Englitazone administration to late pregnant rats produces delayed body growth and insulin resistance in their fetuses and neonates

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The level of maternal circulating triacylglycerols during late pregnancy has been correlated with the mass of newborns. PPARγ (peroxisome-proliferator-activated receptor γ) ligands, such as TZDs (thiazolidinediones), have been shown to reduce triacylglycerolaemia and have also been implicated in the inhibition of tissue growth and the promotion of cell differentiation. Therefore TZDs might control cell proliferation during late fetal development and, by extension, body mass of pups. To investigate the response to EZ (englitazone), a TZD, on perinatal development, 0 or 50 mg of englitazone/kg of body mass was given as an oral dose to pregnant rats daily from day 16 of gestation until either day 20 for the study of their fetuses, or until day 21 of gestation for the study of neonates. EZ decreased maternal triacylglycerol levels at day 20 of gestation and neonatal mass, but not fetal mass. Fetuses and neonates from EZ-treated mothers exhibited high levels of insulin and were found to be hyperglycaemic. The apparent insulin-resistant state in neonates from EZ-treated pregnant rats was corroborated, since they showed higher plasma NEFA [non-esterified ('free') fatty acid] levels, ketonaemia and liver LPL (lipoprotein lipase) activity and lower plasma IGF-I (type 1 insulin-like growth factor) levels, in comparison with those from control mothers. Moreover, at the molecular level, an increase in Akt phosphorylation was found in the liver of neonates from EZ-treated mothers, which confirms that the insulin pathway was negatively affected. Thus the response of fetuses and neonates to maternal antidiabetic drug treatment is the opposite of what would be expected, and can be justified by the scarce amount of adipose tissue impeding a normal response to PPARγ ligands and by hyperinsulinaemia as being responsible for a major insulin-resistant condition.

Key words: englitazone, insulin, lipoprotein lipase (LPL), peroxisome-proliferator-activated receptor (PPAR), thiazolidinedione, triacylglycerol.

INTRODUCTION

PPARs (peroxisome-proliferator-activated receptors) are members of the steroid nuclear receptor superfamily, which is a large class of ligand-activated transcription factors that regulate gene expression. These receptors, after binding peroxisome proliferator compounds or diverse ligands (see below), are activated and regulate the expression of genes related to lipid metabolism (see [1] for a review), such as peroxisomal fatty acid β-oxidation, gluconeogenesis, lipid transport and ketogenesis. So far, three PPAR subtypes have been identified in rat: PPARα, PPARβ and PPARγ. The isoform α, being involved in the modulation of fatty acid oxidation, is primarily expressed in tissues that have a high level of fatty acid catabolism, such as liver. The isoform γ was initially reported for its regulatory roles in insulin sensitization and adipocyte differentiation. Furthermore, studies have shown that PPARγ plays an important role in cell proliferation and differentiation [2]. Activators of PPAR can be classified as natural substances, such as fatty acids and prostaglandins, and synthetic substances, such as the hypolipidaemic drugs, fibrates, and the antidiabetic agents, TZDs (thiazolidinediones).

Elevated plasma triacylglycerol levels have been shown to be an independent risk factor for coronary heart disease [3]. Thus fibrates, acting as hypotriacylglycerolaemic agents, have been effectively used to reduce that factor [4]. By activating PPARα in liver, fibrates increase LPL (lipoprotein lipase) activity, decrease apolipoprotein C-III, and increase acyl-CoA synthetase, fatty acid transport protein, apolipoprotein AI and AII gene expression (see [5] and references therein). Therefore fibrates regulate lipid homoeostasis by modulating fatty acid oxidation. On the other hand, TZDs have also been shown to decrease triacylglycerolaemia in rodents [6] and humans [7]. By activating PPARγ, which is mainly expressed in white adipose tissue, TZDs increase fatty acid transporters, adipocyte lipid-binding protein, phosphoenolpyruvate carboxykinase and LPL expression. Thus TZDs participate in lipid homoeostasis by increasing fatty acid accumulation and adipogenesis (see [5] and references therein).

During late pregnancy, hypertriacylglycerolaemia is consistently developed [8] as a consequence of enhanced adipose tissue lipolytic activity [9], enhanced liver production of VLDLs (very-low-density lipoproteins) [10] and decreased extrahepatic LPL activity [11]. Although treatment with hypocholesterolaemic drugs in pregnant rats has been shown to impair fetal growth [12], few studies have been undertaken to determine the effects of pharmacological reductions of circulating triacylglycerols, despite the proposed role of maternal hypertriacylglycerolaemia on fetal growth in humans [13].

In relation to the above, in a previous study [14], pregnant rats were treated for 4 days with two different doses of fenofibrate. Pregnant rats treated with 200 mg of fenofibrate/kg of body mass per day showed hypotriacylglycerolaemia during the first 2 days, followed by levels of triacylglycerols similar to those found in control pregnant rats. At such a dose, fetal body mass and triacylglycerolaemia were unaffected by fenofibrate. However, pregnant

Abbreviations used: EIA, enzyme immunoassay; EZ, englitazone; GLUT-2, glucose transporter 2; IGF-I, type 1 insulin-like growth factor; ILK, integrin-linked kinase; IRS, insulin receptor substrate; LPL, lipoprotein lipase; MAPK, mitogen-activated protein kinase; NEFA, non-esterified ('free') fatty acid; PDK1, 3-phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase; PPAR, peroxisome-proliferator-activated receptor; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TZD, thiazolidinedione; VLDL, very-low-density lipoprotein.

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rats treated with 400 mg of fenofibrate/kg of body mass per day showed hypotriacylglycerolaemia during the first 2 days, followed by intense hypertriacylglycerolaemia, even higher than those found in control pregnant rats. Curiously, those fetuses showed higher triacylglycerolaemia and lower body mass than fetuses from control pregnant rats [14]. A direct effect of the drug on the fetus and/or a response of the fetus to maternal alterations in lipoprotein metabolism were not ruled out.

Bearing in mind the proposed role of maternal hypertriacylglycerolaemia on fetal growth and that TZDs have been implicated in growth regulation and cellular differentiation, the present work was carried out to determine how treatment with EZ (englitazone), as a hypotriacylglycerolaemic agent, affects fetal growth during late pregnancy in rats.

MATERIALS AND METHODS

Animals, drug administration and collection of the samples

The experimental protocol was approved by the Animal Research Committee of the University San Pablo-CEU in Madrid, Spain. Female Sprague–Dawley rats weighing 180–210 g were mated, and day 0 of pregnancy was determined as when spermatozoids were found in vaginal smears. From day 16 of gestation, rats were given daily at 09:00 h one dose of 50 mg of EZ (kindly supply by Pfizer, Groton, CT, U.S.A.)/kg of body mass by oral gavage, suspended in 2 % (v/v) Tween 80. Controls only received the medium by oral gavage. On the morning of the 20th day of pregnancy, corresponding to 4 days of treatment, rats were killed, and blood was collected in tubes containing Na_2-EDTA. The conceptus was dissected, and, after being weighed, fetuses were counted and weighed. Fetuses were also decapitated, and blood from all pups of the same mother was collected and pooled into receptacles containing Na_2-EDTA for immediate separation of plasma at 4 °C.

Another set of pregnant rats which received the same daily treatment with EZ from day 16 of gestation for 5 days were allowed to deliver and, on the day of birth, neonates were decapitated, and blood from all pups of the same mother was collected and pooled into Na_2-EDTA-containing tubes. The livers of the neonates were dissected, and those coming from the same mother were pooled, and placed in liquid nitrogen to be stored at − 80 °C until processed for Western blot analysis as described below.

Determinations

Plasma aliquots, kept at − 30 °C, were used to measure glucose by an enzymatic colorimetric test [GOD/PAP (glucose oxidase–phenol 4-aminophenazone peroxidase) method; Roche Diagnostics, Barcelona, Spain) [15]. NEFA [non-esterified ('free') fatty acids] (Wako, Neuss, Germany), cholesterol and triacylglycerols were measured using commercial kits (Menarini, Florence, Italy). For measuring β-hydroxybutyrate levels, plasma was previously deproteinized [16] and then a fluorimetric method was used [17]. Insulin was determined in plasma samples using a specific ELISA kit for rats (Mercodia, Uppsala, Sweden); the values within the detection range of the assay being 0.07–5.5 μg of insulin/ml (1.8 % intra-assay variation; 3.8 % inter-assay variation). Leptin was assayed by ELISA in diluted plasma samples as instructed by the manufacturer, using a commercially available kit specific for rat leptin (Assay Designs, Ann Arbor, MI, U.S.A.). Leptin concentrations were within the detection range of the kit, i.e. 0.06–3.6 ng of leptin/ml (11.6 % intra-assay variation; 11.0 % inter-assay variation). IGF-I (type 1 insulin-like growth factor) was measured by a competitive-binding EIA (enzyme immunoassay) using a rat IGF-I EIA kit (DRG Diagnostics, Mountainside, NJ, U.S.A.); the values being above the minimum detection limit, i.e. 30 ng/ml (7.7 % intra-assay variation; 9.4 % inter-assay variation).

Protein extraction and immunoblotting

Frozen liver (50 mg) was powdered in liquid nitrogen in a mortar pre-cooled to − 80 °C, and lysed in an ice-cold 30 mM Hepes buffer, pH 7.4, containing 5 mM EDTA, 1 % Triton X-100, 0.5 % sodium deoxycholate and 2 mM protease inhibitor (Pefablock; Roche) for 30 min. Cellular debris was pelleted and discarded after centrifugation at 17 000 ***g*** for 30 min at 4 °C. The supernatant was collected, and the protein concentration was determined by the BCA (bicinchoninic acid) protein assay from Pierce (Rockford, IL, U.S.A.).

Protein (25 μg) from each experimental condition was subjected to SDS/7.5 % PAGE and electrophoretically transferred on to PVDF membranes (Amersham Biosciences, Barcelona, Spain). The blots were probed with primary antibodies [anti-insulin receptor and anti-GLUT-2 (glucose transporter 2) from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), anti-IRS-1 (IRS is insulin receptor substrate), anti-IRS-2, anti-PI3K (phosphoinositide 3-kinase), anti-Akt1, anti-pAkt1 (Ser^{473}) and anti-MAPK1/2 (MAPK is mitogen-activated protein kinase) from Upstate Biotechnology (Lake Placid, NY, U.S.A.), anti-PPAR-α from Chemicon (Temecula, CA, U.S.A.) and anti-β-actin from Sigma (St. Louis, MO, U.S.A.)] followed by corresponding secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized using the ECL® (enhanced chemiluminescence) system (Amersham Biosciences) and quantified by densitometry (Bio-Rad, Madrid, Spain). The intensity of each protein was corrected by the values obtained from the immunodetection of β-actin.

LPL activity

LPL activity was measured in acetone powders from frozen liver aliquots by the method described previously [18].

Expression of results and statistical evaluation

Results are expressed as means ± S.E.M. for four to ten animals/group. Data were analysed for homogeneity of variance with the Levene test. Values were log-transformed to equalize the variance between conditions. Statistical comparisons between two groups were made using Student's t test with 95 % confidence limits using the SPSS program (version 9.0.1).

RESULTS AND DISCUSSION

Maternal treatment with EZ reduces neonatal body mass and insulin sensitivity in fetuses and neonates

As shown in Table 1, EZ at similar doses as used by others [19,20] and administered for 4 days, effectively decreased plasma triacylglycerolaemia in 20-day-pregnant rats. In contrast, EZ did not produce any effect in fetal body mass, but produced a significant decrease in neonatal body mass (Table 1). Thus, in late pregnancy, EZ-treated pregnant rats showed lower circulating triacylglycerols and reduced mass in newborns than found in the corresponding control mothers. Thus the pharmacological reduction of triacylglycerolaemia by TZD in late gestation is associated with a diminution in the mass of neonates, in accordance with previous studies carried out under both physiological

Table 1 Effect of EZ on maternal triacylglycerolaemia and body mass of fetuses and neonates

Plasma triacylglycerol (TG) levels in rats at day 20 of gestation receiving medium (control) or EZ for 4 days. Body mass of fetuses and neonates from mothers treated with the medium or EZ. Values are means ± S.E.M.; $n = 4$–10 for mothers and litters, and $n = 80$–100 for fetuses and neonates. Statistically significant differences between groups receiving different treatments are indicated (*, $P < 0.05$; **, $P < 0.01$).

	Control	EZ-treated
Maternal TG (mg/dl)	212.35 ± 28.61	113.53 ± 20.77*
Fetal (day 20) body mass (g)	4.24 ± 0.08	4.15 ± 0.05
Neonatal body mass (g)	6.62 ± 0.06	6.16 ± 0.04**
Size of litter (number)	12.12 ± 0.65	12.20 ± 0.58

and pathological conditions [13]. Nevertheless, since pioglitazone and rosiglitazone have been shown to cross the placenta [21], EZ is also thought to do the same. Therefore a direct drug effect cannot be discarded, and, in fact, retarded fetal development and impaired postnatal growth in rats have already been described for pioglitazone and rosiglitazone [21].

In late gestation, maternal glycaemia is a predominant factor regulating fetal growth [22]. In accordance with this, it has also been shown that fetal glucose is related directly to maternal glucose under both physiological (fasted, after meal) and pathological situations (diabetes) [23,24]. The fetal glucose level is lower than the maternal one, and so, by a positive gradient, the net flux of glucose is from the mother to the fetus [25]. Thus, since EZ is considered to be an insulin-sensitizer in tissues [20], it could be conjectured that EZ-treated pregnant rats would preferentially transfer glucose from the mother to the fetus through the placenta, and, consequently, the fetal pancreas would overproduce insulin to maintain normoglucaemia. However, EZ produced no effect in maternal plasma glucose when administered to pregnant rats (112.2 ± 5.5 and 116.5 ± 12.6 mg/dl for control and EZ-treated rats respectively). Accordingly, fetuses from EZ-treated pregnant rats showed similar plasma glucose levels to those observed in control rats (Figure 1A). However, as shown in Figure 1(A), neonates from treated mothers were clearly hyperglycaemic as compared with those from control pregnant rats. Surprisingly, as shown in Figure 1(B), fetuses and neonates from EZ-treated mothers showed higher levels of insulin than their respective controls, despite receiving an antidiabetic drug. These findings indicate a higher insulin secretion by the fetal pancreas in order to maintain the normoglycaemia, suggesting a decreased insulin sensitivity in fetuses from EZ-treated mothers. After birth, a clear metabolic decompensation is manifested, and the elevated insulinaemia present in neonates from EZ-treated mothers was found to be unable to maintain normoglycaemia, and therefore neonates from EZ-treated mothers were hyperglycaemic compared with the control ones. These data seem to indicate that pups from mothers treated with the antidiabetic drug, developed an insulin-resistant condition. Thus decreased body mass observed in neonates from EZ-treated pregnant rats (Table 1) could be related to an impaired entry of glucose into cells that do not seem to respond to insulin. Although further direct studies are needed, decreased insulin sensitivity in neonates from EZ-treated mothers would enhance gluconeogenic activity [26], therefore contributing to their hyperglycaemia.

On the other hand, it has been demonstrated that in the absence of adipose tissue, the liver is a primary site for TZD action [27]. In a previous study, it was suggested that white adipose tissue is required for the antidiabetic effect of TZDs [28]. If it is assumed that EZ crosses the placenta, the fact that fetuses and

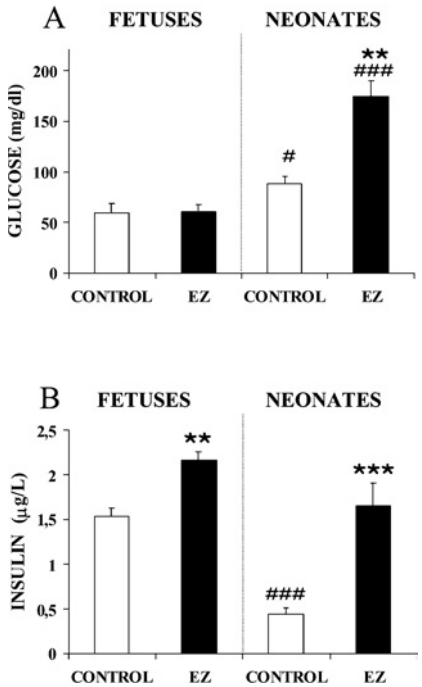

Figure 1 Effect of maternal treatment with EZ on plasma glucose and insulin of fetuses and neonates

Plasma glucose (**A**) and insulin (**B**) levels in fetuses and neonates from mothers receiving medium (control) or EZ for 4 (fetuses) or 5 days (neonates). Values are means ± S.E.M.; $n = 4$–8. Statistically significant differences between groups receiving different treatments are indicated (***, $P < 0.001$). Statistical significant differences between fetuses and neonates within each group of treatment are also indicated (#, $P < 0.05$; ###, $P < 0.001$).

neonates scarcely present adipose tissue might explain the lack of hypoglycaemic effect of EZ in pups from EZ-treated mothers. Nevertheless, a possible role of brown adipose tissue, which is more abundant in fetuses and neonates than in adults and, moreover, a target tissue for PPARγ ligands [29], cannot be discarded.

Maternal treatment with EZ increases plasma NEFA, ketonaemia and liver LPL activity in neonates

Decreased body mass observed in neonates from EZ-treated pregnant rats (Table 1) might also be related to a diminished milk intake, since neonates used in the present study had already fed when they were killed. However, it has been reported that PPARγ activation increases food intake [30]. In order to investigate such a possibility, plasma lipid parameters were determined in neonates and fetuses. As shown in Table 2, triacylglycerolaemia did not change in fetuses and neonates from EZ-treated mothers in comparison with those from control pregnant rats. In both cases, triacylglycerolaemia increased in the transition from the fetal to the neonatal state (Table 2). Since plasma triacylglycerol concentration had been reported previously [31] to increase during the first hours of life in newborns only if they were fed, the increase in plasma triacylglycerols in the same proportion in both groups (approx. 45 %) would indicate that milk intake was also similar in neonates from EZ-treated mothers and in control rats. On the other hand, it is known that newborn rats mobilize their triacylglycerol stores immediately after birth. Although the body fat content in rats at birth is very low, the lipolytic effect produced by the fall in plasma insulin concentration produces an increase in plasma NEFAs [32]. Accordingly, as shown in Table 2, plasma NEFAs increased after birth in pups from control mothers and,

Table 2 Effect of maternal treatment with EZ on plasma lipids of fetuses and neonates

Plasma triacylglycerol (TG), NEFA and ketone body levels in fetuses and neonates from mothers receiving medium (control) or EZ for 4 (fetuses) or 5 days (neonates). Values are means ± S.E.M.; $n = 4$–10. Statistically significant differences between groups receiving different treatments are indicated (**, $P < 0.01$). Statistically significant differences between fetuses and neonates within each group of treatments are also indicated ($^{\#}$, $P < 0.05$; $^{\#\#\#}$, $P < 0.001$).

	Fetus		Neonate	
	Control	EZ-treated	Control	EZ-treated
TG (mg/dl)	69.43 ± 4.23	81.86 ± 4.14	101.52 ± 16.34$^{\#}$	118.34 ± 16.12
NEFA (μM)	195.14 ± 16.28	181.07 ± 20.60	390.00 ± 49.05$^{\#\#\#}$	545.72 ± 51.27**$^{\#\#\#}$
Ketone bodies (μM)	94.55 ± 9.77	119.59 ± 13.25	627.42 ± 174.34$^{\#}$	908.37 ± 145.07$^{\#\#\#}$

surprisingly, even more so in those from EZ-treated mothers in spite of insulinaemia (Figure 1B). Given the well-known anti-lipolytic effect of insulin [33], this finding emphasizes further the insulin-resistant condition of the neonates from EZ-treated mothers. Interestingly, a trend similar to that reported for NEFA was also found in plasma ketone bodies (Table 2) and, although the difference between the two groups did not become significant, this finding indicated an elevated hepatic ketogenesis in neonates from EZ-treated mothers, even higher than the augmented ketogenesis typically found in control neonates after birth [34]. In relation to that, recent data indicated that prolonged exposure to elevated ketone body concentration impaired insulin-stimulated glucose uptake [35].

In previous studies, circulating insulin and LPL activity showed inverse correlations in fed neonates [31]. Curiously, LPL activity increased significantly in the liver of neonates from EZ-treated mothers compared with neonates from control pregnant rats (3.59 ± 0.82 and 11.32 ± 2.01 pkat/mg of protein for control and EZ-treated rats respectively; $P < 0.01$). Thus insulinaemia and LPL activity were not inversely interrelated in neonates from EZ-treated rats, again suggesting an insulin-resistant condition. Hepatic triacylglycerol content remained unchanged in neonates from treated mothers in comparison with those from control pregnant rats (results not shown). Thus an enhanced LPL activity in neonates from EZ-treated mothers would be facilitating the uptake of fatty acids, derived from plasma triacylglycerols contained in circulating triacylglycerol-rich lipoproteins, for their oxidation rather than for deposit. Thus the fatty acid entry into hepatocytes favoured by an EZ-stimulated LPL activity along with an activated gluconeogenesis, as suggested above, would potentiate ketone body production in neonates from EZ-treated mothers (Table 2). In a previous report using transgenic mice expressing LPL exclusively in liver, it was postulated that liver LPL expression at times of metabolic stress, such as the perinatal period, shunts circulating triacylglycerol to the liver to provide more energy for liver-specific functions such as VLDL and ketone body production, and subsequently spares glucose [36]. If that situation was occurring in neonates from EZ-treated mothers, it could contribute to their hyperglycaemia (Figure 1A) and explain the non-effectiveness of the drug on their triacylglycerolaemia, despite having activated hepatic LPL.

Maternal treatment with EZ reduces plasma IGF-1 in neonates

A further explanation for the changes discussed above is that it could be related to a higher milk intake in neonates from EZ-treated mothers, which would provide plasma with triacylglycerols, and so compensate the effect of activated LPL in the liver. Since leptin has been proposed as a factor regulating food ingestion [37], even in the early stages of life, the levels of this hormone were also measured. As shown in Table 3, leptin levels were similar between the two groups, in both fetuses and neonates. Thus, in spite of reports that PPARγ ligands inhibit leptin expression [38], the levels of this hormone did not change in fetuses from EZ-treated mothers compared with those from control rats. Furthermore, as the main source for plasma leptin in neonates is maternal milk [37], and as no change was found in leptin levels between neonates from EZ-treated mothers and those from the control, this would confirm there were no differences in food intake between pups from treated and control animals. On the other hand, leptin has been proposed as a mitogenic factor able to regulate intrauterine growth [39]. However, in the present study, plasma leptin was unaffected by treatment with EZ in neonates (Table 3), despite the neonates from EZ-treated mothers showing significantly lower body mass (Table 1).

Table 3 Effect of maternal treatment with EZ on plasma IGF-1 and leptin of fetuses and neonates

Plasma leptin and IGF-1 levels in fetuses and neonates from mothers receiving medium (control) or EZ for 4 (fetuses) or 5 days (neonates). Values are means ± S.E.M.; $n = 4$–10. Statistically significant differences between groups receiving different treatments are indicated (*, $P < 0.05$). Statistically significant differences between fetuses and neonates within each group of treatments are also indicated ($^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$).

	Fetus		Neonate	
	Control	EZ-treated	Control	EZ-treated
Leptin (ng/ml)	5.34 ± 0.56	5.03 ± 0.59	2.71 ± 0.54$^{\#\#}$	2.78 ± 0.64$^{\#}$
IGF-1 (ng/ml)	67.67 ± 16.03	47.79 ± 9.80	111.26 ± 7.97$^{\#}$	78.99 ± 9.78*

The decreased body mass could be connected to a diminished level of plasma IGF-I observed in pups from EZ-treated mothers (Table 3), since it has been postulated that this factor promotes growth and differentiation in a variety of tissues [40]. Thus, as shown in Table 3, the plasma IGF-I of fetuses from EZ-treated mothers showed a trend to decrease that was confirmed and became significant in neonates. Thus plasma IGF-I levels are significantly lower in the neonates from mothers treated for 5 days with EZ than in pups from control rats (Table 3). Several studies have previously shown the importance of plasma IGF-I in body size at birth (see [41], and references therein). Related to this, in a previous study, the specific deletion of the gene encoding IGF-I in murine liver [42] produced transgenic mice with a marked reduction in circulating IGF-I levels, which were insulin-resistant and hyperinsulinaemic [41]. It is also interesting to note that the most important regulator of fetal IGF-I concentrations is insulin [43], the secretion of which may be enhanced by adequate glucose transfer across the placenta [41]. However, in the present study, fetuses and neonates from EZ-treated pregnant rats showed elevated insulinaemia, but their IGF-1 levels were reduced in comparison with neonates from control mothers (Table 3), which agreed with the insulin-resistant condition in the offspring from

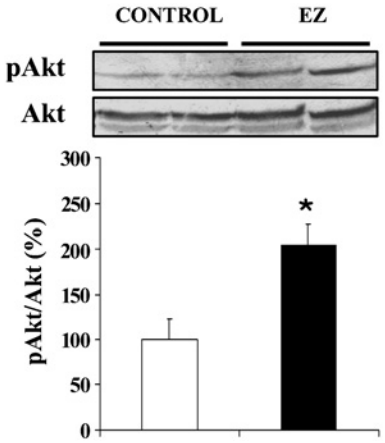

Figure 2 Effect of maternal treatment with EZ on Akt phosphorylation of liver of neonates

Basal Akt phosphorylation in liver of neonates from mothers receiving medium (control) or EZ. The autoradiographs shown identify the hepatic Akt protein (lower part) and the corresponding (Ser^{473})-phosphorylated Akt (upper part). Autoradiographs were quantified by scanning densitometry, and so the Figure represents the signal due to the phosphorylation corrected by the Akt protein determined by Western immunoblotting, as described in the Materials and methods section. Results are means ± S.E.M for four animals per group. Statistically significant differences between groups receiving different treatments are indicated (*, $P < 0.05$).

EZ-treated mothers. Consequently, if EZ actually crossed the placenta, the present results would be in accord with the capability of PPARγ ligands to reduce IGF-1 concentrations, as suggested previously by Stoll [44]. Recently, however, it has been shown that, at least in white adipose tissue, IGF-1 is not a direct target gene of PPARγ [45].

Maternal treatment with EZ increases basal Akt phosphorylation in liver of neonates

In order to understand the molecular events that induce the insulin-resistant state observed in the neonates from EZ-treated mothers, the insulin signalling pathway was determined. Thus the hepatic expression of insulin receptor, PI3K, IRS-1, IRS-2, MAPK, and its phosphorylation, GLUT-2 and PPARα were determined, and no differences were found between neonates from control mothers and pups from EZ-treated mothers (results not shown). However, as shown in Figure 2, a clear and significant increase of Akt phosphorylation in liver of neonates from EZ-treated mothers was found compared with those from control pregnant rats, confirming a notable change in the mitogenic insulin/IGF-I pathway. This constitutively active form of Akt found in the liver of neonates from mothers receiving EZ might be related to their hyperinsulinaemic state (Figure 1). In fact, this enhanced phosphorylation of Akt found in basal conditions in neonates from EZ-treated mothers could prevent further phosphorylation of the enzyme in response to insulin, and therefore it could act as a negative-control mechanism of that pathway. This would explain why these animals, although having elevated insulin levels, were hyperglycaemic. Previous reports, where constitutive activation of Akt has been produced by mechanisms that mimic insulin actions, i.e. overexpression of PI3K catalytic subunit, have also shown insulin resistance [46]. Contrary to this, several reports have shown that TZDs potentiate insulin signalling by acting at the Akt phosphorylation level [47,48]. In that sense, it has been reported that, in human macrophages, PPARγ agonists produced a reduction in the amount of phosphorylated Akt that correlated with an increase on PTEN (phosphatase and tensin homologue deleted on chromosome 10) levels [49]. However, PPARβ agonists in keratinocytes were shown to up-regulate ILK (integrin-linked kinase) and PDK1 (3-phosphoinositide-dependent kinase-1) directly and to down-regulate PTEN, leading to activation of Akt [50]. In the present study, hepatic levels of PTEN, ILK and PDK1 were not different between neonates from EZ-treated and control mothers (results not shown). If all these observations are valid for neonates, it would indicate that the lack of response to insulin found in neonates from EZ-treated mothers would be due to the hyperinsulinaemia itself rather than because of a direct effect of the drug. Moreover, since it has been reported in culture cells that elevated levels of ketone bodies did not modify basal Akt phosphorylation [35], the constitutive activation of Akt in the liver of neonates from EZ-treated mothers might discard hyperketonaemia as responsible for the insulin-resistant state found in these animals. Eventually, EZ is an insulin-sensitizer agent, and maternal insulin resistance is necessary in pregnancy to guarantee a correct nutrient supply from the mother to the fetus [8]. Therefore EZ could be exerting transient reductions on maternal glycaemia which would produce perturbations in fetal development (Table 1), in addition to increases in the plasma insulin level, each time more prolonged (Figure 1B).

In conclusion, the TZD-induced reduction in maternal triacylglycerolaemia could contribute to the decreased neonatal mass. Nevertheless, a diminution in the nutrient supply does not appear to be implicated. In fact, pups from EZ-treated mothers present adequate amounts of fuels, such as glucose, NEFAs and ketone bodies in plasma to support their regular growth. Accordingly, since it is well-known that insulin and IGF-1 are important mitogenic factors in fetal growth, it is proposed that the retarded growth observed here in pups from EZ-treated mothers is mainly related to both their reduced levels of IGF-I and their insulin-resistance state. Moreover, the constitutive Akt phosphorylation observed in the liver of neonates from EZ-treated mothers, would be sufficient to generate the above-mentioned insulin resistance state. Finally, although PPARγ ligands have been shown to produce antidiabetic effects, the scarcity of adipose tissue present in rat neonates would have impeded EZ to exert its antidiabetic effects.

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