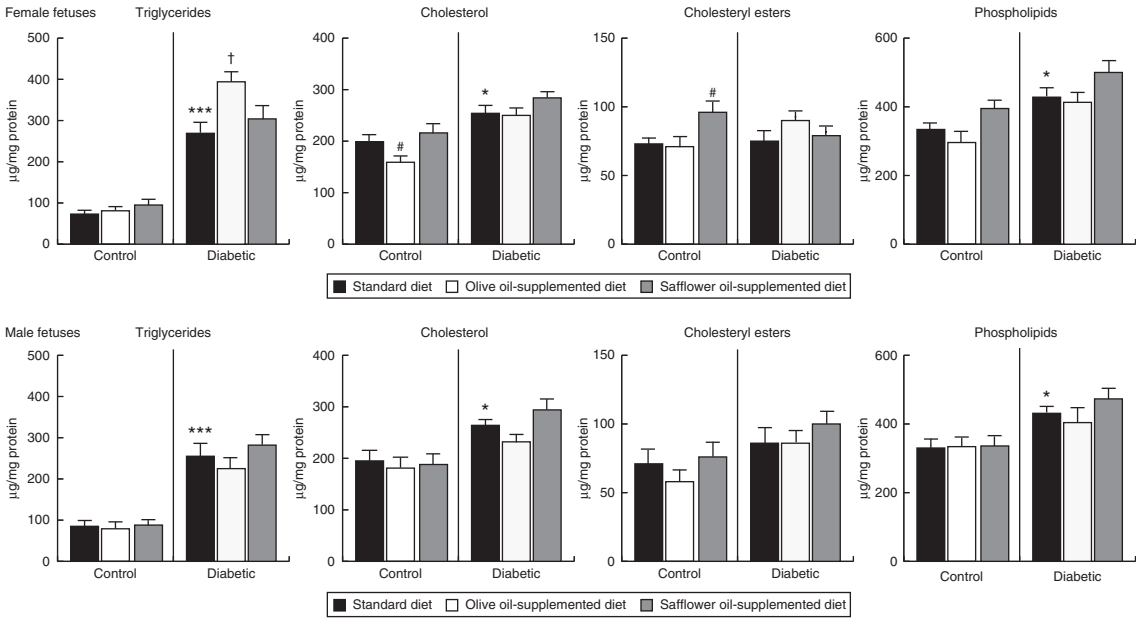

Figure 2
Lipid concentrations in the hearts of fetuses from control and diabetic rats fed with a standard, a 6% olive oil-supplemented or a 6% safflower oil-supplemented diet from days 1 to 21 of pregnancy. Values represent mean ± S.E.M., obtained from female or male fetuses from $n=9$ rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's test was performed. *$P<0.05$, ***$P<0.001$ diabetic with standard diet vs control with standard diet; $^{\#}P<0.05$ control with oil-supplemented diets vs control with standard diet; and $^{\dagger}P<0.05$ diabetic with oil-supplemented diets vs diabetic with standard diet.

production of NO can affect embryo and fetal development and is a marker of a pro-inflammatory state (Lappas *et al*. 2011). In this work, we found increased nitrates/nitrites concentrations, an index of NO production, in the hearts of female and male fetuses of diabetic rats when compared with controls ($P<0.01$, Fig. 4). We also evidenced the ability of the maternal diets supplemented with olive oil to decrease nitrates/nitrites (stable NO metabolites) in the hearts of male fetuses in the diabetic group ($P<0.01$, Fig. 4), and the ability of the maternal diets supplemented with 6% safflower oil to decrease nitrates/nitrites in the hearts of both female and male fetuses in the diabetic group ($P<0.01$, Fig. 4).

Table 2 Maternal and fetal metabolic parameters in control and diabetic rats from experimental group 2 fed with standard diet supplemented with or without 6% olive oil or 6% safflower oil from days 1 to 21 of pregnancy. Values represent mean ± S.E.M., obtained from mothers and female or male fetuses from $n=9$ rats in experimental group 2. Two-way ANOVA in conjunction with Bonferroni's test was performed

	Control			Diabetic		
	Standard diet	Standard diet supplemented with 6% olive oil	Standard diet supplemented with 6% safflower oil	Standard diet	Standard diet supplemented with 6% olive oil	Standard diet supplemented with 6% safflower oil
Maternal data						
Glycemia (mg/dl)	93 ± 7	105 ± 9	98 ± 12	230 ± 15[a]	225 ± 15	230 ± 14
Triglyceridemia (g/l)	2.1 ± 0.15	2.03 ± 0.25	2.01 ± 0.15	4.15 ± 0.20[a]	3.87 ± 0.22	3.92 ± 0.16
Fetal data (combined males/females)						
Glycemia (mg/dl)	46 ± 7	45 ± 9	44 ± 6	146 ± 11[a]	129 ± 15	120 ± 23
Triglyceridemia (g/l)	0.52 ± 0.05	0.59 ± 0.06	0.48 ± 0.05	0.98 ± 0.07[a]	1.05 ± 0.10	0.90 ± 0.08

[a]$P<0.01$ diabetic with standard diet vs control with standard diet.

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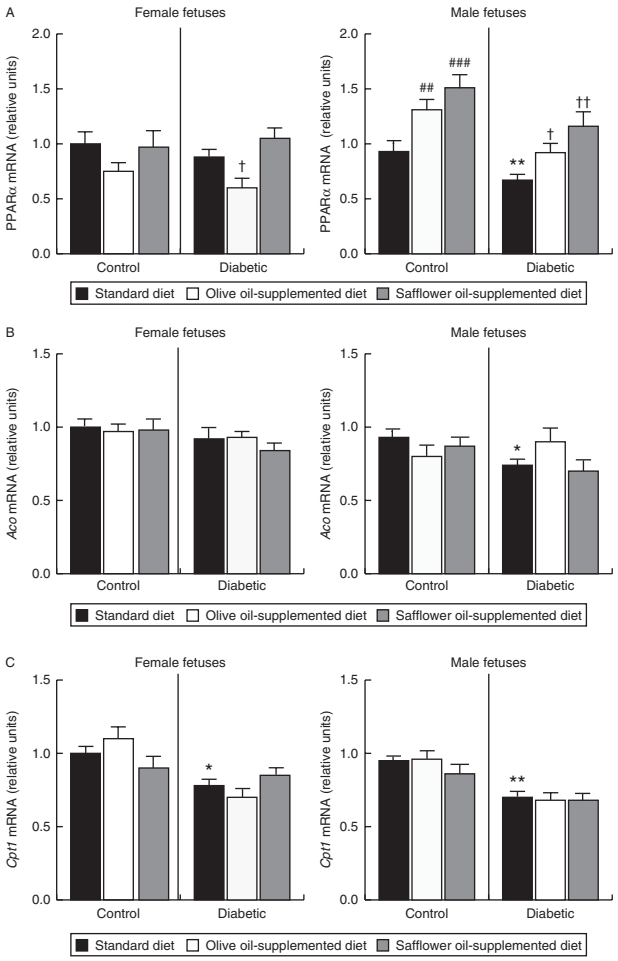

Figure 3
(A) PPARα expression, (B) *Aco* expression, and (C) *Cpt1* expression in hearts of fetuses from control and diabetic rats fed with a standard, a 6% olive oil-supplemented or a 6% safflower oil-supplemented diet from days 1 to 21 of pregnancy. Values represent mean ± S.E.M., obtained from female or male fetuses from $n=9$ rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's test was performed. $^{*}P<0.05$, $^{**}P<0.01$ diabetic with standard diet vs control with standard diet; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ control with oil-supplemented diets vs control with standard diet; and $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ diabetic with oil-supplemented diets vs diabetic with standard diet.

Effect of maternal diets enriched with olive oil or safflower oil on lipoperoxidation

As PPARα activation can regulate anti-oxidant pathways in different tissues during development (Martinez *et al.* 2011*a*,*b*) and lipoperoxidation is a marker of oxidative stress previously found to be increased in the fetuses and placentas of diabetic rats (Jawerbaum & White 2010), we next evaluated lipoperoxidation in the heart of fetuses from control and diabetic rats that were treated with or without diets supplemented with 6% olive oil or 6% safflower oil from days 1 to 21 of pregnancy. Lipoperoxidation was observed to be increased in the hearts of male fetuses ($P<0.01$), although not in the hearts of female fetuses from diabetic rats when compared with controls (Fig. 5). We also evidenced the ability of the maternal diets supplemented with 6% safflower oil, although not of those supplemented with 6% olive oil, to decrease lipoperoxidation in the hearts of male fetuses in the diabetic group ($P<0.01$, Fig. 5).

Discussion

In this study, we found sex-dependent increases in lipid content, NO production, and lipoperoxidation, together with decreased expression of PPARα and rate-limiting enzymes involved in lipid oxidation in the hearts of term fetuses of diabetic rats. Fetal or maternal treatments with PPAR activators regulated most of the parameters evaluated in a sex-dependent way. PPARα expression was upregulated both by fetal treatments with PPARα ligands and by maternal treatments with diets enriched with PPAR ligands during gestation, thus indicating the ability of these treatments to amplify PPARα signaling pathways in the fetal heart.

PPARα is a master regulator of lipid oxidation in metabolic tissues such as liver, kidney, and heart (Lefebvre *et al.* 2006). In this study, we observed a decrease in PPARα and its target genes *Aco* and *Cpt1* in the hearts of male fetuses from diabetic rats, although only a decrease in *Cpt1* in the hearts of female fetuses in the diabetic group when compared with controls. These decreases, together with

Figure 4
Nitric oxide (NO) production, evaluated through the concentration of NO stable metabolites nitrates/nitrites, in the hearts of fetuses from control and diabetic rats fed with a standard, a 6% olive oil-supplemented, or a 6% safflower oil-supplemented diet from days 1 to 21 of pregnancy. Values represent mean ± S.E.M., obtained from female or male fetuses from $n=9$ rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's test was performed. $^{**}P<0.01$, $^{***}P<0.01$ diabetic with standard diet vs control with standard diet and $^{\dagger\dagger}P<0.01$ diabetic with oil-supplemented diets vs diabetic with standard diet.

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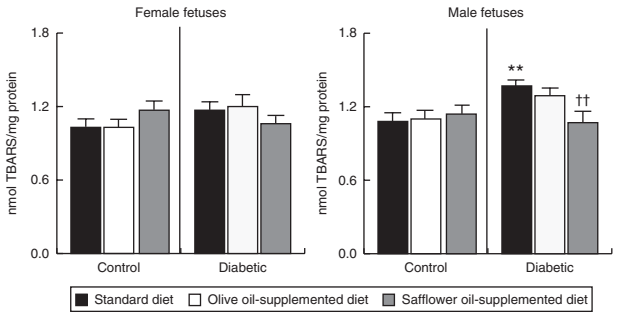

Figure 5
Lipoperoxidation, examined through the concentrations of TBARS, in the hearts of fetuses from control and diabetic rats fed with a standard, a 6% olive oil-supplemented or a 6% safflower oil-supplemented diet from days 1 to 21 of pregnancy. Values represent mean ± S.E.M., obtained from female or male fetuses from $n=9$ rats in each experimental group. Two-way ANOVA in conjunction with Bonferroni's test was performed. $^{**}P<0.01$ diabetic with standard diet vs control with standard diet and $^{††}P<0.01$ diabetic with oil-supplemented diets vs diabetic with standard diet.

the increased maternal and fetal triglyceridemia, may be involved in the overaccumulation of lipids in the hearts of fetuses in the diabetes experimental model evaluated. Previous studies performed in Akita mice, a more severe genetic model of diabetes, showed no changes in triglyceride content but a decrease in lipid transporters and in the expression of PPARα in the fetal hearts (Lindegaard & Nielsen 2008). It is known that PPARα regulates lipid oxidation in the hearts of neonates and adults (Finck 2007), but its function as a lipid metabolic sensor is not yet clear in the fetuses. In this work, we found that the PPARα ligand LTB_4 is able to increase the expression of the lipid-oxidizing enzymes ACO and CPT1 in the fetal heart. Nevertheless, maternal diets enriched with PPAR ligands did not increase the expression of ACO and CPT1, and did not lead to a decreased content of lipid species in the fetal heart. It is known that PPAR ligands decrease lipid content in the fetal liver (Martinez *et al.* 2011*a*) and that the liver is the main target in treatment with PPARα ligands in adult animals (Finck 2007). However, in this study, we found that both the maternal diets enriched with PPAR ligands and the fetal treatments with the PPARα agonist LTB_4 led to an increased expression of PPARα in the fetal heart, indicating that PPARα signaling is stimulated in this fetal organ by the given treatments. Several studies have shown that different PPAR ligands can induce different conformational changes in PPARs, as well as different biological responses (Hostetler *et al.* 2005, Gregoire *et al.* 2009). Indeed, ligand activation stabilizes the interaction between PPAR and RXR, enhances the formation of functional PPAR–RXR heterodimers, and can also regulate the heterodimerization with different partners (Harmon *et al.* 2011, Balanarasimha *et al.* 2014). The resulting interaction plays a key role leading to the formation of complexes, which result in gene activation or repression (Harmon *et al.* 2011). Thus, it is possible that transactivation pathways are those mainly activated by LTB_4 in the fetal heart, leading to the expression of regulation of lipid oxidizing enzymes, whereas transrepression pathways are those mainly activated by the maternal dietary treatments with unsaturated fatty acids. However, further studies are needed to address this point, as this work was limited due to the evaluation of PPAR ligands in different *in vivo* approaches. The formation of PPAR complexes and the resulting activity are tissue-dependent, and PPARs' capacity to activate or repress metabolic and pro-inflammatory pathways was similarly evidenced using different *in vivo* and *in vitro* approaches in the fetal liver and lung (Harmon *et al.* 2011, Martinez *et al.* 2011*a*, Kurtz *et al.* 2012).

In the heart, relevant extra-metabolic functions related with the control of the pro-inflammatory and pro-oxidant environment can be exerted by PPARα activation (Palomer *et al.* 2013). We found that NO production, which is increased in pro-inflammatory states (Lappas *et al.* 2011), was increased in the hearts of male and female fetuses from diabetic rats. Previous research has demonstrated that diabetic embryopathy is related with NO overproduction, and that different fetal organs such as liver, lung, and placenta have increased NO production when being developed in a diabetic mother (Martinez *et al.* 2011*a*,*b*, Kurtz *et al.* 2012). Moreover, in the presence of oxidative stress, NO overproduction leads to the formation of peroxynitrites, which induce severe damage to different organs, including the heart (Mungrue *et al.* 2002, Lappas *et al.* 2011). Thus, it was interesting to find that maternal diets enriched with 6% olive oil (only in male fetuses) and 6% safflower oil (in both male and female fetuses) prevented the overproduction of NO in the hearts of fetuses from diabetic rats. Future research is needed to identify whether putative epigenetic changes are involved in the fetal changes observed, and to analyze the effects of these treatments in the heart of neonates.

It is known that cardiac dysfunction is sex-dependent, possibly in part due to the differential ability of estrogens to regulate lipid metabolism (Kautzky-Willer & Handisurya 2009, Oosthuyse & Bosch 2012). PPARα signaling is also sex-dependent due to the effects of estrogens and androgens on PPARα expression and due to the use of common coactivators by different nuclear

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receptors (Collett *et al*. 2000, Yoon 2009, Benz *et al*. 2012). Indeed, we have previously found sex-dependent effects of PPARα agonists in different tissues even at fetal stage (Kurtz *et al*. 2014). Interestingly, in this study, we found that only the heart of male fetuses showed increased lipoperoxidation in the diabetic group. Increased oxidative stress, related with impairments in energy metabolism, stress in endoplasmic reticulum, and apoptosis, has been described in the heart of adult diabetic animals (Li *et al*. 2007, Palomer *et al*. 2013).

Previous research has identified the ability of maternal diets enriched with PPAR ligands to reduce oxidative stress during embryo development and to reduce pro-inflammatory markers in the placenta and fetal lungs (Higa *et al*. 2012, Kurtz *et al*. 2012, Martinez *et al*. 2012). A limitation of this study was that, due to the lack of sufficient biological material, we were unable to measure NO production and lipid peroxidation in the LTB_4-treated group. Although the 6% olive oil-supplemented diet is enriched with both oleic acid and polyphenols that can exert antioxidant properties (Martin-Pelaez *et al*. 2013), only the maternal treatments supplemented with 6% safflower oil were able to decrease lipoperoxidation in the hearts of male fetuses from diabetic rats. This may be due to the increased ability of the safflower oil-supplemented diet to amplify PPARα signaling pathways. Indeed, we showed that this diet leads to a greater increase in PPARα expression in the hearts of fetuses from diabetic rats. In addition, safflower oil is enriched with linoleic acid, which can activate PPARs by itself and by the production of eicosanoids that can further activate PPARs (Jawerbaum & Capobianco 2011).

In conclusion, the fetal hearts evaluated in the mild diabetic rat model were profoundly affected, as evidenced by overaccumulation of lipid, increased NO, and lipoperoxidation, which may lead to heart dysfunction later in life. These alterations were more marked in the hearts of male fetuses, which showed reduced expression of PPARα and target genes involved in fatty acid oxidation. Moreover, PPARα signaling in the fetal heart was mainly activated by the maternal dietary treatment enriched with 6% safflower oil, leading to the regulation of lipoperoxidation and NO production, by ameliorating the pro-oxidant and pro-inflammatory environment and thus may prevent the future development of cardiac dysfunction.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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