# Englitazone Delays Fetal Growth in Late Gestation in the Rat

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## Summary

The mechanisms regulating hepatocyte proliferation are relevant to liver development, carcinogenesis, and regeneration. Studies of hepatocyte proliferation control during late foetal and postnatal development have been used as a model to understand such mechanisms. Since peroxisome proliferator-activated receptor gamma (PPARγ) ligands have been implicated in the inhibition of growth and differentiation of certain human cancers, in the present study, we have investigated the effect of englitazone (EG), a PPARγ ligand, on foetal and postnatal development. Our results indicate that, EG administered semi-chronically to pregnant rats, produced a body weight reduction on the progeny. This effect may be related to the diminished level of plasma IGF-I found in the neonates from treated-mothers. Surprisingly, despite receiving an anti-diabetic drug, foetus and neonates showed high levels of insulin, and were hyperglycemic. The plasma levels of leptin, other putative mitogenic factor, were not affected by the treatment. In the liver of neonates from mothers receiving EG, the expression of PPARα, IR, PI3K and IRS-1 was unchanged, as was the phosphorylation of MAPK. Nevertheless, an increase on Akt phosphorylation was observed on liver of neonates from treated-mothers, confirming a remarkable change on the mitogenic insulin/IGF-I pathway. In conclusion, the growth inhibitory effect reported herein may be associated with the ability of PPARγ ligands to reduce IGF-I concentrations and produce an insulin resistance state on foetus/neonates. These data strengthen the idea that PPARγ ligands have potential benefits on cancer treatment.

## Introduction

The mechanisms regulating hepatocyte proliferation are relevant to liver development, carcinogenesis, and regeneration. Hepatocyte proliferation during late foetal and postnatal development has been used as a model to understand the mitogenic signalling pathways involved in such mechanisms (1). PPARγ is a member of the steroid nuclear receptor superfamily, a large class of ligand-activated transcription

factors regulating gene expression. Their activated receptors regulate the expression of target genes after binding to peroxisome proliferator responsive elements (PPRE). Initially, PPARγ was known for its regulatory roles in insulin sensitization and adipocyte differentiation. More recent studies have shown that PPARγ has an important role in cell proliferation and cancer (2). Thus, thiazolidinediones (TZD), anti-diabetic drugs that activate PPARγ, have been implicated in the growth inhibition and differentiation of certain cancers, *i.e.*, human hepatocellular carcinoma (2). Therefore, in the present study, the effect of EG (a TZD that function as a PPARγ ligand) on foetal and postnatal development was investigated.

## 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 SD rats weighing 180-210 g were mated. Pregnancy day 0 was determined when spermatozoids were found in vaginal smears. From day 16 of gestation, rats were given a daily dose of 50 mg of EG/kg body weight, suspended in 2% Tween-80, by oral gavage, at 9.00 AM. On the morning of the 20$^{th}$ day of pregnancy, corresponding to 4 days of treatment, the rats were decapitated. The conceptus was dissected, weighed, and the fetuses counted and weighed. Fetuses were decapitated, blood was collected from all pups of the same mother, pooled into receptacles containing $Na_2$-EDTA for immediate plasma separation at 4°C. A different set of pregnant rats received the same EG treatment from day 16 of gestation for 5 days. The rats were allowed to deliver. On the day of birth, neonates were decapitated, and blood was collected as described above. Neonate livers were dissected, and those from the same mother were pooled, frozen in liquid N, and stored at -80°C until further analysis.

**Analyses.** Plasma aliquots, kept at –30° C, were used to measure glucose by an enzymatic colorimetric test (Glucose oxidase, GOD/PAP method, Roche Diagnostics, Barcelona, Spain) (3). Plasma insulin was determined using a specific ELISA kit for rats (Mercodia, Uppsala, Denmark) with a detection range of the 0.07-5.5 μg insulin/ml (1.8% intra-assay variation, 3.8% inter-assay variation). Plasma leptin was assayed by ELISA using a commercially available kit specific for rat leptin (Assay Designs, Inc., Ann Arbor, MI) with a detection range of 0.06-3.6 ng leptin/ml (11.6% intra-assay variation, 11.0% inter-assay variation). IGF-I was measured by a competitive binding enzyme immunoassay using a rat IGF-1 EIA kit (DRG Diagnostics) with a detection limit of 30 ng/ml (7.7% intra-assay variation, 9.4% inter-assay variation).

**Protein Extraction and Immunoblotting.** Aliquots (50 mg) of frozen liver were powdered in liquid N in a precooled mortar, and lysed in 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 for 30 min. Cellular debris was pelleted

and discarded by centrifugation at 17,000 g for 30 min at 4 °C. Supernatant fractions were collected and their protein concentration determined by the BCA protein assay. Aliquots (25 μg of protein) from each experimental condition were subjected to 7.5% SDS-PAGE, and electrophoretically transferred to PVDF membranes. The blots were probed with primary antibodies: Anti-insulin receptor -GLUT-2, -IRS-1, -IRS-2; -PI3K, -Akt1, -p Akt1, -MAPK1/2, PPAR-α, and -β-actin, followed by corresponding secondary antibodies conjugated with horseradish peroxidase. Immunoreactive bands were visualized using the enhanced chemiluminescence system (ECL), and quantified by densitometry. The intensity of the proteins studied was corrected by the values obtained from the immunodetection of β-actin.

**Statistical Evaluation.** The results are expressed as the mean ± SEM of 4-10 animals/group. The data were analyzed for homogeneity of variance with Levene test. Values were log-transformed to equalize the variance between conditions. Statistical comparisons between two groups were made using the Student *t* test with 95% confidence limits (4) using the SPSS program (version 9.0.1).

## Results

EG semi-chronically administered to pregnant rats induced a significant decrease in neonatal body weight (Figure 1). Retarded fetal development and impaired postnatal growth in rats have been described for pioglitazone and rosiglitazone (5), using doses similar to the one used herein. Since it has been postulated that IGF-I promotes growth and differentiation in a variety of tissues (6), we determine whether the decreased body weight observed in neonates (Figure 1) may be related to a decreased IGF-I plasma levels in fetuses and neonates.

As shown in Figure 2, foetal plasma IGF-I from EG-treated mothers showed a decrease trend that was confirmed in the EG-treated neonates. Plasma IGF-I was significantly lower in the neonates from mothers treated for five days with EG, than in pups from control rats (Figure 2). Several authors have previously reported the importance of plasma IGF-I in body size at birth (7, and references therein).

Surprisingly, as shown Table 1, despite treatment with an antidiabetic drug, fetuses and neonates from mothers receiving EG showed higher levels of plasma insulin than their respective controls, and the neonates were hyperglycemic. These data suggests that pups from EG-treated mothers have developed an insulin resistant condition. In a recent report, the specific deletion of the gene encoding IGF-I in murine liver (8), produced transgenic mice with a marked reduction in circulating IGF-I levels, insulin resistant, and hyperinsulinaemic (7).

Leptin is a putative mitogenic factor that regulates intrauterine growth (9). However, in the present study fetus and neonate plasma leptin levels were not altered by EG treatment (Table 1), although the neonates from treated-mothers showed significantly lower body weight (Figure 1).

**Figure 1.** Average body weight of fetuses and neonates from mothers receiving medium (Control) or englitazone (EZ) for four (fetuses) or five days (neonates). Values represent the mean ± SEM, n = 80-100. Statistical comparisons between groups receiving different treatment are shown by asterisks (***, $p < 0.001$).

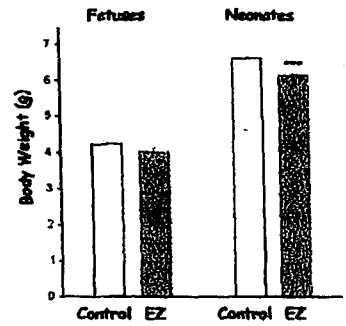


**Figure 2.** Plasma IGF-I levels in fetuses and neonates from mothers receiving medium (Control) or englitazone (EZ) for four (fetuses) or five days (neonates). Values represent the mean ± SEM, n = 4-8. Statistical comparisons between groups receiving different treatment are shown by an asterisk (*, $p < 0.05$).

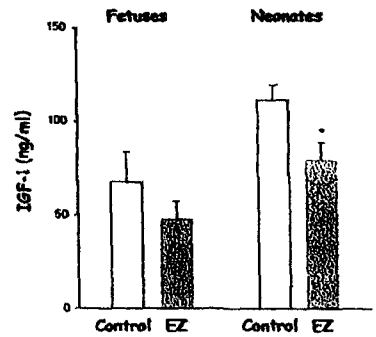


**Table 1.** Plasma Glucose, Insulin and Leptin Levels in Fetuses and Neonates from Mothers Receiving Medium (Control) or Englitazone for Four (Fetuses) or Five Days (Neonates)[1].

| | FETUSES | | NEONATES | |
|---|---|---|---|---|
| | Control | Englitazone | Control | Englitazone |
| Glucose (mg/dl) | 59.45 ± 9.66 | 60.53 ± 7.18 | 88.33 ± 7.8 | 174.02 ± 15.66[2] |
| Insulin (μg/L) | 1.53 ± 0.10 | 2.16 ± 0.101[2] | 0.44 ± 0.07 | 1.65 ± 0.26[3] |
| Leptin (ng/ml) | 5.34 ± 0.56 | 5.03 ± 0.59 | 2.71 ± 0.54 | 2.78 ± 0.64 |

[1] Values represent the mean ± SEM, n = 4-10

[2] Statistically significant, $p < 0.001$ [3] Statistically significant, $p < 0.002$

In order to understand the molecular events inducing the insulin resistant state observed in the neonates from EG-treated mothers, we considered the possibility that it may be influenced by a mitogenic signaling pathway, thus, we examined the hepatic expression of IR, PI3K, IRS-1, IRS-2 and MAPK (ERK-1 and ERK-2). As shown in Figure 3, none of the levels of the proteins examined were modified by EG treatment, neither was the phosphorylation of MAPK (data not shown). Furthermore, the amount of GLUT-2, an important glucose transporter in the liver, was also

unaltered by the EG treatment (Figure 3). In addition, we analyzed the hepatic expression of PPARα, a transcription factor related to mitogenic processes (1), on neonates from mothers receiving EG. No significant changes were observed (Figure 3).

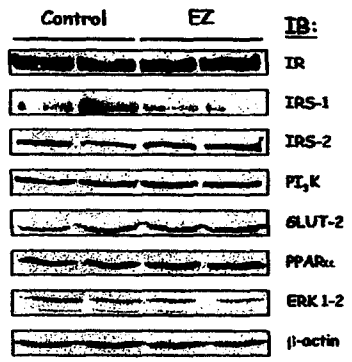


**Figure 3.** Different insulin- and mitogenic-signaling proteins in liver of neonates from mothers receiving medium (Control) or englitazone (EZ). PPARα, IR, PI3K, IRS-1, IRS-2, MAPK (ERK-1 and ERK-2), and GLUT-2 proteins were determined by Western immunoblotting (IB), as described in Materials and Methods. Detection of β-actin was used as a control. Representative autoradiographs from seven animals per group.

Interestingly, as shown in Figure 4, an increase on hepatic Akt phosphorylation in neonates from EG-treated mothers was observed, confirming a remarkable change on the mitogenic insulin/IGF-I pathway. This constitutively active form of Akt found in the liver of neonates from mothers receiving EG may be related to their hyperinsulinemic state (Table 1). This basal phosphorylation of Akt may prevent further phosphorylation of the enzyme in response to insulin, thus acting as a negative control mechanism. In fact, although these animals have elevated insulin levels, they were hyperglycemic.

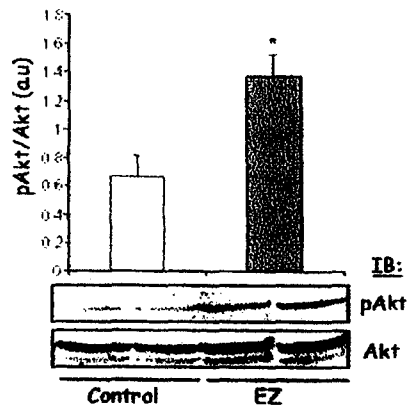


**Figure 4.** Basal Akt phosphorylation in liver of neonates from mothers receiving medium (Control) or englitazone (EZ). The autoradiographs identify the hepatic Akt protein (lower lane) and the corresponding (Ser473)-phosphorylated Akt (upper lane). The data represent the signal due to the phosphorylation corrected by the Akt protein determined by Western immunoblotting. Data represent the mean ± SEM of four animals per group. Statistical comparisons between groups receiving different treatment are shown by asterisks (*, $p < 0.05$).

## Conclusions

The growth inhibition reported herein in the pups from mothers receiving EG during late gestation may be associated with their reduced IGF-1 levels. Actually, the

capability of PPARγ ligands to reduce IGF-1 concentration, has been suggested by Stoll (10). Furthermore, since in the late foetal and postnatal development, the cellular proliferation take great importance, a direct role of PPARγ, which activation has been shown to inhibit cell proliferation and induce differentiation (11), cannot be discarded. Thus, these results may be related to the fact recently reported that acromegalic patients with reduced expression of PPARγ in the colonic mucosa and increased serum IGF-1 levels, present an increased prevalence of colonic polyps (12).

Nevertheless, the retarded growth of the pups from the mothers treated with EG, might be also related to their insulin resistance state, since it is well known that insulin is an important mitogenic factor in the foetal growth (13). This possibility, along with the highest Akt phosphorylation observed in liver of neonates from treated mothers, deserve further study.

Altogether, these data strengthen the idea that PPARγ ligands have potential benefits on cancer treatment, and represent an example of how PPAR γ and IGF-1 are involved in processes related to cell proliferation and cancer (11, 12).

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