PPARα agonists regulate lipid metabolism and nitric oxide production and prevent placental overgrowth in term placentas from diabetic rats

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Abstract

Maternal diabetes impairs fetoplacental metabolism and growth. Peroxisome proliferator-activated receptor α (PPARα) is a nuclear receptor capable of regulating lipid metabolism and inflammatory pathways. In this study, we analyzed whether placental and fetal PPARα activation regulates lipid metabolism and nitric oxide (NO) production in term placentas from diabetic rats. Diabetes was induced by neonatal streptozotocin administration. On day 21 of pregnancy, placentas from control and diabetic rats were cultured in the presence of PPARα agonists (clofibrate and leukotriene B_4 (LTB_4)) for further evaluation of levels, synthesis, and peroxidation of lipids as well as NO production. Besides, on days 19, 20, and 21 of gestation, fetuses were injected with LTB_4, and the placentas were explanted on day 21 of gestation for evaluation of placental weight and concentrations of placental lipids, lipoperoxides, and NO metabolites. We found that placentas from diabetic rats showed reduced PPARα concentrations. They presented no lipid overaccumulation but reduced lipid synthesis, parameters negatively regulated by PPARα activators. Lipid peroxidation and NO production, increased in placentas from diabetic rats, were negatively regulated by PPARα activators. Fetal PPARα activation in diabetic rats does not change placental lipid concentrations but reduced placental weight and NO production. In conclusion, PPARα activators regulate lipid metabolism and NO production in term placentas from diabetic rats, an activation that regulates placental growth and can partly be exerted by the developing fetus.

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Introduction

Maternal diabetes impairs fetal and placental development, metabolism, and growth. Both type 1 and type 2 diabetes increase the risks of miscarriage and malformations (Balsells *et al.* 2009, Michael Weindling 2009). Gestational, type 1, and type 2 diabetes increase the risks of perinatal morbidity and mortality and also program increases in the risks of metabolic and cardiovascular diseases in the offspring's life (Plagemann *et al.* 2008, Melamed & Hod 2009, Reece *et al.* 2009).

A hallmark related to the programming of metabolic diseases is the overgrowth observed in both the fetuses and their placentas at term gestation (Jansson *et al.* 2006, Plagemann *et al.* 2008). Fetal overgrowth is mainly the result of an increase in metabolic substrates transferred through the placenta and of the increase of insulin produced in fetal pancreas as a response to the increased glucose concentrations (Desoye & Shafrir 1994, Jansson *et al.* 2006, Herrera & Ortega-Senovilla 2010). Indeed, together with the increased insulin-like growth factors, these changes may guide the metabolic and proliferative changes that lead to both fetal and placental overgrowth (Hiden *et al.* 2009). In addition, in the placenta, nitric oxide (NO) can dilate placental vessels and induce angiogenesis and trophoblast proliferation (Dulak & Jozkowicz 2003, Valdes *et al.* 2009), and NO overproduction is observed in intrauterine tissues in human diabetic gestations and in experimental diabetic models (Jawerbaum & Gonzalez 2005, San Martin & Sobrevia 2006, Leach *et al.* 2009).

There are several experimental models of diabetes that show similar impairments in fetoplacental development and growth when compared to human diabetic pregnancies (Jawerbaum & White 2010). These models have been valuable to address the causes of these impairments, which are related, at least in part, to the intrauterine metabolic impairments and the consequent generation of an excess of reactive oxygen and nitrogen species, which leads to a pro-inflammatory environment that exerts damage throughout development (Myatt & Cui 2004, Jawerbaum & Gonzalez 2006).

As mechanisms capable of stimulating metabolic and anti-inflammatory pathways could be beneficial to placental development in maternal diabetes, we here

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focus on the activity of the nuclear receptor peroxisome proliferator-activated receptor α (PPARα). This nuclear receptor is one of the three PPAR isotypes, ligand-activated transcription factors that regulate metabolic and inflammatory pathways (Bensinger & Tontonoz 2008). Through its classical pathway, PPARα heterodimerizes with its partner RXR and after ligand binding, they recruit coactivators and release corepressors leading to the expression of target genes (Hihi *et al.* 2002). The huge ligand pocket characteristic of PPARs accepts different ligands, such as different polyunsaturated fatty acids, which can activate the three PPAR isotypes, and the arachidonic acid derivative leukotriene B_4 (LTB_4), which is a specific ligand of the PPARα isotype (Lin *et al.* 1999, Hihi *et al.* 2002, Narala *et al.* 2010). Besides, clofibrate is a pharmacological PPARα activator frequently used to treat dyslipidemias (Desvergne *et al.* 2004, Panadero *et al.* 2009).

PPARα is highly expressed in several organs such as the liver and the heart, in which PPARα ligands are crucial regulators of lipid catabolism (Lefebvre *et al.* 2006, Finck 2007, Rakhshandehroo *et al.* 2007). Besides, PPARα activation prevents lipid peroxidation and regulates anti-inflammatory pathways in several tissues (Cuzzocrea *et al.* 2008, Belfort *et al.* 2010). In addition, when PPARα is activated, NO generation is inhibited in various tissues and cell types, in part by reducing the concentrations of the inducible form of NO synthase (iNOS; Paukkeri *et al.* 2007, Cuzzocrea *et al.* 2008, Ibarra-Lara *et al.* 2010).

PPARα is expressed in the placenta and in different fetal organs during the post-placentation stage (Braissant & Wahli 1998, Wang *et al.* 2002). Our previous studies have shown that PPARα activation regulates lipid catabolism and synthesis in placentas from diabetic rats in an early post-placentation period (day 14 of rat gestation; Martinez *et al.* 2008). Besides, we have recently shown that lipid accumulation and peroxidation in fetal livers from diabetic rats at term gestation can be regulated by PPARα activators (Martinez *et al.* 2011). Interestingly, we have also found that fetal activation with the endogenous ligand LTB_4 can prevent fetal and fetal liver overgrowth in maternal diabetes (Martinez *et al.* 2011). Owing to the relevance in understanding the relationship between fetal growth and placental growth, we consider these findings as a starting point to address fetal effects on placental growth. Therefore, we here investigated the effects of the pharmacological PPARα activator clofibrate and the endogenous PPARα activator LTB_4 on placental lipid concentrations, synthesis, and peroxidation, as well as on placental NO production in term pregnant control and diabetic rats (day 21 of pregnancy). Besides, in both control and diabetic animals, we injected fetuses with the endogenous PPARα activator LTB_4 on days 19, 20, and 21 of pregnancy and analyzed their corresponding placentas on day 21 of gestation to address the placental lipid concentrations, lipoperoxidation, and NO production, as well as PPARα expression and the placental weight.

Materials and methods

Animals

Albino Wistar rats bred in our laboratory were fed *ad libitum* with commercial rat chow (Asociación cooperativa Argentina, Buenos Aires, Argentina). 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 described previously (Portha *et al.* 1979, Capobianco *et al.* 2008). Control animals were injected with citrate buffer alone. The diabetic state was confirmed in 2-month-old rats prior to mating. Rats were considered diabetic when they presented fasting glycemic values higher than 130 mg/dl. Glycemia was determined in blood from the tail central vein by Accu-Chek reagent strips and a glucometer obtained from Accu-Chek, Roche Diagnostics. Control and diabetic female rats were mated with control males. Pregnancy was confirmed by the presence of spermatozoa in vaginal smears and this day was considered day 1 of gestation. 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 number 85-23, revised 1985, http://grants1.nih.gov/grants/olaw/references/phspol.htm).

Placental preparations

Animals were killed in a CO_2 chamber on day 21 of pregnancy and the uterus of each animal was transferred to a Petri dish containing Krebs Ringer bicarbonate (KRB) medium: 5 mM glucose, 145 mM Na^+, 2·2 mM Ca^{2+}, 1·2 mM Mg^{2+}, 127 mM Cl^-, 25 mM HCO_3^-, 1·2 mM SO_4^{2-}, and 1·2 mM PO_4^{3-}. Fetuses and placentas were explanted and weighed. Placentas (100 mg) were frozen at −80 °C for evaluation of protein expression of PPARα. In order to analyze the effect of PPARα on lipid concentrations, *de novo* lipid synthesis, lipid peroxidation, and NO production, placental explants (100 mg) were incubated for 3 h as previously (Martinez *et al.* 2008) in a metabolic shaker under an atmosphere of 5% CO_2 in air at 37 °C, with or without the addition of either LTB_4 (0·1 μM, Cayman Chemical Co., Ann Arbor, MI, USA) or clofibrate (20 μM, Sigma–Aldrich). Thereafter, tissues were frozen at −80 °C for further analysis of lipid concentrations, lipid peroxidation, and NO production. To analyze the *de novo* lipid synthesis,

placentas (100 mg) were incubated as described above, with addition of 1 μCi ^{14}C-acetate (53 mCi/mmol) and then stored at −80 °C until determination of the newly formed radioactive lipids, as described below. Maternal glycemia and triglyceridemia were measured by commercial enzymatic kits from Wiener Lab (Rosario, Argentina) in serum obtained through centrifugation of blood from the aorta artery.

To perform the *in vivo* studies, we proceeded as described previously (Martinez *et al.* 2011). Fetuses from pregnant animals received either a 50 μl s.c. injection of LTB_4 (340 mg/kg dissolved in vehicle) or vehicle alone (17 μmol ethanol/kg, dissolved in saline solution) on days 19, 20, and 21 of pregnancy. For this purpose, the pregnant animals were anesthetized in a CO_2 chamber and a slight anesthesia maintained with ether vapors. An abdominal incision allowed the exposure of the wall of the left uterine horn, through which the fetuses were injected. The entire surgery lasted 15 min and was repeated for three consecutive days. In each animal, fetuses in the left uterine horn were numbered according to their distance to the ovary. Of the fetuses, two were injected with LTB_4 solution for the three consecutive days, whereas two other fetuses were injected with vehicle for the three consecutive days. After the injection, the abdominal incision was sutured. Fetuses from eight control and eight diabetic animals were subjected to this surgery procedure. After 3 h of the last injection (day 21 of pregnancy), animals were killed in a CO_2 chamber. The uterus of each animal was transferred to a Petri dish containing KRB medium. Fetuses and placentas were explanted and weighed. Placentas (100 mg) were stored at −80 °C for further analysis of lipid concentrations, lipid peroxidation, and NO production or preserved in RNA-later stabilization solution (Ambion) for the evaluation of the gene expression of iNOS and PPARα. Fetal blood was obtained from decapitation, collected in heparinized syringes, and the plasma preserved at −80 °C for further evaluation of triglyceridemia and glycemia levels by using commercial kits obtained from Wiener Lab.

PPARα concentrations

PPARα protein expression was evaluated by western blot as described previously (Martinez *et al.* 2008). Placentas were homogenized in 300 μl ice-cold lysis buffer (pH 7·4, 20 mM Tris–HCl, 150 mM NaCl, and 1% Triton X-100) containing 1% protease inhibitor cocktail (Sigma–Aldrich), incubated on ice for 2 h, and centrifuged at 9600 ***g*** for 10 min at 4 °C. Protein concentrations were determined by the Bradford method. An equivalent volume of 100 μg of protein lysates was separated by electrophoresis in 12% SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes, which were blocked with 2% BSA for 24 h and then incubated overnight at 4 °C with a polyclonal rabbit IgG antibody either against PPARα (1:200; Cayman Chemical Co.) or against α-actin (Sigma–Aldrich). After washing with Tris buffer saline and Tween 0·05%, the blots were treated with HRP-conjugated secondary antibody (1:10000) obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA) for 1h and washed several times. The specific signals were visualized using the ECL Western Blotting Analysis System from G. E. Healthcare (Buckinghamshire, UK) enhanced chemiluminescence system. This kit uses Lumigen TMA-6, which is a substrate for the chemiluminescent detection of peroxidase conjugates on membranes. Reaction of the substrate with a HRP label generates sustained luminescence of high intensity. The identity of PPARα was established by the use of molecular weight standards (Invitrogen), which allow the identification of the band revealed at the expected size of 50 kDa, which was absent in the negative control experiments performed in the absence of primary antibody and present in the liver homogenates used as a positive control. Actin reactivity was detected with a phosphatase alkaline-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The relative intensity of protein signals was quantified by densitometric analysis using the ImageJ Software (NIH, Bethesda, MD, USA). Results are expressed as PPARα protein/actin protein ratio.

Lipid level analysis

Placental lipid concentrations were evaluated by thin layer chromatography (TLC) as described previously (Capobianco *et al.* 2008). Placentas were homogenized in PBS. Liver lipids were extracted by three rounds of organic extraction in methanol:chloroform (2:1) and the solvent evaporated under a N_2 stream. Lipids were developed by TLC in thin silica gel 60 plates (Merck) using hexane:ether:acetic acid (80:20:2) as the developing solvent mixture. Lipid species were stained with iodine. Triglycerides, cholesterol, cholesteryl esters, and phospholipids were identified and quantified by comparison with known amounts of pure lipid standards obtained from Nu-Chek-Prep (Elysian, MN, USA), on the same plate. The plates were scanned and analyzed by densitometry using the ImageJ software. Results are expressed as μg/mg protein.

Evaluation of the *de novo* lipid synthesis

Placental lipid synthesis from ^{14}C-acetate was evaluated as described previously (Capobianco *et al.* 2008). Briefly following the incubations performed in the presence of

^{14}C-acetate (53 mCi/mmol) either with or without the addition of LTB_4 (0·1 μM) or clofibrate (20 μM), lipids were separated by TLC as described above. The radioactive spots were identified with a STORM scanner (G. E. Healthcare). Thereafter, the radioactive spots corresponding to the different ^{14}C-labeled lipid species were scrapped into vials and counted in a liquid scintillation counter. Results are expressed as dpm/μg protein.

Lipid peroxidation evaluation

Lipid peroxidation was estimated by measuring the concentrations of thiobarbituric acid reactive substances (TBARS), as described previously (Kurtz *et al.* 2010), a method widely used to assess peroxidation of fatty acids (Ohkawa *et al.* 1979). Approximately 100 mg of tissue was homogenized in 100 mM Tris–HCl buffer, pH 7·6. The homogenate was added to trichloroacetic acid (40%) and centrifuged at 1·8 ***g*** for 10 min. The supernatant was added with an equal volume of thiobarbituric acid (46 mM), and the solution was heated at 95 °C for 15 min. Then, the samples were cooled and quantified spectrophotometrically at 530 nm. Malondialdehyde (Sigma–Aldrich) subjected to the same conditions as the tissue homogenates was used as a standard. TBARS are expressed as nmol/mg protein.

NO production analysis

NO production was evaluated by the determination of the concentration of its stable metabolites nitrates/nitrites, as described previously (Higa *et al.* 2010), by using a commercial assay kit (Cayman Chemical Co.). For that, placental tissues were homogenized in 1ml Tris–HCl buffer pH 7·6 and an aliquot was separated for protein determination. Nitrates in the supernatant were reduced to nitrites using nitrate reductase, and total nitrites were measured by the Griess method (Green *et al.* 1982). Optical densities were measured at 540 nm in a microliter plate using $NaNO_3$ and $NaNO_2$ as standard. Results are expressed as nmol/mg of protein.

Assessment of *iNOS* and *PPARα* mRNA

iNOS and PPARα expression was evaluated by RT-PCR, as described previously (Kurtz *et al.* 2010). Placental RNA was extracted with Tri reagent (Genbiotech, Buenos Aires, Argentina) in accordance with the manufacturer's instructions. cDNA was synthesized incubating 1 μg extracted placental RNA with 20 μl reverse transcription buffer containing 7·5 mM random primer hexamers (Promega), 0·5 mM each of all four dNTPs (Invitrogen) and 200 U MMLV reverse transcriptase (Invitrogen) at 25 °C for 10 min, then at 37 °C for 50 min, and then at 70 °C for 15 min. cDNA (2 μl) was used to perform the amplification in a 25 μl reaction buffer containing dNTPs (0·2 mM), magnesium chloride solution (2 mM), 1·25 U Taq polymerase (GoTaq Flexi Polymerase, Promega), and 0·45 μM of each specific primer.

Primers for iNOS were forward: 5′-TGGTGAAGGGTGTCGTGAAA-3′ and reverse: 5′-CTCACTGGGACTGCACAGAA-3′, whose amplification product is a 128 bp fragment (Nowicki *et al.* 2003). Primers for PPARα were forward: 5′-TCACACAATGCAATCCGTTT-3′ and reverse: 5′-GGCCTTGACCTTGTTCATGT-3′, whose amplification product is a 177 bp fragment (Yeon *et al.* 2004). The primers for the ribosomal protein L30, used as an internal control, were forward: 5′-CCATCTTGGCGTCTGATCTT-3′ and reverse: 5′-GGCGAGGATAACCAATTTC-3′, designed to obtain a 201 bp fragment, using Primer 3 Software (http://frodo.wi.mit.edu/primer3). The initial step in the reaction was 95 °C for 5 min, followed by 28 cycles for L30, 34 cycles for iNOS, and 36 cycles for PPARα. 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 (10 μl) were separated on a 2% agarose gel and stained with ethidium bromide. The density of the bands was quantified by image analysis with ImageJ Software and normalized to L30.

Statistical analysis

Data are presented as the mean ± S.E.M. Groups were compared using Student's *t*-test or one-way ANOVA in conjunction with Tukey's test where appropriate. Differences between groups were considered significant when P value $<0·05$.

Results

PPARα in term placentas from control and diabetic rats

Diabetic animals at term gestation showed increased glycemia (control: 101±9; diabetic: 282±31 mg/dl, $P<0·001$) and triglyceridemia (control: 199±15; diabetic: 554±61 mg/dl, $P<0·001$). Together with these metabolic changes, there was an increase in placental weight in the diabetic group (control: 561±11 mg; diabetic: 670±18 mg, $P<0·001$). As a putative regulator of metabolic pathways in term placentas, we measured the protein expression of PPARα in placentas from control and diabetic rats on day 21 of gestation. We found that PPARα concentrations in placentas from diabetic animals were reduced ($P<0·05$) when compared with those from healthy rats (Fig. 1).

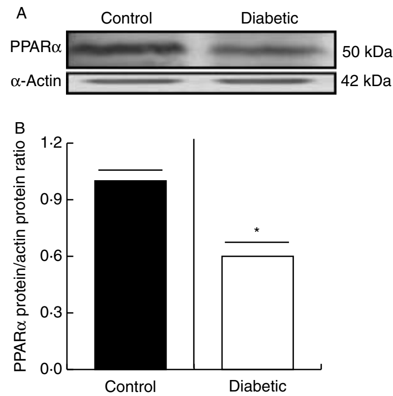

Figure 1 Protein expression of PPARα is reduced in term placentas from diabetic rats. Antibodies used were rabbit anti-PPARα and rabbit anti-actin. (A) Representative western blots exhibiting PPARα protein expression in placentas from control and diabetic rats. (B) Densitometric analysis of PPARα concentrations, normalized against actin, used as an internal control. Values are mean ± S.E.M. for placentas obtained from eight rats on day 21 of gestation. Student's *t*-test was performed. $^{*}P<0{\cdot}05$ denotes differences between diabetic versus control groups.

Lipid concentrations and *de novo* synthesis in term placentas from control and diabetic rats: effect of PPARα activation

The placentas from diabetic rats on day 21 of gestation showed no changes in the concentrations of triglycerides, cholesteryl esters, phospholipids, and cholesterol when compared with controls (Fig. 2). To analyze the effects of PPARα agonists on placental lipid concentrations, three placentas from control ($n=8$) and three from diabetic ($n=8$) rats were explanted on day 21 of gestation and cultured for 3 h in the presence or absence of the PPARα agonists LTB_4 (0·1 μM) or clofibrate (20 μM), and placental lipid concentrations were then evaluated. We found that LTB_4 did not modify the concentrations of the evaluated lipids in placentas from both control and diabetic rats, while clofibrate reduced triglycerides ($P<0{\cdot}01$) and cholesterol ($P<0{\cdot}05$) concentrations in control placentas, and reduced the concentrations of cholesteryl esters ($P<0{\cdot}05$) and phospholipids ($P<0{\cdot}05$) in placentas from diabetic rats (Fig. 2).

When the *de novo* lipid synthesis was evaluated in the placentas on day 21 of gestation, we found that the *de novo* synthesis of triglycerides ($P<0{\cdot}001$) and phospholipids ($P<0{\cdot}01$; Fig. 3) was reduced and that there were no changes in the *de novo* synthesis of cholesterol and cholesteryl esters in the placentas from diabetic animals when compared with controls. To analyze the effects of PPARα agonists on placental *de novo* lipid synthesis, three placentas from control ($n=8$) and three from diabetic ($n=8$) rats were explanted on day 21 of gestation and cultured for 3 h in the presence of ^{14}C-acetate as a tracer either with or without addition of LTB_4 (0·1 μM) or clofibrate (20 μM), and concentrations of radioactive lipids in the placentas were then analyzed. We found that neither clofibrate nor LTB_4 modified the *de novo* synthesis of the evaluated lipids in the placentas from control rats (Fig. 3). Differently, in the placentas from diabetic animals, the endogenous ligand LTB_4 reduced the *de novo* synthesis of phospholipids ($P<0{\cdot}001$) and cholesterol ($P<0{\cdot}05$), whereas the pharmacological ligand clofibrate reduced the *de novo* synthesis of triglycerides ($P<0{\cdot}05$), phospholipids ($P<0{\cdot}05$), and cholesterol ($P<0{\cdot}05$; Fig. 3).

Lipid peroxidation and NO production in placentas from control and diabetic rats at term gestation: effect of PPARα activation

The placentas from diabetic rats on day 21 of gestation showed increased lipid peroxidation, as indicated by an increase in the concentrations of TBARS ($P<0{\cdot}01$, Fig. 4A), as well as increased NO production, as indicated by an increase in its stable metabolites nitrates/nitrites ($P<0{\cdot}05$, Fig. 4B). To analyze the effects of PPARα agonists on placental lipid peroxidation and NO production, three placentas from

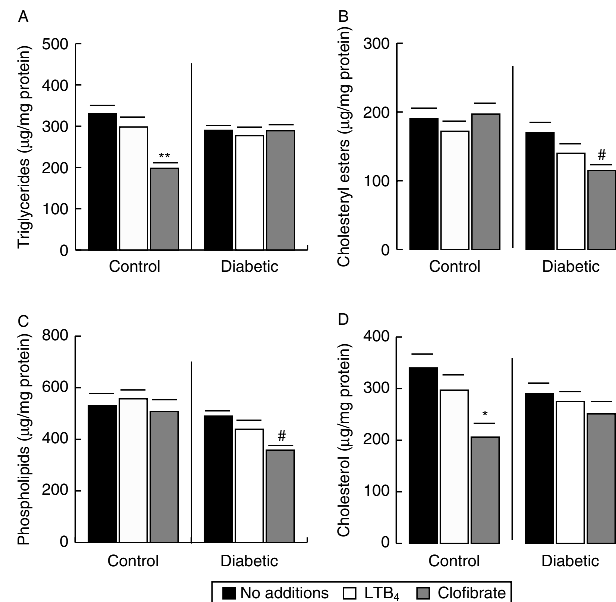

Figure 2 PPARα activators regulate lipid concentrations in term placentas from diabetic rats. Effects of the PPARα agonists LTB_4 (0·1 μM) and clofibrate (20 μM) on lipid concentrations in placentas obtained from control and diabetic rats on day 21 of gestation. (A) Triglycerides, (B) cholesteryl esters, (C) phospholipids, and (D) cholesterol. Values are mean ± S.E.M. for placentas obtained from eight rats on day 21 of gestation. ANOVA followed by Tukey's test was performed. $^{*}P<0{\cdot}05$ and $^{**}P<0{\cdot}01$ denote differences between treated control group versus non-treated control group. $^{\#}P<0{\cdot}05$ denotes differences between treated diabetic group versus non-treated diabetic group.

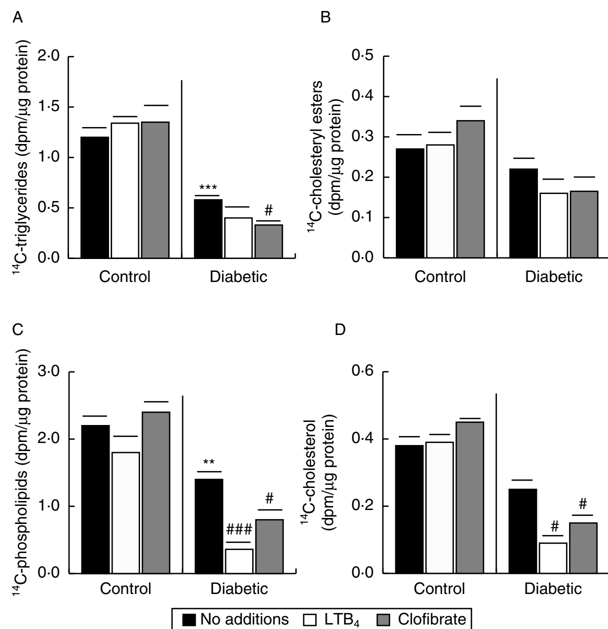

Figure 3 PPARα activators regulate the *de novo* lipid synthesis in term placentas from diabetic rats. Effects of the PPARα agonists LTB_4 (0·1 μM) and clofibrate (20 μM) on the *de novo* lipid synthesis in term placentas obtained from control and diabetic rats. (A) Triglycerides, (B) cholesteryl esters, (C) phospholipids, and (D) cholesterol. Values are mean±S.E.M. for placentas obtained from eight rats on day 21 of gestation. ANOVA followed by Tukey's test was performed. $^{**}P<0·01$ and $^{***}P<0·001$ denote differences between non-treated diabetic group versus non-treated control group. $^{\#}P<0·05$ and $^{\#\#\#}P<0·001$ denote differences between treated diabetic group versus non-treated diabetic group.

control (n=8) and three from diabetic (n=8) rats were explanted on day 21 of gestation and cultured for 3 h in the presence or absence of the PPARα agonists LTB_4 (0·1 μM) or clofibrate (20 μM), and placental lipid peroxidation and NO production were then analyzed. We found that neither clofibrate nor LTB_4 modified TBARS concentrations in placentas from control rats. Differently, both LTB_4 ($P<0·05$) and clofibrate ($P<0·01$) were able to reduce the concentrations of TBARS in term placentas from diabetic animals (Fig. 4A). Moreover, the NO stable metabolites nitrates/nitrites were negatively regulated by both clofibrate and LTB_4 in the placentas from both control ($P<0·05$) and diabetic ($P<0·01$) rats, with the PPARα agonists in the diabetic group being capable of reducing NO production to control values (Fig. 4B).

Lipid concentrations in placentas from control and diabetic rats at term gestation: effect of fetal LTB_4 administration

Considering that we previously found profound effects of *in vivo* fetal PPARα activation on fetal liver lipid concentrations and fetal growth (Martinez *et al.* 2011), we tested the hypothesis that fetal PPARα activation could influence placental lipid concentrations, NO production, and placental growth. For this purpose, fetuses from control and diabetic rats were injected with either LTB_4 (0·1 μM) or vehicle through the uterine wall on days 19, 20, and 21 of gestation. On day 21 of gestation, fetal blood was obtained to measure fetal glycemia, triglyceridemia, and cholesterolemia, and the placental weight and placental lipid concentrations were analyzed. Fetal glycemia ($P<0·001$), triglyceridemia ($P<0·05$), and cholesterolemia ($P<0·05$) were increased in the vehicle-injected fetuses from diabetic animals, when compared with the vehicle-injected control group (Fig. 5). Fetal administration of the natural PPARα agonist LTB_4 in late gestation did not change fetal glycemia, triglyceridemia, and cholesterolemia in both control and diabetic animals when compared with their respective vehicle-injected groups (Fig. 5). In control animals, the fetal administration of

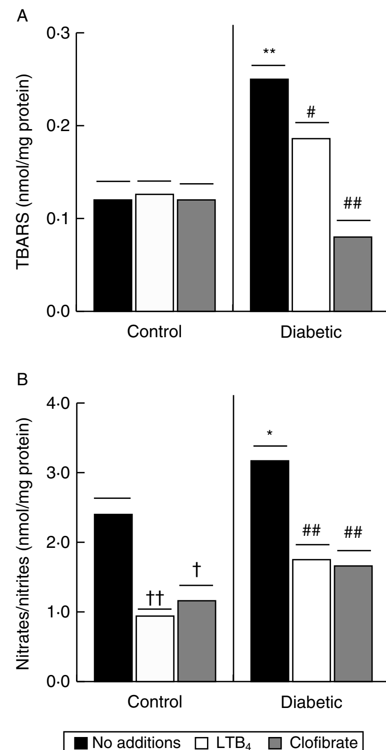

Figure 4 PPARα activators regulate lipid peroxidation and NO production in term placentas from diabetic rats. Effect of the PPARα agonists LTB_4 (0·1 μM) and clofibrate (20 μM) on (A) lipid peroxidation and (B) NO production in term placentas from control and diabetic rats. Values are mean±S.E.M. for placentas obtained from eight rats on day 21 of gestation. ANOVA followed by Tukey's test was performed. $^{*}P<0·05$ and $^{**}P<0·01$ denote differences between non-treated diabetic group versus non-treated control group. $^{\dagger}P<0·05$ and $^{\dagger\dagger}P<0·01$ denote differences between treated control group versus non-treated control group. $^{\#}P<0·05$ and $^{\#\#}P<0·01$ denote differences between treated diabetic group versus non-treated diabetic group.

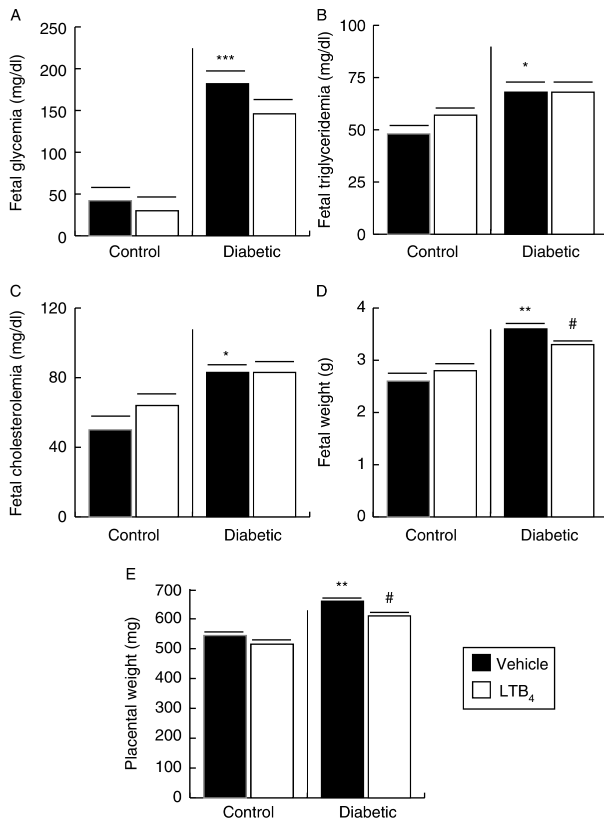

Figure 5 Fetal PPARα activation reduces fetal and placental weight and does not modify serum lipid profile in diabetic rats on day 21 of gestation. Fetuses from control and diabetic rats were injected with LTB_4 (0·1 μM) or vehicle on days 19, 20, and 21 of pregnancy. Fetal glycemia (A), triglyceridemia (B), and cholesterolemia (C), as well as fetal weight (D) and placental weight (E), were evaluated on day 21 of pregnancy. Values are mean ± s.e.m.; n=8 rats per group. ANOVA followed by Tukey's test was performed. *$P<0{\cdot}05$, **$P<0{\cdot}01$, and ***$P<0{\cdot}001$ denote differences between vehicle-treated diabetic versus vehicle-treated control groups. #$P<0{\cdot}05$ denotes differences between LTB_4-treated diabetic group versus vehicle-treated diabetic group.

LTB_4 in late gestation did not change fetal or placental weight. Differently, in diabetic animals, a reduction in both fetal growth and placental growth was found when LTB_4 was injected in the fetuses compared with those injected with vehicle ($P<0{\cdot}05$, Fig. 5). On the other hand, no changes in the concentrations of the lipid species analyzed were found in the placentas corresponding to LTB_4-injected fetuses from control and diabetic rats when compared with their respective vehicle-injected controls (Fig. 6).

Lipid peroxidation and NO production in placentas from control and diabetic rats at term gestation: effect of fetal LTB_4 administration

In order to analyze whether fetal PPARα activation regulates lipid peroxidation and NO production in rat term placentas, fetuses from control and diabetic rats were injected with either LTB_4 (0·1 μM) or vehicle (controls) through the uterine wall on days 19, 20, and 21 of gestation. On day 21 of gestation, the placentas were explanted and TBARS and nitrates/nitrites concentrations were analyzed as respective indexes of lipid peroxidation and NO production. In addition, expression of the inducible isoform of iNOS was analyzed in the placentas corresponding to LTB_4- and vehicle-injected fetuses.

When we studied lipid peroxidation, there were no changes in this parameter in the placentas corresponding to LTB_4-injected fetuses when compared with those corresponding to vehicle-injected fetuses in both control and diabetic rats (Fig. 7A). When we analyzed NO production, we found that in both control and diabetic groups, nitrates/nitrites concentrations were reduced in the placentas corresponding to LTB_4-injected fetuses when compared with their respective vehicle-injected groups ($P<0{\cdot}05$, Fig. 7B). Interestingly, in the vehicle-injected diabetic group, which showed an increase in NO stable metabolites nitrates/nitrites in the placenta when compared with the vehicle-injected control group, there was also an increase in the placental expression of iNOS ($P<0{\cdot}05$, Fig. 7C). Moreover, in the diabetic group,

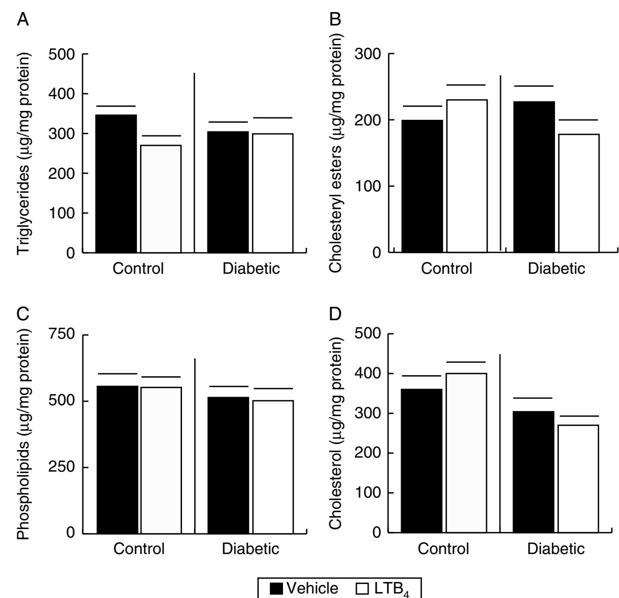

Figure 6 Fetal PPARα activation does not modify lipid concentrations in the placenta. Fetuses from control and diabetic rats were injected with LTB_4 (0·1 μM) or vehicle on days 19, 20, and 21 of pregnancy and their corresponding placentas analyzed on day 21 of pregnancy. Concentrations of (A) triglycerides, (B) cholesteryl esters, (C) phospholipids, and (D) cholesterol were evaluated in term placentas obtained from control and diabetic rats. Values are mean ± s.e.m. for placentas obtained from eight rats on day 21 of pregnancy. ANOVA followed by Tukey's test was performed.

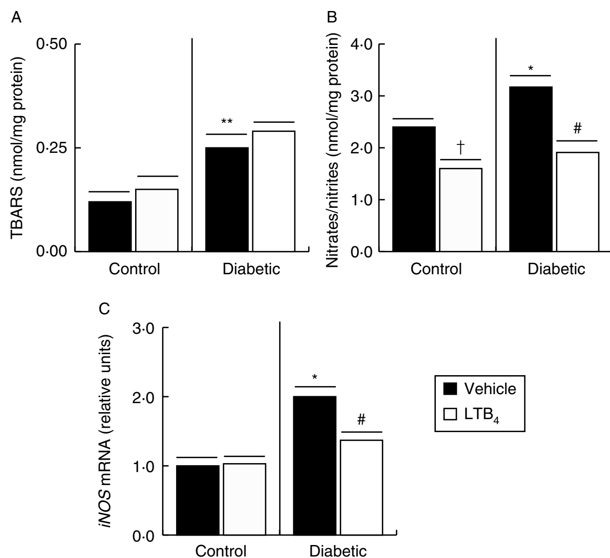

Figure 7 Fetal PPARα activation does not modify lipid peroxidation but negatively regulates NO production and iNOS expression in the placenta from diabetic rats. Fetuses from control and diabetic rats were injected with LTB_4 (0·1 μM) or vehicle on days 19, 20, and 21 of pregnancy and their corresponding placentas analyzed on day 21 of pregnancy. Concentrations of (A) TBARS, an index of lipid peroxidation, (B) nitrates/nitrites, stable NO metabolites, and (C) iNOS expression were evaluated in term placentas obtained from control and diabetic rats. Values are mean ± S.E.M. for placentas obtained from eight rats on day 21 of gestation. ANOVA followed by Tukey's test was performed. *$P<0·05$ and **$P<0·01$ denote differences between vehicle-treated diabetic group versus vehicle-treated control group. $^{†}P<0·05$ denotes differences between LTB_4-treated control group versus vehicle-treated control group. $^{\#}P<0·05$ denotes differences between LTB_4-treated diabetic group versus vehicle-treated diabetic group.

together with the reduction of nitrate/nitrite concentrations to control values, the expression of iNOS was also reduced in the placentas corresponding to LTB_4-injected fetuses when compared with the placentas corresponding to the vehicle-injected fetuses ($P<0·05$, Fig. 7C).

PPARα expression in placentas from control and diabetic rats at term gestation: effect of fetal LTB_4 administration

In order to analyze whether fetal administration of the endogenous PPARα agonist LTB_4 regulates the expression of PPARα in rat term placentas, fetuses from control and diabetic rats were injected with either LTB_4 (0·1 μM) or vehicle (controls) through the uterine wall on days 19, 20, and 21 of gestation. On day 21 of gestation, the placentas were explanted and PPARα expression was analyzed.

A reduction in PPARα expression was found in placentas from diabetic animals injected with vehicle when compared with those from control animals injected with vehicle ($P<0·05$, Fig. 8). Interestingly, when we studied the effect of fetal LTB_4 administration, there were significant increases in placental PPARα expression in the LTB_4-injected fetuses from both control and diabetic rats when compared with those corresponding to vehicle-injected fetuses in both control and diabetic groups ($P<0·01$, Fig. 8).

Discussion

PPARs signaling pathways are important in placental development and function, and their altered concentrations have relevance in several gestational diseases (Giaginis *et al.* 2008, Wieser *et al.* 2008, Arck *et al.* 2010). In this study, we found reduced PPARα concentrations in term placentas from diabetic rats. Similarly, protein expression of PPARα is reduced in term placentas from gestational diabetic patients (Holdsworth-Carson *et al.* 2010). These changes seem to be dependent on the developmental stage, as PPARα concentrations are enhanced in placentas from diabetic rats in the early post-placentation period (Martinez *et al.* 2008). In this regard, changes in concentrations and function of the different PPAR isoforms occur throughout pregnancies both in humans and in experimental models (Capobianco *et al.* 2005, Wieser *et al.* 2008, Holdsworth-Carson *et al.* 2009, Lappas & Rice 2009, Pustovrh *et al.* 2009). In this research, in the *in vitro* experiments performed in placentas at term gestation, we found some effects

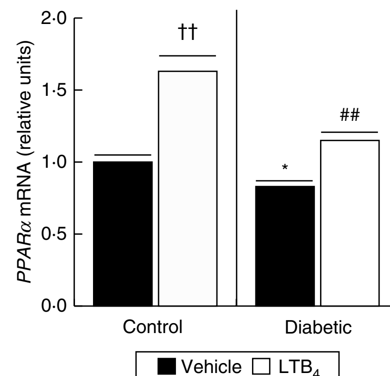

Figure 8 Fetal LTB_4 upregulates PPARα expression in placentas from diabetic rats. Fetuses from control and diabetic rats were injected with LTB_4 (0·1 μM) or vehicle on days 19, 20, and 21 of pregnancy and their corresponding placentas analyzed on day 21 of pregnancy. PPARα expression was evaluated in term placentas obtained from control and diabetic rats. Values are mean ± S.E.M. for placentas obtained from eight rats on day 21 of gestation. ANOVA followed by Tukey's test was performed. *$P<0·05$ denotes differences between vehicle-treated diabetic group versus vehicle-treated control group. $^{††}P<0·01$ denotes differences between LTB_4-treated control group versus vehicle-treated control group. $^{\#\#}P<0·01$ denotes differences between LTB_4-treated diabetic group versus vehicle-treated diabetic group.

related to the capacity of lipid oxidation of clofibrate, a potent PPARα pharmacological activator, but no changes in the presence of the endogenous ligand LTB_4, capable of oxidizing lipids in placentas at midgestation (Martinez *et al.* 2008). The capacity of lipid accretion of the placenta changes throughout gestation. Indeed, at midgestation, placentas from diabetic rats show a marked accumulation of triglycerides and cholesteryl esters (Martinez *et al.* 2008) whereas term placentas from the same diabetic rat model showed no lipid overaccumulation. These findings suggest that changes in lipid metabolism occur at term gestation in parallel to important changes in the concentrations of PPARα. On the other hand, although both clofibrate and LTB_4 can activate lipid metabolism through PPARα signaling pathways in several tissues including the placenta (Lefebvre *et al.* 2006, Finck 2007, Martinez *et al.* 2008), different effects can be achieved due to the high potency of clofibrate and the different conformational changes that each ligand can induce, leading to the recruitment of different co-activators and to the release of different co-repressors (Schaiff *et al.* 2000, Martinez *et al.* 2008, Panadero *et al.* 2009, Yoon 2009). Therefore, a limitation of the present work that needs to be addressed in further studies is the evaluation of the activation of PPARα achieved by each ligand.

Regarding the placental *de novo* lipid synthesis, we here found that PPARα activation regulates the synthesis of triglycerides, phospholipids, and cholesterol in diabetic tissues. Indeed, although mainly a regulator of lipid catabolism, PPARα can also regulate lipid synthesis in different tissues (Rakhshandehroo *et al.* 2007, Panadero *et al.* 2009), an effect that may be related to PPARs interaction with the liver receptor X (LXR), a main regulator of this anabolic pathway (van Straten *et al.* 2009, Hein *et al.* 2010).

Both the classical PPARs activation pathway and the transactivation of other nuclear transcription factors such as NF-κB have been related to PPARs anti-inflammatory effects (Straus & Glass 2007, Bensinger & Tontonoz 2008). Indeed, PPARs activation both stimulates the expression of the antioxidant enzyme superoxide dismutase and catalase, and regulates glutathione metabolism in several cell types (Lin *et al.* 2007, Abdelmegeed *et al.* 2009, Ibarra-Lara *et al.* 2010). Activation of PPARα also regulates lipid peroxidation, a regulation evident in plasma and tissues in different pathologies and inflammatory conditions including the diabetic state (Inoue *et al.* 1997, Anwer *et al.* 2007, Belfort *et al.* 2010). NO production is negatively regulated by PPARα activation in different tissues, through a mechanism in part related to the reduction of iNOS concentrations (Paukkeri *et al.* 2007, Cuzzocrea *et al.* 2008, Ibarra-Lara *et al.* 2010). In this study, we found that PPARα activation negatively regulates lipid peroxidation and NO production in the placenta, an effect that is particularly relevant in maternal diabetes, in which the excess of reactive oxygen and nitrogen species are clearly related to the damaging effects in placental tissues (Jawerbaum & Gonzalez 2006, Webster *et al.* 2008).

An important question addressed in this work is whether the fetus can actively regulate placental function and growth in an adverse environment such as maternal diabetes. Our experimental approach allowed the evaluation of a fetal role in regulating placental lipid metabolism, NO production, and growth. We here found that fetal administration of the endogenous PPARα ligand LTB_4 does not change lipid concentrations and peroxidation in the placenta, but specifically regulates the production of placental NO, through a mechanism that seems to depend, at least in part in the diabetic animals, on a down-regulation of the expression of the inducible isoform of NOS. Although further research is needed to understand the direct or indirect nature of the effect of fetal LTB_4 on the placenta, the observed responses to fetal LTB_4 in this tissue may be due to its effect on the placental endothelial cells, which have a high capacity of NO synthesis and are likely to be the placental cells most exposed to fetal signaling molecules (Giannubilo *et al.* 2008).

A limitation of this study was that it was not possible to address the *in vivo* effects of the pharmacological ligand clofibrate, as vehicles needed to dissolve this drug impaired development in rat fetuses at term. Nevertheless, our recent studies performed in term pregnant rats have shown potent effects of the PPARα agonist LTB_4 on fetal liver lipid catabolism when the drug was given to the fetuses for three consecutive days, even more potent than those achieved *in vitro* by both LTB_4 and clofibrate in fetal liver tissues (Martinez *et al.* 2011).

In this work, we found that PPARα expression was reduced in the placentas from diabetic rats when compared with controls. Moreover, fetal administration of the PPARα ligand LTB_4 highly increased PPARα expression in the placentas from both control and diabetic rats. This finding provides direct evidence of an LTB_4-mediated increase in the PPARα signaling pathway. As previous studies have shown that PPARα dietary ligands in the mother can increase PPARα-mediated pathways in the fetuses (Ringseis *et al.* 2007), this suggests that PPARα agonists are relevant as both mother-to-fetus and fetus-to-mother signaling molecules.

Together with the changes in placental NO production, iNOS expression, and PPARα expression, a reduction in placental growth was observed in the placentas corresponding to the LTB_4-injected fetuses in diabetic animals. This is important because of the

placental overgrowth characteristic in maternal diabetes and the relationship between placental growth and function and the abnormal intrauterine programming in the fetuses (Catalano & Kirwan 2001, Gatford *et al.* 2010, Thornburg *et al.* 2010). Besides, our results suggest that the fetus has an active role in the regulation of growth in their placentas, an effect evident in a context in which the fetus is exposed to an adverse environment due to maternal diabetes. Although it is difficult to pinpoint the mechanism involved in the observed reduction of placental growth, our results suggest that it may be related to normalization of the production of NO, a pro-angiogenic and vasodilator agent (Dulak & Jozkowicz 2003, Valdes *et al.* 2009), and not to changes in lipid concentrations and peroxidation, parameters that do not change when PPARα is activated in the fetuses. On the other hand, studies performed in trophoblasts and different cell types have shown that PPARs activation affects cell proliferation, differentiation, and apoptosis (Lin *et al.* 2007, Barak *et al.* 2008, Fournier *et al.* 2008, Parast *et al.* 2009, Arck *et al.* 2010, Benameur *et al.* 2010), suggesting that there may be many pathways related to placental growth that could be regulated by activating fetal PPARα that deserves further study.

In conclusion, our findings on PPARα signaling in term placentas from diabetic rats provide evidence of its role as a regulator of lipid metabolism, lipid peroxidation, and NO production in term placentas, signaling pathways specially relevant in maternal diabetes. Besides, we here demonstrate that fetal administration of LTB_4 can modulate placental NO production, iNOS expression, and PPARα expression, as well as placental weight, suggesting a specific role of this endogenous PPARα activator in the fetus in the regulation of placental functions and growth.

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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