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PPAR activation as a regulator of lipid metabolism, nitric oxide production and lipid peroxidation in the placenta from type 2 diabetic patients

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ABSTRACT

Peroxisome proliferator activated receptors (PPARs) are ligand activated transcription factors with crucial functions in lipid homeostasis, anti-inflammatory processes and placental development. Maternal diabetes induces a pro-inflammatory environment and alters placental development. We investigated whether PPARs regulate lipid metabolism and nitric oxide (NO) production in placental explants from healthy and type 2 diabetic (DM2) patients. We found decreased PPARα and PPARγ concentrations, no changes in PPARδ concentrations, and increased lipids, lipoperoxides and NO production in placentas from DM2 patients. PPARα agonists reduced placental concentrations of triglycerides and both PPARα and PPARδ agonists reduced concentrations of phospholipids, cholesteryl esters and cholesterol. PPARγ agonists increased lipid concentrations in placentas from DM2 patients and more markedly in placentas from healthy patients. Endogenous ligands for the three PPAR isotypes reduced NO production and lipoperoxidation in placentas from DM2 patients. We conclude that PPARs play a role in placental NO and lipid homeostasis and can regulate NO production, lipid concentrations and lipoperoxidation in placentas from DM2 patients.

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1. Introduction

Maternal type 2 diabetes challenges fetal development and outcome. It increases the risks of spontaneous abortions, malformations, stillbirth, macrosomia, perinatal morbidity and mortality and intrauterine programming of metabolic and cardiovascular diseases (Balsells et al., 2009; Melamed and Hod, 2009; Michael Weindling, 2009). The placenta is crucial for fetal development and growth, and is profoundly affected by maternal diabetes (Desoye et al., 2011). Morphological and vascular alterations, as well as a pro-oxidative state, have been identified in the placentas from gestational diabetes mellitus (GDM) patients (Gauster et al., 2012; Lappas et al., 2011). Besides, our recent results have shown increased markers of a pro-oxidant and a pro-inflammatory state in the placenta from type 2 diabetic patients (Capobianco et al., 2012). On the other hand, metabolic derangements and impaired lipid homeostasis characterize both the maternal and the intrauterine compartment in GDM and type 2 diabetic pregnancies (Desoye et al., 2011; Herrera and Ortega-Senovilla, 2010; Scifres et al., 2011). Peroxisome proliferator activated receptors (PPARs) are nuclear receptors that heterodimerize with retinoid X receptors to modulate, in response to ligand activation, the expression of target genes with crucial functions in developmental, metabolic and anti-inflammatory processes (Bensinger and Tontonoz, 2008; Wieser et al., 2008). There are three PPAR isotypes, named PPARα, PPARδ and PPARγ. They are activated by certain unsaturated fatty acid species and arachidonic acid derivatives that function as ligands for PPARs such as leukotriene B_4 (LTB_4), prostaglandin I_2 (PGI_2) and 15deoxy$\Delta^{12,14}$$PGJ_2$ (15dPGJ_2), respective ligands of PPARα, PPARδ and PPARγ (Forman et al., 1997). Fibrates are antilipidemic drugs that activate PPARα (Desvergne et al., 2004; Forman et al., 1997). Regarding its function, PPARα is highly involved in fatty acid oxidation and plays a critical role in transcriptional

Abbreviations: GDM, gestational diabetes mellitus; PPAR, peroxisome proliferator activated receptor; PG, prostaglandin; PGI_2, prostacyclin or prostaglandin I_2; $cPGI_2$, carbaprostacyclin; LTB_4, leukotriene B_4; 15dPGJ_2, 15deoxy$\Delta^{12,14}$$PGJ_2$; NO, nitric oxide; TBARS, thiobarbituric acid reactive substances; Clof, clofibrate; Rosi, rosiglitazone; GW, GW501516.

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regulatory response to fasting (Leone et al., 1999). Ubiquitously expressed, PPARδ is involved in cell differentiation, myelination and lipid metabolism (Barak et al., 2002). Carbaprostacyclin ($cPGI_2$) and iloprost are drugs that activate PPARδ (Desvergne et al., 2004). PPARγ plays a pivotal role in adipogenesis, fatty acid uptake, lipid storage and systemic energy homeostasis, as well as in the control of inflammation (Bensinger and Tontonoz, 2008). Glitazones, a family of antidiabetic drugs with insulin-sensitizing activity, are synthetic ligands for PPARγ (Lehmann et al., 1995).

Regarding their roles in reproduction, in PPARα null mice, there are increased spontaneous abortion, and the viable offspring have increased inflammatory responses and hepatic steatosis (Yessoufou et al., 2006). Although PPARδ functions are less documented, there is evidence of a relevant role in embryonic, decidual and placental function (Higa et al., 2007; Kurtz et al., 2010; Wang et al., 2007). PPARδ and PPARγ null mice are not viable due to defects in placental development (Barak et al., 2008), and PPARγ roles in trophoblast differentiation and invasion are well established (Fournier et al., 2011). PPARγ has anti-oxidant and anti-inflammatory responses in intrauterine tissues (Jawerbaum and Capobianco, 2011). Indeed, we have previously found that PPARγ activators negatively regulate the production of nitric oxide (NO) in the placenta from control and type 1 diabetic patients, although this regulatory mechanism is altered in the placenta from GDM patients (Jawerbaum et al., 2004). PPARs protein expression has been found altered in the placenta from experimental diabetic models and from GDM patients (Holdsworth-Carson et al., 2010; Jawerbaum et al., 2004). Nevertheless, PPARs have not been previously evaluated in placentas from type 2 diabetic patients and PPARs effects on lipid concentrations and peroxidation in human placentas are unknown. We hypothesized that activation of PPARs could prevent diabetes-induced lipid overaccumulation, increased lipid peroxidation and NO overproduction in the placenta. Therefore, the aim of this work was to analyze placental explants from healthy and type 2 diabetic patients and determine (a) the concentrations of the three PPAR isotypes and (b) the effect of ligands for the three PPAR isotypes on lipid concentrations, nitric oxide production and lipid peroxidation.

2. Materials and methods

2.1. Patients

Following ethics approval and informed consent and based on clinical history, women with type 2 diabetes and without other co-morbidities and women with no pregnancy complications (controls) were recruited in the third trimester of pregnancy. The placentas in both the control ($n = 13$) and the diabetic ($n = 13$) groups were obtained at term (up to 38 weeks of gestation) by Caesarean section delivery, carried out before the onset of labor due to obstetric reasons. All diabetic patients were both insulin- and diet-treated. Medical and obstetric criteria for exclusion were vascular/renal complications, preeclampsia, anemia with total hemoglobin $\leqslant$8%, preterm labor, premature rupture of membranes, chorioamnionitis, intrauterine growth restriction, thrombophilia, positive test for HIV, syphilis or hepatitis B, placental abruption and acute fetal distress.

2.2. Placental preparations

Placental villous explants from control and type 2 diabetic patients were obtained after the basal and the chorial plates were dissected out from central cotyledons. Placental villous explants (100 mg) were immediately frozen at −80 °C for further determination of PPAR isoform protein expression levels by Western blot or cultured for 3 h in 1 mL Krebs Ringer bicarbonate (KRB) medium: 145 mM Na^+, 5.9 mM K^+, 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^{2-} with the addition of 5 mM glucose, as previously described (White et al., 2006), under an atmosphere of 5% CO_2 in air at 37 °C. PPAR ligands or vehicle alone (controls) were added to incubation media (PPARα ligands: LTB_4 0.1 μM (Cayman Chemical Co, Ann Arbor, MI, USA) and clofibrate 20 μM (Sigma–Aldrich, St. Louis, MO, USA); PPARδ ligands: the PGI_2 analog carbaprostacyclin ($cPGI_2$) 1 μM (Cayman Chemical Co) and GW501516 100 nM (Enzo Life Sciences International Inc., NY, USA); PPARγ ligands: $15dPGJ_2$ 2 μM (Cayman Chemical Co.) and rosiglitazone 3 μM (Sigma–Aldrich)), for further determination of lipid concentrations, NO production and lipid peroxidation, as previously described (Capobianco et al., 2008; Kurtz et al., 2010; Martinez et al., 2011a). The viability of the tissue explants was assessed by evaluating lactate dehydrogenase (LDH) concentrations by using a commercial kit (Wiener Labs, Buenos Aires, Argentina) that showed no changes in LDH concentrations in the presence of the evaluated agonists.

2.3. Western blot analysis

Placental explants were homogenized in 500 μl of ice-cold lysis buffer (pH 7.4, 20 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100) containing 1% protease inhibitor cocktail and the homogenates were incubated on ice for 2 h for controlled lysis. Homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C and the supernatant removed. Protein concentrations were determined by Bradford method using a protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, California, USA). Equal amounts of protein samples (75 μg per lane) were separated with the use of 10% SDS–PAGE. The gel preparation, combs, membranes and blotting system used allowed loading and transferring up to 100 μg protein. Proteins were then transferred onto nitrocellulose membranes, which were blocked with 1% of BSA for 1.5 h at 37 °C and then incubated overnight at 4 °C with primary antibodies as follows: rabbit polyclonal IgG antibody against either PPARα (1:1000) (Cayman Chemical Co.) or PPARγ (1:1000) (Santa Cruz Biotechnology, CA, USA); a goat polyclonal IgG antibody against PPARδ (1:1000) (Santa Cruz Biotechnology); or a rabbit polyclonal IgG antibody against actin (1:500) (Sigma–Aldrich) used as an internal control. After washing with Tris buffer saline (TBS) and TBS–Tween 20 (0.05%, TTBS), the blots were treated with goat anti-rabbit or bovine anti-goat IgG conjugated to horseradish peroxidase secondary antibody (1:15000) (Jackson Immuno Research Laboratories, Inc., Baltimore, USA) for 1 h at room temperature and washed several times with TBS and TTBS. The specific signals were visualized using the enhanced chemiluminescence system (Amersham Biosciences, Arlingtoon Height, IL, USA). The identity of each PPAR isotype was established by the use of molecular weight standards and cell lysates from rat liver (PPARα), lung (PPARδ) and adipose tissue (PPARγ) as positive controls, which allow the identification of the band revealed at the expected size of 57, 50 and 51 kDa respectively. The negative control was performed avoiding the incubation with the antibody of interest. The intensity of protein signals was quantified by densitometric analysis using the Image J Software. Results were expressed as the relative intensity of each PPAR isotype and normalized against actin, as previously performed (Martinez et al., 2011a).

2.4. Analysis of lipid concentrations

Placental lipids were extracted in methanol–chloroform 2:1 (v/v) and developed by thin layer chromatography (TLC, Merck and Co, Inc., New Jersey, USA) as previously (Capobianco et al., 2005), with a solvent system consisting of hexane:ethyl ether:

acetic acid 80:20:2 v/v. After development, the TLC plate was dried for 5 min under a N_2 stream and the lipids were stained with iodine vapors. Lipid species levels were quantified by comparison with known amounts of pure lipid standards run on the same plate. The plates were scanned and analyzed by densitometry using the Image J software. Results are expressed as μg/mg protein.

2.5. Analysis of nitric oxide production

The concentration of NO stable metabolites nitrates/nitrites was determined as previously described (Capobianco et al., 2005), by using a commercial assay kit (Cayman Chemical Co.). Briefly, 100 mg of placental tissue was homogenized in Tris–HCl solution (0.1 mM, pH: 7.4) and an aliquot was separated to determine protein content. Nitrates in the supernatant were reduced to nitrites using nitrate reductase, and total nitrites were then quantified by the Griess reaction. Different amounts of sodium nitrate were used as standards. The resulting optical densities were measured at 540 nm in a microliter plate. Results are expressed as nmol/mg of protein.

2.6. Analysis of lipid peroxidation

Lipid peroxidation was evaluated as previously described (Kurtz et al., 2010), 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, 100 mg of placental tissue was homogenized in 100 mM Tris–HCl buffer (0.1 mM, pH: 7.4). Trichloroacetic acid (40%) (Merck and Co, Inc., New Jersey, USA) was added to the homogenate and centrifuged at 3000 rpm for 10 min. The supernatant was added to an equal volume of thiobarbituric acid (46 mM) (Sigma–Aldrich), and the solution was heated at 95 °C for 15 min. Then, the samples were cooled and quantified spectrophotometrically at 540 nm. Different concentrations of malondialdehyde (Sigma–Aldrich) subjected to the same conditions as the tissue homogenates were used as standards. TBARS are expressed as nmol/mg protein.

2.7. Analysis of gene expression

The expression of genes encoding PPARα and PPARγ, Carnitine Palmitoyl Transferase 1, liver isoform (CPT1), Fatty acid synthase (FASN), Manganese superoxide dismutase 2 (SOD2), Catalase (CAT) and Ribosomal protein L30 (L30) were evaluated by RT-PCR. Placental RNA was extracted with Tri reagent (Genbiotech, Buenos Aires, Argentina) in accordance with the manufacturer's instructions. To synthesize the cDNA, 2 μg of extracted human placenta RNA and 1 μg of random primers were incubated at 70 °C for 5 min, and the reverse transcription buffer containing 0.5 mM of each of all four dNTPs (Invitrogen, Buenos Aires, Argentina) and 200 U MMLV reverse transcriptase (Promega, Buenos Aires, Argentina) was added for further incubation at 37 °C for 60 min and then at 70 °C for 15 min. Then, 5 μl of cDNA was amplified in a reaction buffer containing dNTPs (0.25 mM), magnesium chloride solution (2 mM), 0.625 U of Taq polymerase (GoTaq Polymerase, Promega), and 1 μM of each specific primer. The nucleotide sequences for forward and reverse primers of the human transcripts were: PPARα: forward: 5′ ATCGGCGAGGATAGTTCT 3′, reverse: 5′ AATCGCGTTGT GTGACAT 3′; PPARγ: forward: 5′ GTCAGCGGACTCTGGATT 3′, reverse: 5′ CAGATCCAGTGGTTGCAG 3′; CPT1: forward: 5′ GTCCCGGC TGTCAAAGACA 3′, reverse: 5′ CCGACAGCAAAATCTTGAGCA 3′; FASN: forward: 5′ CTCCATGAAGTAGGAGTGGAAG 3′, reverse: 5′ CATCCAGATAGGCCTCATAGAC 3′; SOD2: forward: 5′ GACAAACCT CAGCCCTAACG 3′, reverse: 5′ CTGATTTGGACAAGCAGCAA 3′; CAT: forward: 5′ GCCTGGGACCCAATTATCTT 3′, reverse: 5′ GAATCTC CGCACTTCTCCAG 3′; L30: forward: 5′ CCGCAAAGAAGACGAAAAAG 3′, reverse: 5′ AAAGCTGGGCAGTTGTTAGC 3′. The PCR conditions were: an initial step of 95 °C for 5 min, followed by 34 cycles for PPARα, 32 cycles for PPARγ, 35 for CPT1, 36 cycles for FASN, 36 cycles for SOD2, 34 cycles for CAT and 31 cycles for L30. The condition for each cycle was 95 °C for 15 s, 58 °C for 30 s and 72 °C for 15 s. The resulting products were separated on a 2% agarose gel and stained with Syber Safe (Invitrogen). The density of the bands was quantified by image analysis with ImageJ software and the expression of the genes was normalized to the human ribosomal protein L30.

2.8. Statistical analyses

Data are presented as the mean ± SEM. Differences among groups were compared using Student's *t* tests or a two-way ANOVA with Bonferroni post hoc tests. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Patients and placental PPAR concentrations

There were no significant differences in maternal age, body mass index (BMI) and parity between type 2 diabetic patients and healthy controls (Table 1). In type 2 diabetic patients, metabolic control was achieved by insulin therapy and dietary treatment, and fasting glucose was similar when compared to controls (Table 1). Placental ($p < 0.05$) and neonatal ($p < 0.001$) weight were increased in the type 2 diabetic patients when compared to controls (Table 1). Regarding neonatal complications, we observed two cases of macrosomia and one case of respiratory distress syndrome in newborns from type 2 diabetic patients. Evaluation of PPARs protein in the placenta showed reduced protein expression of PPARα ($p < 0.01$) and PPARγ ($p < 0.05$) and similar protein expression of PPARδ in placentas from type 2 diabetic patients when compared to healthy controls (Fig. 1).

3.2. Effect of PPAR ligands on placental lipid concentrations

Increased concentrations of phospholipids ($p < 0.05$), cholesteryl esters ($p < 0.001$), triglycerides ($p < 0.01$) and cholesterol ($p < 0.01$) were found in the placentas from type 2 diabetic patients when compared to controls (Fig. 2A). In order to analyze the influence of PPARs activation on placental lipid levels, placental explants obtained from type 2 and healthy patients were cultured

Table 1
Patients data.

	Control ($n = 13$)	Diabetic ($n = 13$)
Maternal age	28.1 ± 1.6^a	32.0 ± 1.7^a
Parity		
Primiparous	1	1
Multiparous	12	12
Maternal BMI (kg/m^2)	27.7 ± 1.5^a	29.5 ± 1.6^a
Treatments		
Diet (kcal)	–	1800–2000
Insulin (units per day)	–	24–238
Fasting glucose (mg/dl)	<92^a	75–100^a
Gestational age (wk)	38.6 ± 0.3^a	38.3 ± 0.3^a
Placental weight (g)	546 ± 22^a	629 ± 22^b
Weight at birth (g)	3257 ± 99^a	3842 ± 103^b
Neonatal complications	None	RDS ($n = 1$) Macrosomia ($n = 2$)

Data are shown as means ± SEM. Student's *t*-test was performed on the data. Each letter denotes significant differences with the experimental group denoted with a different letter ($p < 0.05$). RDS: respiratory distress syndrome.

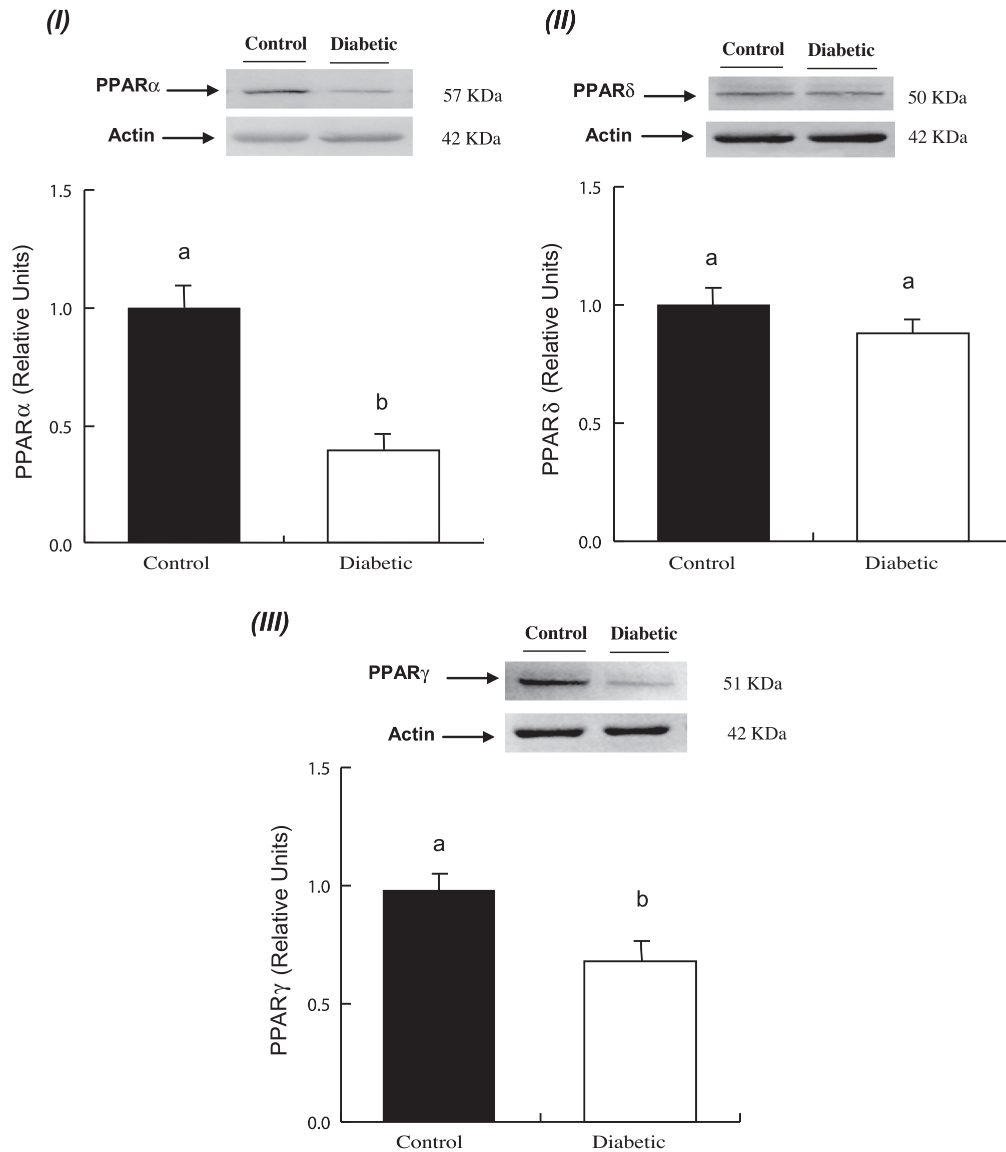

Fig. 1. Representative Western blot and densitometric analysis of PPARs concentrations in placentas from type 2 diabetic patients and healthy controls. The relative values of each PPAR isotype normalized against actin in each sample are shown as means ± SEM, $n = 13$ in each experimental group. Student's t-test was performed on the data. Each letter denotes significant differences with the experimental group denoted with a different letter ($p < 0.05$). Antibodies used: (I) anti-PPARα and anti-actin, (II) anti-PPARδ and anti-actin and (III) anti-PPARγ and anti-actin.

for 3 h in the presence or absence of the following PPAR ligands: LTB_4 0.1 μM and clofibrate 20 μM (PPARα ligands), $cPGI_2$ 1 μM and GW501516 100 nM (PPARδ ligands), and $15dPGJ_2$ 2 μM and rosiglitazone 3 μM (PPARγ ligands), and then placental lipid levels were analyzed. When we analyzed the effects of the PPAR ligands evaluated on lipid species content in placentas from control and diabetic patients, we found significant changes related to the diabetic state, the PPAR ligand and/or their interaction depending on the lipid species and the PPAR isotype analyzed ($p < 0.05$, Fig. 2). When we analyzed the effect of PPARα ligands, we found that LTB_4 negatively regulated the concentrations of all lipid species analyzed ($p < 0.05$) and clofibrate diminished cholesteryl esters concentrations ($p < 0.05$) in the placentas from healthy patients, whereas LTB_4 negatively regulated the concentrations of all lipid species analyzed ($p < 0.01$) and clofibrate decreased the concentrations of the neutral lipids evaluated ($p < 0.05$) in the placentas from type 2 diabetic patients (Fig. 2A). When we analyzed the effect of PPARδ ligands, we found that $cPGI_2$ negatively regulated the concentrations of phospholipids, cholesteryl esters and cholesterol ($p < 0.05$), whereas GW501516 diminished cholesteryl esters and cholesterol concentrations ($p < 0.05$) in the placentas from healthy patients (Fig. 2B). Differently, PPARδ activators were unable to modify the concentrations of the lipid species evaluated in the placentas from type 2 diabetic patients (Fig. 2B). When we analyzed the effect of PPARγ ligands, we found that $15dPGJ_2$ positively regulated the concentrations of the neutral lipid species analyzed ($p < 0.001$) and rosiglitazone increased the concentrations of all lipid species evaluated ($p < 0.05$) in the placentas from healthy patients. Differently, $15dPGJ_2$ only increased the concentrations of cholesteryl esters ($p < 0.05$) and rosiglitazone increased phospholipid and cholesteryl ester concentrations ($p < 0.01$) in the placentas from type 2 diabetic patients (Fig. 2C).

To validate the capacity of PPAR ligands to regulate gene expression in the villous tissue explants, the effect of LTB_4 0.1 μM on the gene expression of PPARα and of the lipid metabolizing enzyme CPT-1, as well as the effect of $15dPGJ_2$ 2 μM on the gene expression of PPARγ and of the enzyme Fatty acid synthase (FASN) were determined. Although no changes in PPARα

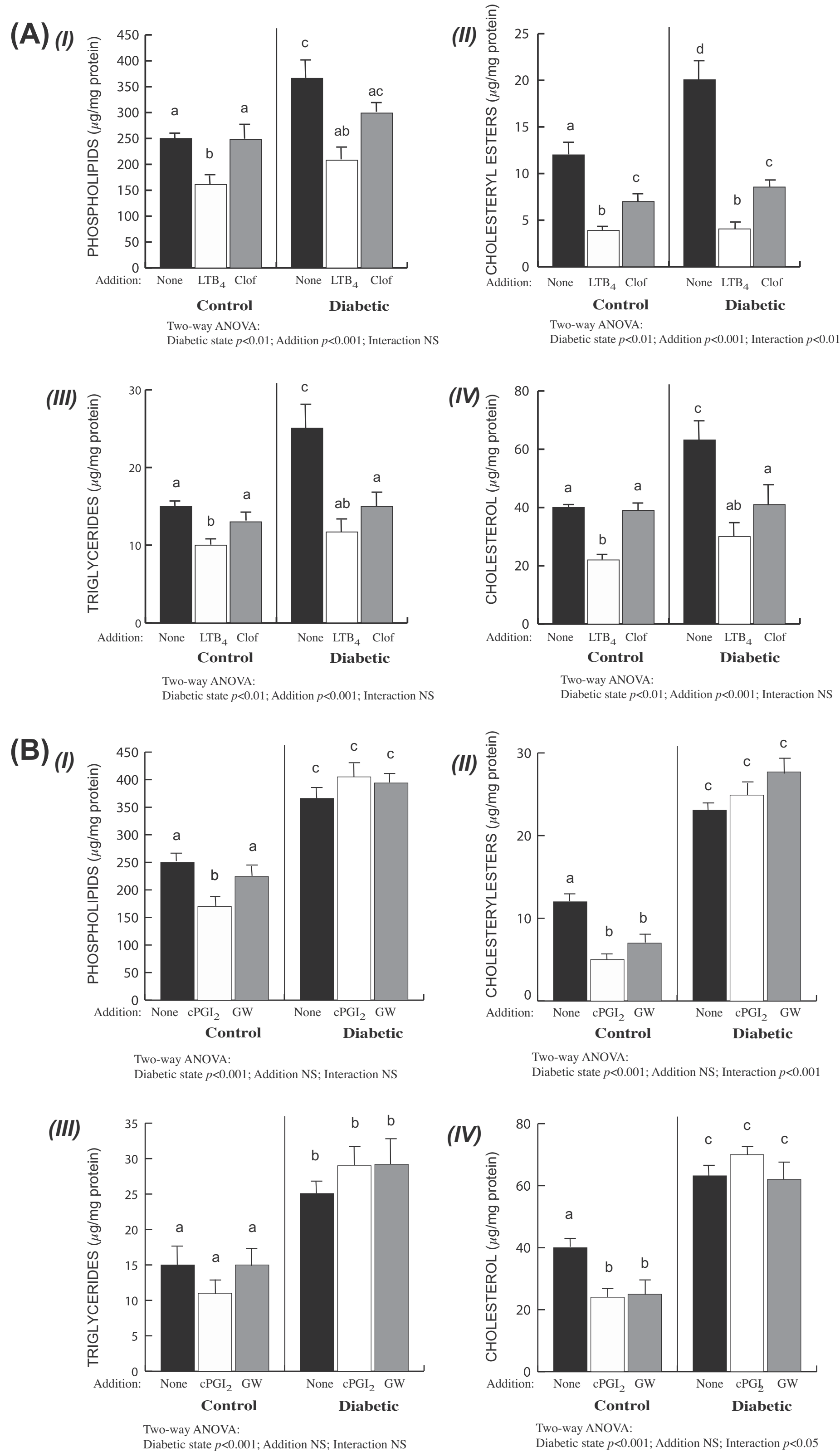

Fig. 2. Effect of PPARs agonists on the levels of (I) phospholipids, (II) cholesteryl esters, (III) triglycerides and (IV). cholesterol in term placentas from type 2 diabetic patients and healthy controls. Placental explants were incubated for 3 h with or without additions of (A) PPARα agonists: LTB_4 0.1 μM and clofibrate (Clof) 20 μM; (B) PPARδ agonists: $cPGI_2$ 1 μM and GW501516 (GW) 100 nM; and (C, see next page) PPARγ agonists: $15dPGJ_2$ 2 μM and rosiglitazone (Rosi) 3 μM; followed by lipids evaluation. Values are means ± SEM; $n = 13$ in each experimental group. Two-way ANOVA followed by Bonferroni's test was performed on the data. Each letter denotes significant differences with the experimental groups denoted with different letters ($p < 0.05$).

expression were observed in placental explants incubated with LTB_4 (D: 1 ± 0.06 D + LTB_4: 1.00 ± 0.07 relative units to L30), an increase in CPT-1 expression was observed (D: 1 ± 0.05 D + LTB_4: 1.16 ± 0.03 relative units to L30, $p < 0.05$, $n = 8$). Besides, incuba-

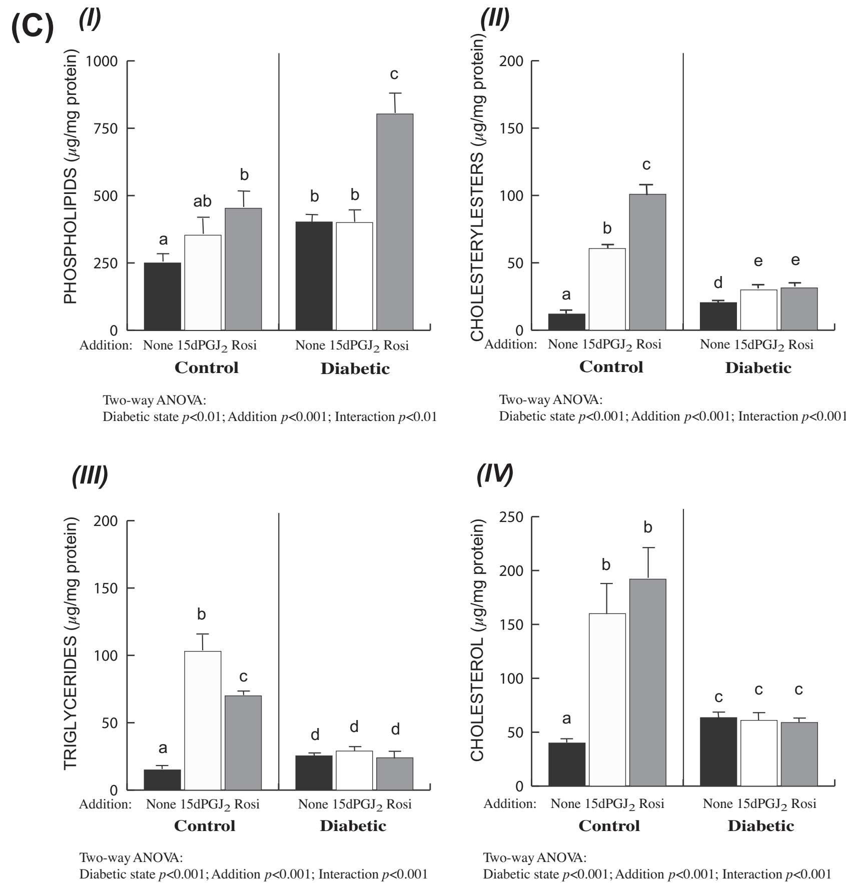

Fig. 2 (*continued, see legend in previous page*)

tions with $15dPGJ_2$ led to an increase in the expression of both PPARγ (D: 1 ± 0.05 D + $15dPGJ_2$: 1.22 ± 0.06 relative units to L30, $p < 0.05$, $n = 8$) and FASN (D: 1 ± 0.03 D + $15dPGJ_2$: 1.18 ± 0.07 relative units to L30, $p < 0.05$, $n = 8$).

3.3. Effect of PPAR ligands on placental nitric oxide production and lipoperoxidation

In order to analyze the influence of PPARs activation on placental NO production and lipid peroxidation, placental explants obtained from type 2 and healthy patients were cultured for 3 h with or without additions of the ligands of the three PPAR isoforms. We found increased NO production and lipid peroxidation, markers of a pro-inflammatory and pro-oxidant state, in the placentas from type 2 diabetic patients ($p < 0.01$, Figs. 3 and 4). When we analyzed the effects of the PPAR ligands evaluated on NO production in the placentas from control and diabetic patients, we found significant changes related to the diabetic state, the PPAR ligands and/or their interaction depending on the PPAR isotype analyzed ($p < 0.01$, Fig. 3). When we address the effect of the PPARα ligands, we found that LTB_4 negatively regulated NO production in the placentas from type 2 diabetic patients ($p < 0.05$), whereas no other effects on the concentrations of NO stable metabolites were observed in the presence of both PPARα and PPARδ ligands (Fig. 3). Differently, the PPARγ ligand $15dPGJ_2$ significantly reduced NO production in the placentas from healthy patients ($p < 0.05$), and in the placentas from diabetic patients to values similar to the control group ($p < 0.001$) (Fig. 3).

When we analyzed the effect of PPAR ligands evaluated on lipid peroxidation in the placentas from control and diabetic patients, we found significant changes related to the diabetic state, the PPAR ligand and/or their interaction depending on the PPAR isotype analyzed ($p < 0.05$, Fig. 4). When we address the effects of PPARα ligands we found that they did not regulate lipid peroxidation in the placentas from healthy patients, but that the PPARα ligand LTB_4 negatively regulated lipid peroxidation in the placentas from type 2 diabetic patients ($p < 0.05$, Fig. 4). PPARδ ligands $cPGI_2$ and GW501516 negatively regulated lipid peroxidation in the placentas from healthy and type 2 diabetic patients ($p < 0.05$, Fig. 4). Moreover, although the PPARγ ligands evaluated were unable to regulate lipid peroxidation in the placenta from healthy patients, both $15dPGJ_2$ and rosiglitazone highly reduced lipoperoxidation in the placentas from type 2 diabetic patients, to values similar to those found in the control group ($p < 0.001$, Fig. 4).

To validate the capacity of PPAR ligands to regulate the expression of anti-oxidant genes in the incubations performed, the effects of LTB_4 on CAT gene expression and of $15dPGJ_2$ on SOD2 expression were determined. We found that LTB_4 increased CAT expression (D: 1 ± 0.09 D + LTB_4: 1.24 ± 0.05 relative units to L30, $p < 0.05$, $n = 8$) and that $15dPGJ_2$ increased SOD2 expression (D: 1 ± 0.1 D + $15dPGJ_2$: 1.67 ± 0.06 relative units to L30, $p < 0.001$, $n = 8$).

4. Discussion

The findings of the present work demonstrate that there are lipid overaccumulation, increased lipoperoxidation and NO overproduction, as well as significant changes in PPARs protein expression in term placentas from type 2 diabetic patients when compared to the healthy controls. Besides, we found that in the human placenta, lipid concentrations, NO production and lipid peroxidation can be

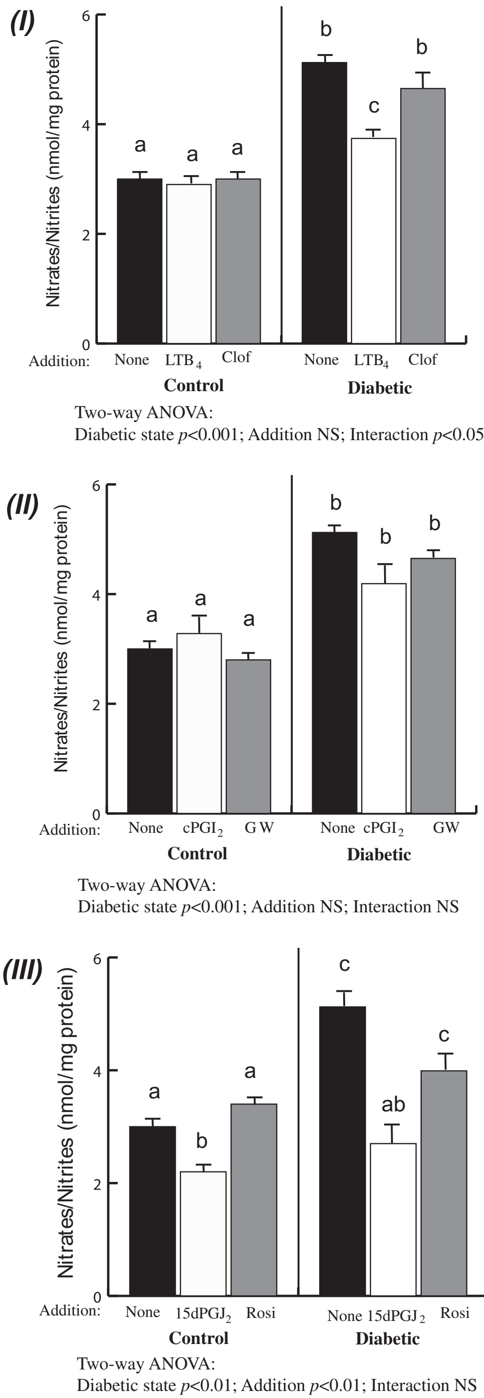

Fig. 3. Effect of PPARs agonists on NO production, evaluated through the measurement of the concentrations of its stable metabolites nitrates/nitrites, in term placentas from type 2 diabetic patients and healthy controls. Placental explants were incubated for 3 h with or without additions of (I) PPARα agonists: LTB_4 0.1 μM and clofibrate (Clof) 20 μM; (II) PPARδ agonists: $cPGI_2$ 1 μM and GW501516 (GW) 100 nM; and (III) PPARγ agonists: $15dPGJ_2$ 2 μM and rosiglitazone (Rosi) 3 μM; followed by nitrates/nitrites measurement. Values are means ± SEM; $n = 13$ in each experimental group. Two-way ANOVA followed by Bonferroni's test was performed on the data. Each letter denotes significant differences with the experimental groups denoted with different letters ($p < 0.05$).

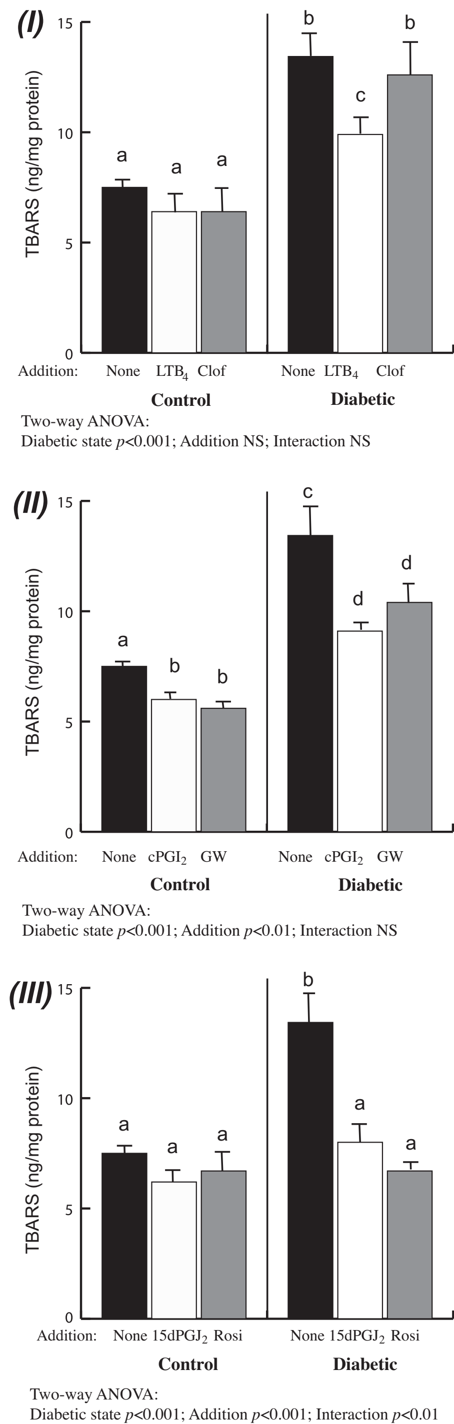

Fig. 4. Effect of PPARs agonists on lipid peroxidation, evaluated through the measurement of TBARS concentrations in term placentas from type 2 diabetic patients and healthy controls. Placental explants were incubated for 3 h with or without additions of (I) PPARα agonists: LTB_4 0.1 μM and clofibrate (Clof) 20 M; (II) PPARδ agonists: $cPGI_2$ 1 M and GW501516 (GW) 100 nM; and (III) PPARγ agonists: $15dPGJ_2$ 2 M and rosiglitazone (Rosi) 3 M; followed by TBARS measurement. Values are means ± SEM; $n = 13$ in each experimental group. Two-way ANOVA followed by Bonferroni's test was performed on the data. Each letter denotes significant differences with the experimental groups denoted with different letters ($p < 0.05$).

regulated by PPARs activation in a PPAR isotype-, PPAR ligand- and diabetic state-dependent fashion.

In pregnancy, type 2 diabetes mellitus has adverse consequences for the mother, the placenta and the fetus (Balsells et al., 2009; Melamed and Hod, 2009; Vambergue and Fajardy, 2011). In this work, despite adequate control of blood glucose concentrations, we found increased placental and fetal weight in the type 2 diabetic group, as well as neonatal complications in three of the thirteen type 2 diabetic pregnancies studied. This suggests that factors other than glucose metabolism, such as alterations in lipid homeostasis and a pro-oxidant/pro-inflammatory environment may have a role in the induction of feto-placental alterations in

type 2 diabetes. Nuclear receptor PPARs have been largely involved in metabolic and anti-inflammatory pathways, as well as in feto-placental development (Bensinger and Tontonoz, 2008; Wieser et al., 2008). Our previous works have shown relevant roles for PPARs in the regulation of lipid homeostasis, anti-oxidant and anti-inflammatory pathways in animal models of diabetes and pregnancy (Jawerbaum and Capobianco, 2011). Besides, changes in the concentrations of PPARs have been found in the placenta from both diabetic experimental models and in the placenta from GDM patients (Capobianco et al., 2005; Holdsworth-Carson et al., 2010; Jawerbaum et al., 2004; Martinez et al., 2011a). Similarly to that previously found in placentas from diabetic rats and from GDM patients, in this work we found reduced PPARα and PPARγ concentrations and no changes in PPARδ concentrations in the placenta from type 2 diabetic patients. Differently, previous studies have shown no changes in placental PPARγ expression in type 1 diabetic patients, but reductions in the PPARγ endogenous ligand $15dPGJ_2$ in placentas from type 1 and GDM patients (Jawerbaum et al., 2004).

Regulation of lipid transfer to the fetus is a crucial role of the placenta and depends on lipid incorporation from maternal circulation as well as from the lipid metabolic pathways within the placenta (Herrera et al., 2006). Indeed, the placenta synthesizes, incorporates and oxidizes lipids, which are prone to lipid peroxidation and can lead to tissue damage in a pro-oxidant environment (Negre-Salvayre et al., 2010). In this work, we demonstrated that placentas from type 2 diabetic patients have increased lipid concentrations and peroxidation, reflecting an imbalance between lipids incorporation and metabolization and a pro-oxidant state. Interestingly, we found that lipid homeostasis is differentially regulated by the activation of the three PPAR isotypes in the human placenta.

PPARα capacity to regulate lipid catabolism has been addressed in multiple organs and tissue types (Desvergne et al., 2006). In this work, we demonstrated that its ligands are effective negative regulators of both polar and neutral lipid species in the human placenta, and capable of preventing lipid overaccumulation in the placenta from type 2 diabetic patients. Thus, the observed reductions in PPARα concentrations may be related to the lipid overaccumulation found in the placenta from type 2 diabetic patients.

It has been shown that PPARα agonists reduce both triglyceridemia and HDL-cholesterol in patients with dyslipidemia (Katsiki et al., 2013). In addition, PPARα and PPARγ agonists play an important role in cholesterol metabolism and are involved in the reverse transport of cholesterol in macrophages (Chinetti et al., 2001). Nevertheless, recent studies have shown that neither PPARα nor PPARγ activators have an effect on the target genes ABCA1 and ABCG1 involved in cholesterol reverse transport in trophoblasts (Aye et al., 2010), and thus putative mechanisms underlying the changes here observed in cholesterol concentrations in the presence of PPAR activators remain to be elucidated.

On the other hand, although PPARδ activators were found to negatively regulate phospholipids, cholesteryl esters and cholesterol concentrations in the placenta from healthy patients, they did not reduce lipid concentrations in the placenta from type 2 diabetic patients. This impairment may be related to the increased lipid concentrations found in the placenta from type 2 diabetic patients, although the mechanisms involved in this impaired signaling will require further research.

PPAR signaling is complex, and methods to evaluate direct PPAR activity are limited to cellular studies, constituting a limitation in studies performed in placental explants such as this one. Similar to our previous findings (Martinez et al., 2011a; Martinez et al., 2011b), we observed differences when evaluating the effects of different PPAR ligands for the same isotype as well as differences in the responses in normal healthy and diabetic placentas. This is accordance with the conformational adaptation of the different ligands to their binding pocket, the different availability/recruitment capacity of multiple PPAR coactivators, and/or a different availability/release capacity of multiple PPAR corepressors that may occur under the activation of different ligands or under pathological conditions (Bensinger and Tontonoz, 2008; Hostetler et al., 2005; Panadero et al., 2009). In addition, as a limitation of this study, possible off-target effects of the ligands tested cannot be excluded.

Previous studies have shown that unlike PPARα and PPARδ, which play roles related to lipid oxidation, PPARγ increases free fatty acid uptake in cultured human trophoblasts through mechanisms that involve increases in the formation of lipid droplets and fatty acid transporters (Bildirici et al., 2003; Schaiff et al., 2005). Accordingly, we here found that PPARγ increases the concentrations of polar and neutral lipids in the placenta from healthy patients, although only positively regulates the concentrations of phospholipids and cholesteryl esters in the placenta from diabetic patients. This, together with the reduced concentrations of PPARγ observed, suggests that PPARγ is not a main mechanism responsible for increased lipid concentrations in the placenta from type 2 diabetic patients.

Regarding NO production, NO is a relevant regulator of placental blood flow, needed to regulate nutrient transport through the placenta, although its overproduction is highly related to a pro-inflammatory state (Leach et al., 2009; San Martin and Sobrevia, 2006). Indeed, both a pro-inflammatory and a pro-oxidant state alter placental function in maternal diabetes, and we have recently found increased NO production, lipoperoxidation and matrix metalloproteinases overactivity (a marker of the pro-inflammatory state) in the placenta from type 2 diabetic patients (Capobianco et al., 2012). Following these observations, here we found that PPARα and PPARγ ligands reduced NO overproduction in the placenta from type 2 diabetic patients. Moreover, activation of the three PPAR isotypes negatively regulated lipid peroxidation in the placenta from type 2 diabetic patients. Indeed, we observed that the PPARγ isotype is the one that exerts most potent effects in the regulation of NO overproduction and pro-oxidant pathways. In agreement, several works have reported anti-inflammatory and anti-oxidant effects of PPARγ activation in different tissues and organs, including the placenta (Bensinger and Tontonoz, 2008; Lappas et al., 2006; Pustovrh et al., 2009).

In conclusion, the results of this work provide novel evidence of the involvement of the three PPAR isotypes in lipid homeostasis, anti-oxidant pathways and NO overproduction in the human placenta. Moreover, our data reveal a relevant role of PPARs in the placenta from type 2 diabetic patients, where reduced concentrations of PPARα and PPARγ were demonstrated.

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