REPRODUCTION

Peroxisome proliferator-activated receptor α activation regulates lipid metabolism in the feto-placental unit from diabetic rats

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Abstract

Maternal diabetes promotes an overaccumulation of lipids in the feto-placental unit and impairs feto-placental development and growth. Here, we investigated the role played by the nuclear receptor peroxisome proliferator-activated receptor (PPAR)α in lipid metabolism in fetuses and placentas from control and neonatal streptozotocin-induced diabetic rats. Placentas and fetuses were studied on day 13.5 of gestation. The concentrations of PPARα (by Western blot) and its endogenous agonist leukotriene B_4 (LTB_4) (by enzyme immunoassay) were analysed. Placental explants and fetuses were cultured with LTB_4 or clofibrate, and then lipid metabolism analysed (concentrations and synthesis from ^{14}C-acetate of triglycerides, phospholipids, cholesterol and cholesteryl esters; release of glycerol and free fatty acids (FFAs)). We found that maternal diabetes led to increases in placental concentrations of triglycerides and cholesteryl esters, and fetal concentrations of phospholipids. PPARα agonists downregulated fetal and placental lipid concentrations in control and diabetic rats. The synthesis of lipids was reduced in the diabetic placenta but increased in fetuses from diabetic animals. PPARα agonists reduced the synthesis of lipids in control placenta and in the fetuses from control and diabetic rats. Glycerol and FFA release was enhanced in the diabetic placenta and in control placenta cultured with PPARα agonists. Maternal diabetes led to reductions in fetal and placental LTB_4 concentrations and to increases in placental PPARα concentrations. Overall, these data support a novel role of PPARα as a regulator of lipid metabolism in the feto-placental unit, relevant in maternal diabetes where fetal and placental PPARα, LTB_4 and lipid concentrations are altered.

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Introduction

The incidence of diabetes in women in reproductive ages, as well as the complications that arise for both the mother and the fetus as a result of this pathology, is elevated (Schwartz & Teramo 2000). Maternal diabetes-induced alterations seem to be the result of major impairments in the components of maternal metabolic fuels, and the resulting toxicities of both hyperglycaemia and hyperlipidaemia (Eriksson *et al.* 2003, Jawerbaum & Gonzalez 2006). Fetal fat accretion in maternal diabetes is the result of increased fetal lipid synthesis (Kasser *et al.* 1981), increased fetal substrate availability, which stimulates insulin levels and fetal growth (Jansson *et al.* 2006), and increased free fatty acids (FFAs) derived from maternal sources (Herrera & Amusquivar 2000). The involvement of lipids from maternal sources in fetal fat accretion is indicated by increases in lipoprotein lipase activity in diabetic placentas (Lindegaard *et al.* 2006) and increases in the transplacental gradient of FFAs towards the fetus, which is proportional to maternal FFA concentrations (Knopp *et al.* 1986). Indeed, there is a

significant correlation between maternal triglyceridaemia and fetal triglyceride content (Desoye & Shafrir 1996). Triglycerides are hydrolysed into their constituents, re-esterified, stored and re-hydrolysed within the placenta, which can regulate the nature of lipids to be transferred to the fetus (Herrera *et al.* 2006). However, the mechanisms that regulate placental lipid metabolism are largely unknown, and revealing that these pathways may help to understand the aetiology of feto-placental lipid alterations induced by maternal diabetes.

Peroxisome proliferator-activated receptors (PPARs) are crucial nuclear receptors controlling transcription of a variety of genes involved in lipid metabolism and cellular differentiation (Desvergne *et al.* 2006). Three isoforms named PPARα, PPARβ/δ and PPARγ, which exhibit different ligand specificities, functions and tissue distribution, have been identified in several tissues, including the placenta (Braissant & Wahli 1998, Fournier *et al.* 2007).

Both PPARγ and PPARβ/δ have essential roles in placental development, and their inactivation induces severe trophoblast differentiation defects that lead to

fetal mortality after midgestation (Barak *et al*. 1999, 2002). However, although PPARα- null mice do not show placental developmental abnormalities, an increased abortion and neonatal mortality rate is observed in these mice, suggesting that PPARα may be involved in mother-to-fetus nutrient exchange (Peraza *et al*. 2006, Yessoufou *et al*. 2006). PPARα is mainly expressed in metabolically active tissues including the liver, heart and skeletal muscle, and is crucial in the regulation of lipid catabolism (Desvergne *et al*. 2006, Lefebvre *et al*. 2006). Also, PPARα is highly expressed both in the labyrinth and junctional zones of the rat placenta, and in human villous trophoblasts (Wang *et al*. 2002). In rat fetuses, PPARα transcripts are found from day 13 of gestation, and expressed in several tissues including the nervous system, digestive track, liver and heart (Braissant & Wahli 1998).

Fibrates are PPARα pharmacological agonists that efficiently lower triglyceridaemia in experimental models of obesity and diabetes, as well as in patients with hyperlipidaemia (Watts & Dimmitt 1999, Desvergne *et al*. 2004). PPARα endogenous ligands are lipophylic molecules such as long-chain polyunsaturated fatty acids and the eicosanoid leukotriene B_4 (LTB_4), which is synthesised in placenta and fetuses through the activity of lipoxygenases (Lin *et al*. 1999, Xu *et al*. 2007). Treatment of rats with pharmacological PPARα activators during pregnancy causes proliferation of peroxisomes and induction of peroxisomal enzyme activities in both maternal and fetal livers (Cibelli *et al*. 1988, Peraza *et al*. 2006). Reduction in maternal hypertriglyceridaemia has been found when rats are treated with fibrates during the whole pregnancy but not when treated only at term (Soria *et al*. 2002, Ringseis *et al*. 2007). These results suggest that PPARα is involved in the regulation of maternal lipid metabolism; however, direct effects of PPARα activation in lipid metabolism in the fetus and the placenta have not been reported yet and may be relevant in maternal diabetes.

We have previously characterised several embryonic and placental alterations in a mild experimental rat model of diabetes induced by neonatal administration of streptozotocin (Jawerbaum & Gonzalez 2006). In embryos and placentas from these diabetic rats, there are alterations in lipid metabolism, as well as in the protein expression of PPARβ/δ and PPARγ, PPAR isoforms that have specific regulatory roles in embryonic and placental lipid metabolism (Capobianco *et al*. 2005, Higa *et al*. 2007).

The aim of the present study was to evaluate whether PPARα agonists regulate lipid metabolism in the feto-placental unit from control and diabetic rats after placentation. In addition, we analysed whether maternal diabetes alters the protein expression of PPARα and the levels of LTB_4, its endogenous agonist, in both fetuses and placentas.

Table 1 Glycaemia, triglyceridaemia and placental and fetal leukotriene B_4 (LTB_4) concentrations in control and diabetic rats on day 13.5 of gestation.

	Control	Diabetic
Glycaemia (mg/dl)	99±10	229±20[†]
Triglyceridaemia (g/l)	1.0±0.2	2.1±0.3*
Placental LTB_4 concentrations (pg/mg protein)	3.4±0.5	1.4±0.3*
Fetal LTB_4 concentrations (pg/mg protein)	0.6±0.1	0.3±0.06*

Values are means±S.E.M., $n=8$ rats in each experimental group. Student's *t*-test was performed. *$P<0.05$ [†]$P<0.01$ denotes differences between the control and diabetic groups.

Results

Rat glycaemia, triglyceridaemia and feto-placental concentrations of LTB_4 and PPARα

Elevated concentrations of both glucose and triglycerides were observed in diabetic rats on day 13.5 of gestation when compared with controls (Table 1). The concentrations of the endogenous PPARα agonist LTB_4 and the protein expression of PPARα were analysed in placentas and fetuses from control and diabetic rats on day 13.5 of gestation. LTB_4 concentrations were reduced in both the placentas and the fetuses from diabetic animals when compared with controls (Table 1). PPARα protein expression was enhanced in the placenta from diabetic rats in comparison with controls, while there were no changes in PPARα concentrations in the fetuses from diabetic animals when compared with controls (Fig. 1).
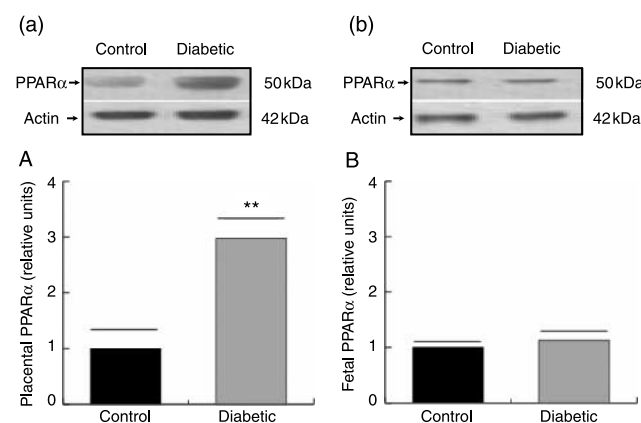

Figure 1 (a and b) Representative Western blots exhibiting PPARα protein expression in (a) placentas and (b) fetuses from control and diabetic rats on day 13.5 of gestation. Antibodies employed: anti-PPARα and anti-actin. (A and B) PPARα concentrations relative to those of actin for each sample in (A) placentas and (B) fetuses from control and diabetic rats on day 13.5 of gestation. Values are means±S.E.M., $n=8$ rats in each experimental group. Student's *t*-test was performed. **$P<0.01$ denotes differences between the control and diabetic groups.

Effect of PPARα agonists on placental and fetal lipid concentrations

Fetuses and placentas obtained from control and diabetic rats on day 13.5 of gestation were cultured for 3 h in the presence or absence of the PPARα agonists LTB$_4$ (0.1 μM) or clofibrate (20 μM), and placental and fetal lipid concentrations were analysed. We found an increase in triglycerides and cholesteryl esters, but no differences in the levels of phospholipids and cholesterol in the placenta from diabetic animals when compared with controls without additions (Fig. 2).

When the effect of PPARα agonists was analysed in the placenta from control and diabetic rats, we found that the pharmacological agonist clofibrate led to a reduction in all lipid species analysed in diabetic tissues and to a reduction in the levels of triglycerides, phospholipids and cholesteryl esters in control tissues (Fig. 2). On the other hand, although the endogenous agonist LTB$_4$ did not significantly change any lipid species analysed in the placenta from control rats, in the placenta from diabetic rats LTB$_4$ specifically reduced the concentrations of phospholipids and cholesterol, those lipid species that were not overaccumulated in these tissues (Fig. 2).

When lipid mass was analysed in the fetuses (Fig. 3), we found that phospholipids were the lipid species overaccumulated in the fetuses from diabetic animals, whereas no changes in the concentrations of triglycerides, cholesteryl esters and cholesterol were found when fetuses from diabetic rats were compared with controls. When the effects of PPARα agonists were analysed in the fetuses from control and diabetic rats, we found that both clofibrate and LTB$_4$ had similar effects, and were able to reduce the concentrations of triglycerides, cholesteryl esters and cholesterol in the fetuses from both the control and diabetic animals. In contrast, neither clofibrate nor LTB$_4$ was able to modify the concentrations of phospholipids, the only lipid species that was overaccumulated in the fetuses from diabetic animals (Fig. 3).

Effects of PPARα agonists on placental and fetal lipid syntheses

The *de novo* lipid synthesis was analysed in fetuses and placentas from control and diabetic rats on day 13.5 of gestation that were cultured in the presence of ^{14}C-acetate as a tracer either with or without the addition of LTB$_4$ (0.1 μM) or clofibrate (20 μM). When the effect of PPARα agonists on the *de novo* lipid synthesis was analysed in the placenta from control rats (Fig. 4), a reduction in the synthesis of triglycerides, cholesteryl esters and cholesterol was found in the presence of LTB$_4$, while the synthesis of all lipid species analysed was decreased in the presence of clofibrate. In contrast, there were no effects of either LTB$_4$ or clofibrate on the synthesis of the lipids analysed in the placenta from diabetic rats,
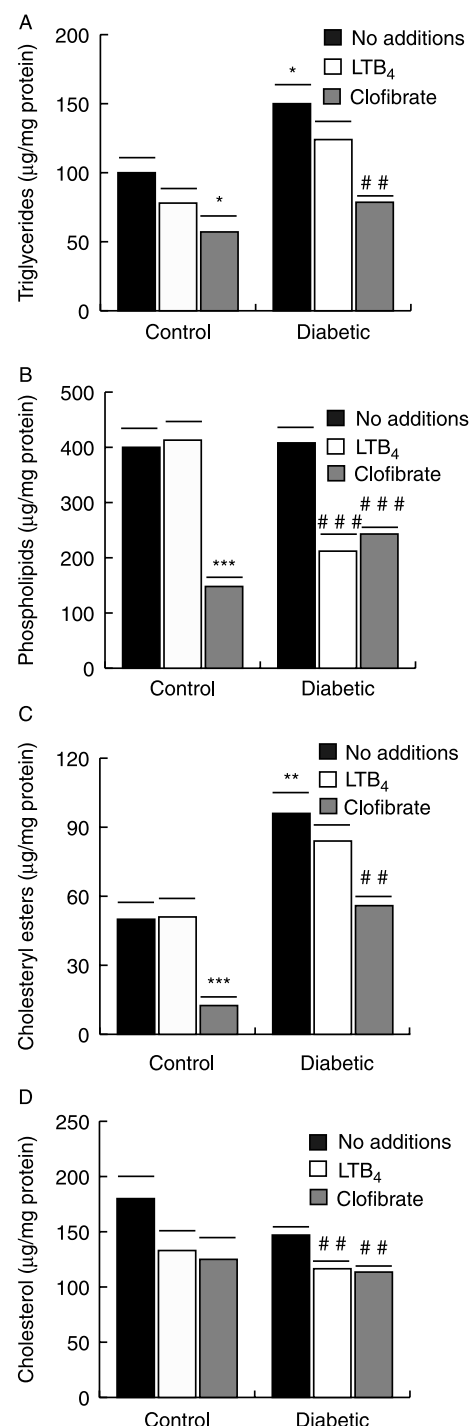

Figure 2 Effect of LTB$_4$ and clofibrate additions on the concentrations of (A) triglycerides, (B) phospholipids, (C) cholesteryl esters and (D) cholesterol in placentas obtained from control and diabetic rats on day 13.5 of gestation. Placentas were incubated either with or without the addition of 0.1 μM LTB$_4$ and 20 μM clofibrate for 3 h in KRB followed by lipids evaluation. Values are means ± s.e.m.; n=9 rats per group. ANOVA followed by Tukey's test was performed. *P<0.05 and ***P<0.001 denote differences from control group without additions; ##P<0.01 and ###P<0.001 denote differences from diabetic group without additions.
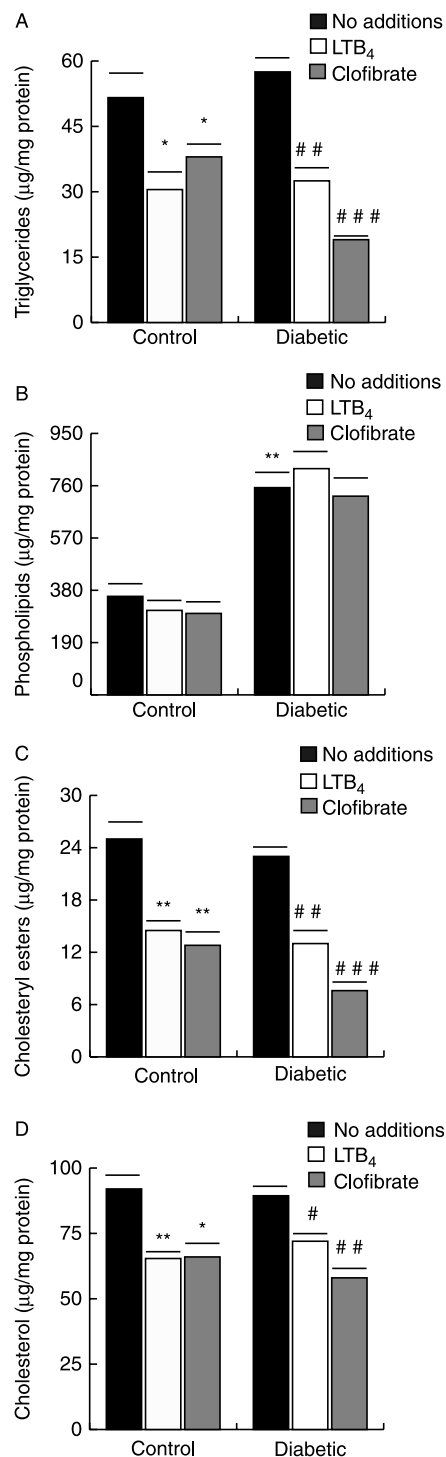
Figure 3 Effect of LTB$_4$ and clofibrate additions on the concentrations of (A) triglycerides, (B) phospholipids, (C) cholesteryl esters and (D) cholesterol in fetuses obtained from control and diabetic rats on day 13.5 of gestation. Fetuses were incubated either with or without the addition of 0.1 μM LTB$_4$ and 20 μM clofibrate for 3 h in KRB followed by lipids evaluation. Values are means±s.e.m.; *n*=8 rats per group. ANOVA followed by Tukey's test was performed. *$P<0.05$ and **$P<0.01$ denote differences from control group without additions; #$P<0.05$, ##$P<0.01$ and ###$P<0.001$ denote from diabetic group without additions.
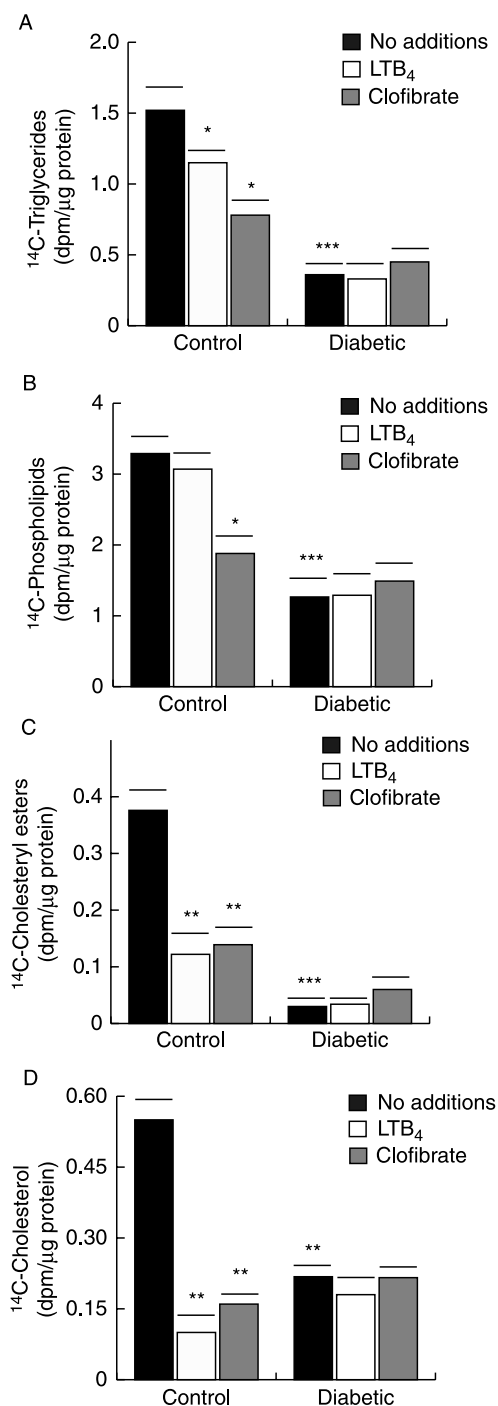
Figure 4 Effect of LTB$_4$ and clofibrate additions on the *de novo* synthesis of (A) triglycerides, (B) phospholipids, (C) cholesteryl esters and (D) cholesterol in placentas obtained from control and diabetic rats on day 13.5 of gestation. Placentas were incubated either with or without the addition of 0.1 μM LTB$_4$ and 20 μM clofibrate for 3 h in KRB in the presence of 1 μM ^{14}C-acetate (53 mCi/mmol) followed by evaluation of the incorporation of the tracer to lipids. Values are means±s.e.m.; *n*=8 rats per group. ANOVA followed by Tukey's test was performed. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ denote differences from control groups without additions.

which showed reduced *de novo* synthesis of all lipid species studied when compared with controls (Fig. 4).

On the other hand, in the fetuses from control animals (Fig. 5), LTB$_4$ did not significantly change the synthesis of any lipid species, while clofibrate reduced the synthesis of triglycerides and cholesteryl esters. In contrast, both LTB$_4$ and clofibrate were able to reduce the synthesis of all lipid species analysed in the fetuses from diabetic animals, which showed a high synthesis rate of these lipid species (Fig. 5).

Effects of PPARα agonists on placental release of glycerol and FFAs

To further address direct lipolytic effects of PPARα agonists, glycerol and FFAs were analysed in the media where placentas and fetuses from control and diabetic rats on day 13.5 of gestation were cultured for 3 h in the presence or absence of the PPARα agonists studied. Glycerol and FFAs were below the limit of detection of the employed techniques in the culture media of the fetuses. When the effects of PPARα agonists on glycerol and FFA release were analysed in the placenta from control rats, we found an increase in glycerol and FFA concentrations in the presence of LTB$_4$ and clofibrate, when compared with the controls without additions (Fig. 6). In contrast, PPARα agonists were devoid of effect in the placenta from diabetic animals, in which both glycerol and FFA concentrations were increased when compared with controls (Fig. 6).

Discussion

The present work describes a novel role of PPARα as a regulator of lipid metabolism in the fetuses and placentas from healthy and diabetic rats. PPARα agonists lead to a reduction in the concentrations of several lipid species in the fetuses and the placenta as a result of their effects on both lipid catabolism and lipid synthesis. The observed effects of PPARα agonists may contribute to the understanding of the impairments in lipid metabolism in fetuses and placentas from diabetic rats, which showed alterations in the concentrations of PPARα and its endogenous agonist LTB$_4$.

We found that LTB$_4$ production was reduced in the placenta and the fetuses from diabetic animals, an alteration that may impair feto-placental development and growth. Consistent with this hypothesis, murine pregnancies predisposed to spontaneous abortion have reduced LTB$_4$ levels in the feto-placental unit (Gendron *et al.* 1992). Interestingly, increased abortion and neonatal mortality rates have also been observed in control and diabetic *Ppara*- null mice, in which an altered balance in Th1/Th2 cytokine response is observed (Yessoufou *et al.* 2006). On the other hand, mice that overexpress PPARα show defects in cardiac development and metabolism (Finck *et al.* 2002), and fetuses from rats
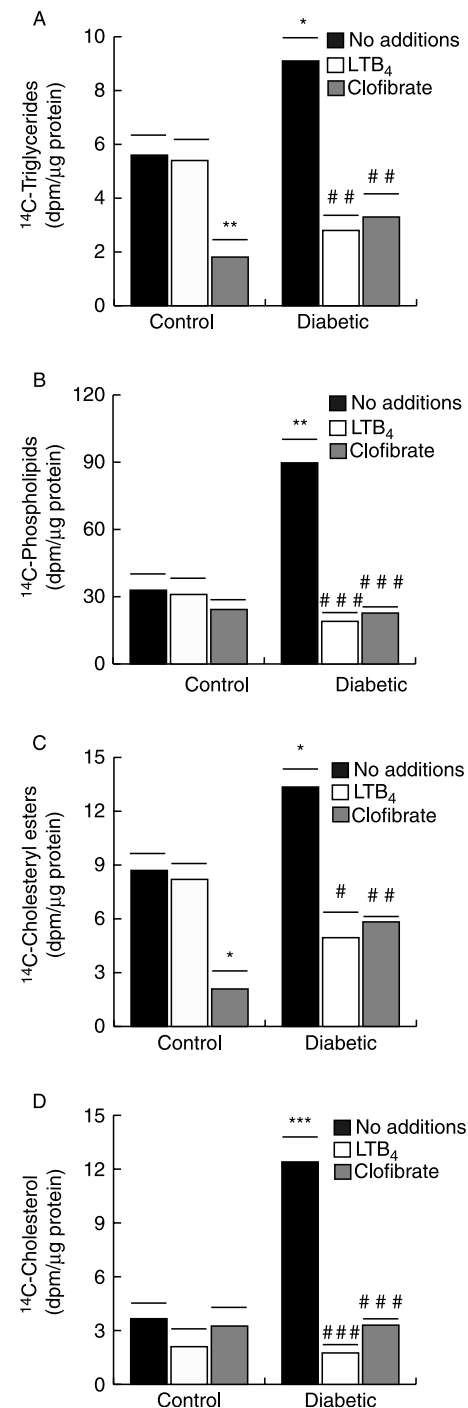

Figure 5 Effect of LTB$_4$ and clofibrate additions on the *de novo* synthesis of (A) triglycerides, (B) phospholipids, (C) cholesteryl esters and (D) cholesterol in fetuses obtained from control and diabetic rats on day 13.5 of gestation. Fetuses were incubated either with or without the addition of 0.1 μM LTB$_4$ and 20 μM clofibrate for 3 h in KRB in the presence of 1 μM ^{14}C-acetate (53 mCi/mmol) followed by evaluation of the incorporation of the tracer to lipids. Values are means ± S.E.M.; $n = 8$ rats per group. ANOVA followed by Tukey's test was performed. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ denote differences from control groups without additions; $^{#}P < 0.05$, $^{##}P < 0.01$ and $^{###}P < 0.001$ denote differences from diabetic group without additions.
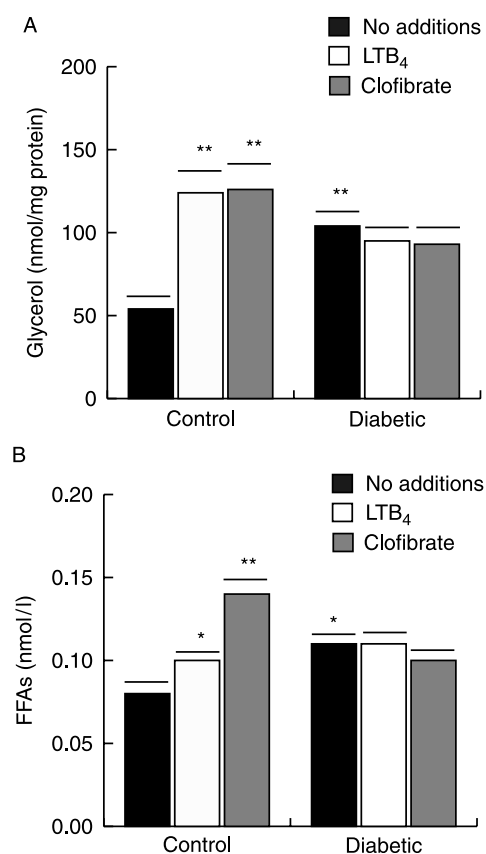
Figure 6 Effect of LTB$_4$ and clofibrate additions on (A) glycerol and (B) FFA release in placentas obtained from control and diabetic rats on day 13.5 of gestation. Placentas were incubated either with or without the addition of 0.1 µM LTB$_4$ and 20 µM clofibrate for 3 h in KRB followed by evaluation of glycerol and FFAs in the culture media. Values are means±S.E.M.; $n=8$ rats per group. ANOVA followed by Tukey's test was performed. *$P<0.05$ and **$P<0.01$ denote differences from control groups without additions.

treated with fibrates do not show malformations but present metabolic alterations (Soria *et al*. 2002, Peraza *et al*. 2006), suggesting that proper concentrations of PPARα and their ligands are needed for a proper development and growth. In this work, we found that PPARα concentrations were similar in fetuses from control and diabetic rats, but were increased in the placenta from diabetic animals, an alteration that may be related to a disregulated lipid utilisation in the feto-placental unit.

Alterations in the feto-placental lipid metabolism associated with maternal diabetes depend on the type and severity of the diabetic condition, and also on the developmental stage (Desoye & Shafrir 1996, Grissa *et al*. 2007). We have previously shown that during early organogenesis lipid synthesis is decreased in embryos from mild and severe diabetic rats whereas triglycerides are overaccumulated in embryos obtained from severe diabetic rats (Jawerbaum *et al*. 2002, Sinner *et al*. 2003). However, in this work, we identified phospholipids as

the lipid species overaccumulated in the fetuses from mild diabetic rats on day 13.5 of gestation, together with an increase in the *de novo* lipid synthesis that is likely to contribute to the increased fetal lipid mass. These differences probably reflect developmental changes occurring after mid-pregnancy in the rat, when the efficient mother-to-fetus nutrient exchange through the chorioallantoic placenta is initiated (Ain *et al*. 2003).

On the other hand, overaccumulation of triglycerides and cholesteryl esters was found in the diabetic placenta, despite the compensatory reduction in the *de novo* lipid synthesis observed. Our previous studies performed in term placenta in the same diabetic model have shown reductions in the *de novo* lipid synthesis but no changes in lipid mass, supporting the concept that the capacity of accretion of lipids in the placenta depends on the developmental stage, and is reduced at term (Capobianco *et al*. 2005).

There is little evidence of PPAR-mediated regulation of lipid metabolism in the placenta (Xu *et al*. 2007). PPARγ has been found to regulate uptake of FFAs, formation of lipid droplets and the *de novo* lipid synthesis in rat trophoblasts and placental tissues (Capobianco *et al*. 2005, Schaiff *et al*. 2007).

In this work, we focused on PPARα, a major regulator of lipid metabolism that regulates the transcription of multiple genes involved in the catabolism and the synthesis of lipids mainly in the liver and also in other tissues (Lefebvre *et al*. 2006). The degree of induction of these genes is specific for each tissue and depends on the levels of PPARα and its different ligands and co-activators (Desvergne *et al*. 2006). Some differences were observed in this work between the effects of PPARα agonists clofibrate and LTB$_4$ on the regulation of feto-placental lipid metabolism, which suggests that clofibrate effects are more potent, while those of LTB$_4$ are more specific. Indeed, although both PPARα agonists were able to reduce lipid concentrations in the placenta and the fetuses from control and diabetic rats, LTB$_4$ was able to reduce only those lipid species that are not overproduced in both the placenta and fetuses from diabetic animals. We speculate that overaccumulation of lipids might be specific for triglycerides and cholesteryl esters in the diabetic placenta and for phospholipids in the fetuses from diabetic rats due to the lack of LTB$_4$ catabolic effects on them.

On the other hand, the observed capacity of PPARα agonists to reduce the *de novo* lipid synthesis in control placentas was not observed in the diabetic placenta probably due to the reduced synthesis of all lipid species found in these diabetic tissues. Whether or not the upregulation of PPARα concentrations observed in the diabetic placenta is involved in the reduced lipid synthesis found in these tissues remains to be established.

The *de novo* lipid synthesis in the fetuses from diabetic animals was highly elevated, an alteration that probably contributes to their increased lipid accumulation (Kasser

et al. 1981), and that was reduced through the activation of PPARα. Similar effects of PPARα agonists on the *de novo* lipid synthesis were observed in the fetuses from control and diabetic animals, consistent with a similar PPARα protein expression. Nevertheless, the degree of activation of PPARα by its agonists will require further research as they may have PPARα-independent effects (Cui *et al.* 2001, Miyahara *et al.* 2006).

Most enzymes required for FFA oxidation are highly expressed in the placenta, an organ capable of both transporting fatty acids to the fetus and using them as metabolic fuels (Shekhawat *et al.* 2003). The capacity of releasing glycerol as an index of lipid catabolism in placental explants has previously been reported (Ramsay *et al.* 1991). In this work, we found that both pharmacological and endogenous PPARα agonists stimulated glycerol and FFA release in the control placenta. In addition, both glycerol and FFA release was elevated in the diabetic placenta, a catabolic pathway that seems to occur despite the low concentrations of LTB_4 and probably as a result of the increased PPARα concentrations. By contrast, PPARα agonists did not change glycerol and FFA release in the diabetic placenta, probably due to a further oxidation of FFAs and glycerol as it occurs in different tissues as a result of the stimulation of the increased PPARα concentrations (Patsouris *et al.* 2004, Desvergne *et al.* 2006).

In conclusion, here we demonstrate an important role of PPARα in fetal and placental lipid metabolism, as PPARα agonists reduce lipid concentrations through the regulation of both a reduction in the *de novo* lipid synthesis and an increase in lipid catabolism in these intrauterine tissues. These PPARα signalling pathways are disregulated and appear to be especially relevant in maternal diabetes, where several alterations in feto-placental lipid metabolism are found, probably as a response to the excess lipids derived from maternal circulation.

Materials and Methods

Animals

Albino Wistar rats were bred in the laboratory with free access to Purina rat chow and water, under a 14 h light:10 h darkness cycle. At 2 days of age, they were injected either with streptozotocin (90 mg/kg s.c.; Sigma–Aldrich) in citrate buffer (0.05 M, pH 4.5) or with buffer alone (controls). The spontaneous evolution of this treatment leads to a diabetic state (Portha *et al.* 1979), characterised by glycaemia values between 180 and 230 mg/dl and marked glucose intolerance, while control rat glycaemia levels were below 100 mg/dl. This experimental model is compatible with the pregnant state, and the reproductive characteristics of this model have previously been reported (Jawerbaum & Gonzalez 2005). In the evening of proestrus, control and diabetic females weighing between 200 and 300 g were caged overnight with control males. The following day was designated as day 0.5 of pregnancy if sperm cells were found in the vaginal smear. The guidelines for the care and use of animals approved by the local institution were followed, according to 'Principles of laboratory animal care' (NIH publication no. 85-23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm).

Tissue preparations

Animals were killed by cervical dislocation on day 13.5 of gestation, after the initiation of mother-to-fetus circulation through the placenta (Ain *et al.* 2003) and when PPARα is expressed in both placental and fetal tissues (Braissant & Wahli 1998). Placentas and fetuses were removed and placed in Petri dishes containing Krebs–Ringer bicarbonate solution (KRB, ionic composition: 5.5 mM glucose, 145 mM Na^+, 2.2 mM Ca^{++}, 1.2 mM Mg^{++}, 127 mM Cl^-, 25 mM HCO_3^-, 1.2 mM SO_4^{2-} and 1.2 mM PO_4^{3-}). Placentas and fetuses were either frozen at $-70\,°C$ for further analyses of LTB_4 and PPARα concentrations or prepared for the evaluation of the effect of PPARα agonists on lipid concentrations, the *de novo* lipid synthesis and lipid catabolism, as follows. Two fetuses or one placenta selected at random from each animal ($n=8$ or 9 animals in each experimental group) were incubated for 3 h as previously done to evaluate regulation of lipid metabolism (White *et al.* 2004, Capobianco *et al.* 2005) in a metabolic shaker under an atmosphere of 5% CO_2 and 95% O_2 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 $-70\,°C$ for further analysis of lipid concentrations, and aliquots of incubation medium were frozen at $-20\,°C$ for further determination of glycerol and FFA concentrations.

To analyse the *de novo* lipid synthesis, fetuses and placentas were incubated as described above, with the addition of 1 μCi ^{14}C-acetate (53 mCi/mmol; Amersham Biosciences), and then stored at $-70\,°C$ until determination of the newly formed radioactive lipids, as described below.

LTB_4 concentrations analysis

LTB_4 concentrations were measured in control and diabetic fetuses and placentas by employing a commercial enzyme immunoassay kit (Cayman Chemical Co). One fetus or one placenta from each rat ($n=8$ rats in each experimental group) was selected at random. Eicosanoids were extracted twice in absolute ethanol. The extracts were dried in a Savant Speed-Vac concentrator (Savant, Hicksville, NY, USA) and reconstituted with 200 μl assay buffer provided by the commercial kit. Briefly, the kit uses a polyclonal antibody against LTB_4 that binds in a competitive manner the eicosanoid in the sample or an acetylcholinesterase molecule, which has LTB_4 covalently attached to it. After a simultaneous incubation, a *p*-nitrophenyl phosphate substrate was added, and the yellow colour generated was evaluated on a microplate reader at 405 nm.

PPARα protein expression assessment

Two fetuses or one placenta from each rat ($n=8$ rats in each experimental group) were selected at random for the determination of PPARα protein expression by Western blot,

as previously performed for other PPAR isoforms (Jawerbaum *et al.* 2004, Higa *et al.* 2007). The tissues were homogenised in 300 µl ice-cold lysis buffer (pH 7.4, 20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100) containing 1% protease inhibitor cocktail and then incubated on ice for 2 h. Tissues were centrifuged at 7200 ***g*** for 10 min at 4 °C and the supernatant removed. Protein concentrations were determined by Bradford method using a protein assay reagent (Bio-Rad, Inc). Equal amounts of protein samples were separated with the use of 15% SDS-PAGE. Proteins were then transferred into nitrocellulose membranes, which were blocked overnight with 1% BSA and then incubated with a polyclonal rabbit IgG antibody either against PPARα (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or actin (Sigma–Aldrich) at 4 °C overnight. After washing with Tris buffer saline and Tween 0.05%, the blots were treated with horseradish peroxidase-conjugated secondary antibody for 1 h and washed several times. The specific signals were visualised using the enhanced chemiluminescence system (Amersham Biosciences). The identity of PPARα was established by the use of molecular weight standards, 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. Actin reactivity was detected with a phosphatase alkaline-conjugated secondary antibody. The relative intensity of protein signals was quantified by densitometry using the Sigma Gel Program (Sigma–Aldrich).

Lipid concentrations assessment

Lipid concentrations were determined by thin layer chromatography (TLC), as previously described (Capobianco *et al.* 2005). Briefly, placental and fetal lipids were extracted in methanol–chloroform in the ratio of 2:1 (v/v) and then concentrated in a Savant Speed-Vac concentrator. Total lipids were chromatographed with a solvent system consisting of hexane:ethyl ether:acetic acid in the ratio of 80:20:2 (v/v). After development, the TLC plate was dried for 5 min under an N_2 stream and the lipids were stained with iodine vapours. Lipid species levels were quantified by comparison with known amounts of pure lipid standards run on the same plate. The plates were scanned and analysed by densitometry using the Sigma Gel Program (Sigma–Aldrich).

Determination of de novo lipid synthesis

Fetal and placental lipid syntheses from ^{14}C-acetate were evaluated as previously (Higa *et al.* 2007). 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. Thereafter, the radioactive spots corresponding to the different ^{14}C-labelled lipid species were scrapped into vials and counted in a liquid scintillation counter.

Determination of FFA and glycerol release

FFAs and glycerol generated during the hydrolysis of triglycerides and phospholipids and released to the culture media of the placental tissues were determined. FFAs were determined by employing a colorimetric commercial kit (Randox Laboratories, Antrim, UK). Glycerol was determined by an enzymatic method as described previously (Young *et al.* 1988, White *et al.* 2006). Briefly, 0.2 ml placental explant incubation medium was allowed to react with 1 ml assay solution containing glycine (15 mg/ml), $MgCl_2$ (0.4 mg/ml), ATP (0.75 mg/ml), NAD^+ (0.36 mg/ml), hydrazine hydrate (0.2 ml/ml), glycerokinase (0.42 units/ml) and glycerol-3-phosphate dehydrogenase (2.5 units/ml; Roche Diagnostics Corporation). After 45 min, optical density was read at 340 nm in a microtitre plate using calibration curves of glycerol standards.

Statistical analysis

Results are expressed as means ± s.e.m. Comparisons between groups were performed employing either one-way ANOVA in conjunction with Tukey's test or Student's *t*-test, where appropriate. The statistical level of significance was defined as $P < 0.05$.

Acknowledgements

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