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

Julio SEVILLANO, Inmaculada C. LÓPEZ-PÉREZ, Emilio HERRERA, María del PILAR RAMOS and Carlos BOCOS

Facultad de Farmacia, Universidad San Pablo-CEU, Montepríncipe, Ctra. Boadilla del Monte Km 5.300, E-28668 Boadilla del Monte (Madrid), Spain

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 peroxisomal 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, glucose-neogenesis, lipid transport and ketogenesis. So far, three PPAR subtypes have been identified in rat: PPARα, PPARβ and PPARγ. The isofrom 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 isofrom 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, phosphoethanolpyruvate 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 hypolipidaemic 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; PKC, phosphokinase C-kinase; PPAR, peroxisome-proliferator-activated receptor; PTEN, phosphatase and tensin homologue deleted on chromosome 10; TZD, thiazolidinedione; VLDL, very-low-density lipoprotein.

1 To whom correspondence should be addressed (email carbocos@ceu.es).
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 spermatozoa 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 supplied 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 Na2-EDTA for immediate separation 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.

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. Therefore, the use of TZD may be contraindicated in late pregnancy, because of the risk of newborn hypoglycaemia and hypotriacylglycerolaemia.
Table 1  Effect of EZ on maternal triacylglycerolaemia and body mass of fetuses and neonates

<table>
<thead>
<tr>
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<th>Control</th>
<th>EZ-treated</th>
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<tbody>
<tr>
<td>Maternal TG (mg/dl)</td>
<td>212.35±28.61</td>
<td>113.53±29.77*</td>
</tr>
<tr>
<td>Fetal (day 20) body mass (g)</td>
<td>4.24±0.08</td>
<td>4.15±0.05</td>
</tr>
<tr>
<td>Neonatal body mass (g)</td>
<td>6.62±0.06</td>
<td>6.16±0.04**</td>
</tr>
<tr>
<td>Size of litter (number)</td>
<td>12.12±0.65</td>
<td>12.20±0.58</td>
</tr>
</tbody>
</table>

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 normoglycaemia. 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 1A, neonates from control mothers were clearly hyperglycaemic as compared with those from EZ-treated pregnant rats. Surprisingly, as shown in Figure 1B, 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 insulinemia 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 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.

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<0.01). Statistical significant differences between fetuses and neonates within each group of treatment are also indicated (#, P<0.05; ###, P<0.001).

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,
surprisingly, even more so in those from EZ-treated mothers in spite of insulinemia (Figure 1B). Given the well-known antilipolytic 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 insulinemia 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).

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 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 hyperinsulinemic [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 insulinemia, but their IGF-I levels were reduced in comparison with neonates from control mothers (Table 3), which agreed with the insulin-resistant condition in the offspring from

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

<table>
<thead>
<tr>
<th></th>
<th>Fetus Control</th>
<th>EZ-treated</th>
<th>Neonate Control</th>
<th>EZ-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (mg/dl)</td>
<td>69.43±4.23</td>
<td>81.86±4.14</td>
<td>101.52±16.34</td>
<td>118.34±16.12</td>
</tr>
<tr>
<td>NEFA (µM)</td>
<td>195.14±16.28</td>
<td>181.07±20.60</td>
<td>390.00±49.06***</td>
<td>545.72±51.27****</td>
</tr>
<tr>
<td>Ketone bodies (µM)</td>
<td>94.55±9.77</td>
<td>119.59±13.25</td>
<td>627.42±174.34^4</td>
<td>908.37±145.07***</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>Fetus Control</th>
<th>EZ-treated</th>
<th>Neonate Control</th>
<th>EZ-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin (ng/ml)</td>
<td>5.34±0.56</td>
<td>5.03±0.59</td>
<td>2.71±0.54***</td>
<td>2.78±0.64^4</td>
</tr>
<tr>
<td>IGF-1 (ng/ml)</td>
<td>67.67±16.03</td>
<td>47.79±9.80</td>
<td>111.26±7.97^4</td>
<td>78.99±9.78*</td>
</tr>
</tbody>
</table>
significant differences between groups receiving different treatments are indicated (*, P < 0.05) in the 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 PPARɛ 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-1 pathway. This constitutively active form of Akt found in the liver of neonates from mothers receiving EZ might be related 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 triacyl-glycerolaemia 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-1 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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