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Troglitazone does not protect rat pancreatic β cells against free fatty acid-induced cytotoxicity

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Abstract

Thiazolidinediones are a novel class of antidiabetic drugs that reduce insulin resistance through interaction with nuclear peroxisome proliferator-activated receptor (PPAR)γ. One of these agents, troglitazone, was also proposed to protect β cells against FFA-induced toxicity, but this effect has not yet been directly demonstrated. We recently reported *in vitro* conditions under which free fatty acids (FFA) cause β cell death by necrosis or apoptosis. The present study investigates whether troglitazone (10 μM) interferes with this FFA-induced toxicity. Addition of this compound did not protect against oleate- or palmitate-induced toxicity. On the contrary, it increased palmitate-induced necrosis during the first two days of culture, and elevated (increase by 10–20%, $P < 0.05$) both oleate- and palmitate-induced apoptosis after 8 days. These results do not support the view that troglitazone exerts a direct protective effect on β cells that are exposed to cytotoxic FFA concentrations. They instead indicate that the agent may sensitize pancreatic β cells to FFA-induced damage, raising the possibility that its use facilitates the deleterious effect of increased FFA levels on the pancreatic β cell mass.

Keywords: Pancreatic β cells; Islets of Langerhans; Free fatty acids; Lipotoxicity; Thiazolidinediones; Troglitazone; Diabetes

1. Introduction

Type 2 diabetes is characterized by a state of insulin-resistance associated with inadequate pancreatic β cell function [1,2]. Among the drugs that are currently used in the treatment of type 2 diabetes are the thiazolidinediones, which improve peripheral insulin sensitivity. This novel class of antidiabetic agents interacts with the nuclear receptor PPARγ [3]. This action mechanism may also allow thiazolidinediones to improve β cell functions [4,5], independent of their effects at the level of insulin sensitivity. PPARα, -β, and -γ receptors are expressed in rodent [6,7] and human [8] pancreatic islet cells, but have not yet been categorized for the different islet cell-types. In other tissues, PPARα and -γ regulate the uptake, oxidation and/or esterification of FFA [9,10]; the function of ubiquitously expressed PPARβ is not yet identified. In pancreatic β cells, the type and role of PPAR remains to be defined but experiments with the PPARγ ligand troglitazone suggest its potential significance as a target in β cell therapy. This particular ligand was shown to prevent the development of diabetes in Zucker diabetic fatty rats, a model for obesity-induced type 2 diabetes [11,12]. It lowered the triglyceride content of pancreatic islets isolated from both lean and obese Zucker rats by increasing their oxidation of FFA and slightly decreasing their esterification [13]. These observations led to the suggestion that troglitazone can restore β cell functions by exerting a direct protective effect on β cells, in particular against damage caused by their exposure to high levels of FFA [13].

Using a direct cytotoxicity assay, we have recently found that FFA are also cytotoxic to normal rat islet β cells, but the FFA toxicity appeared inversely related to the cellular triglyceride accumulation [14]. In view of these observations, a reduction of triglyceride formation in β cells—as postulated for troglitazone [13]—may not be cytoprotective for normal pancreatic β cells. We, therefore, examined whether troglitazone really exerts a cytoprotective effect on β cells exposed to cytotoxic FFA concentrations.

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Abbreviations: FFA, free fatty acid; PPAR, peroxisome proliferator-activated receptor; UCP, uncoupling protein.

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2. Materials and methods

2.1. Purification and culture of rat β cells

Adult male Wistar rats were housed according to the guidelines of the Belgian Regulations for Animal Care. The protocol was approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel. Rats were sedated and killed with CO_2 followed by decapitation. Pancreatic islets were isolated by collagenase digestion and dissociated into single cells in calcium-free medium containing trypsin and DNAse [15]. Single β cells (more than 90% pure) were purified by autofluorescence-activated sorting using cellular light-scatter and FAD-autofluorescence as discriminating parameters [15]. For viability testing, single cell preparations were cultured in polylysine-coated microtiter plates in Ham's F10 medium containing 6 or 10 mmol/L glucose, 1% BSA pre-treated with charcoal (fraction V, RIA grade, Sigma), 2 mmol/L L-glutamine, 50 μmol/L 3-*iso*butyl-1-methylxanthine, 0.075 mg/mL penicillin and 0.1 mg/mL streptomycin [16,17]. Palmitate and oleate (sodium salt, Sigma) were added after solubilization in 95% ethanol and heating up to 60° (1:100 dilution in culture medium). Troglitazone (Sankyo) was dissolved in the same solvent. Control conditions contained similar dilutions of ethanol, which did not affect β cell viability.

After 48 hr or 8 days of culture with or without these fatty acids, the percent living cells was counted after staining with neutral red [17]. The mode of cell death was determined by a Hoechst 33342 propidium iodide fluorescent assay, which distinguishes the percent dead cells with or without a fragmented nucleus, thus quantifying the percent of apoptotic and necrotic cells [16].

2.2. Data analysis

The cytotoxicity of free fatty acids was calculated from the percent dead (neutral red negative, NRneg) cells counted in the FFA-containing condition x and in the corresponding control c [18] by the following formula:

$$\text{Cytotoxicity}\,(x) = \frac{\%\text{NRneg}\,(x) - \%\text{NRneg}\,(c)}{100\% - \%\text{NRneg}\,(c)} \times 100\%$$

For the propidium iodide assay the cytotoxicity index for necrosis was calculated against the percent necrotic cells in the following control condition:

$$\text{Necrosis index}\,(x) = \frac{\%\text{necrosis}\,(x) - \%\text{necrosis}\,(c)}{100\% - \%\text{necrosis}\,(c)} \times 100\%$$

the apoptosis index was calculated in a similar way.

Results are presented as mean ± SEM. Data are analyzed by ANOVA, followed by group comparisons using Student's paired t-test, with correction of the P-values for multiple comparisons by the Bonferroni method [19].

Table 1
Effect of troglitazone (10 μM) on oleate- and palmitate-induced cytotoxicity in cultured β cells

	Toxicity index			
Exposure time	2 Days		8 Days	
Troglitazone (μM)	0	10	0	10
Oleate				
0.25	$10 \pm 2^{*}$	$11 \pm 3^{*}$	$17 \pm 5^{*}$	$19 \pm 3^{**}$
0.50	$21 \pm 5^{*}$	$21 \pm 3^{**}$	$39 \pm 7^{*}$	$46 \pm 7^{**}$
Palmitate				
0.25	$35 \pm 4^{**}$	$54 \pm 8^{**,\#}$	$58 \pm 6^{**}$	$79 \pm 4^{***,\#}$
0.50	$67 \pm 11^{**}$	$77 \pm 5^{***}$	$98 \pm 2^{***}$	$100 \pm 0^{***}$

β Cells were cultured with or without FFA (0.25 or 0.5 mM), in the presence or absence of troglitazone. Toxicity was evaluated at days 2 and 8 of culture in Ham's F10 medium with 10 mM glucose and 1% BSA. Data represent mean ± SEM for four experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control (without FFA added); $^{\#}P < 0.05$ vs. cells exposed to FFA in the absence of troglitazone (ANOVA, followed by Student's paired t-test with the Bonferroni correction).

3. Results and discussion

Exposure of rat β cells to the FFA (oleic or palmitic acid) resulted in concentration-dependent cell death (Table 1). When compared for equimolar concentrations, cytotoxicity was higher with palmitate than with oleate. Addition of troglitazone (10 μM) did not protect the β cells against oleate (Table 1), while it increased the toxicity of 0.25 mM palmitate ($P < 0.05$ vs. no troglitazone) (Table 1). At 0.5 mM palmitate, virtually all cells were dead in the absence and in the presence of troglitazone (Table 1). In the control condition without oleic and palmitic acid, troglitazone exerted no toxic effect over a period of 8 days ($30 \pm 3\%$ dead cells vs. $25 \pm 1\%$ in its absence; $P > 0.05$).

A Hoechst 342 propidium iodide staining [16] was used to examine the mode of FFA-induced cell death. As reported previously [14], both oleate and palmitate induced apoptosis of 23–26% β cells over an 8-day culture period (Table 2). The presence of troglitazone did not increase this percentage at 0.5 mM FFA, but lowered the FFA concentration at which the FFA cytotoxicity occurs: the apoptotic effects of oleate and palmitate were now detected at 0.25 mM instead of 0.5 mM (Table 2). Similarly, troglitazone sensitized the β cells to a mixture of oleate and palmitate, that otherwise exerts negligible cytotoxicity [14]—at 0.5 mM, this mixture induced apoptosis in the presence of the drug, but not in the absence it (Table 2).

Palmitate caused also necrosis in 32% of the β cells. This effect occurred within 48 hr and did not increase with longer culture periods (Tables 2 and 3). Addition of troglitazone also slightly increased palmitate-induced necrosis within 48 hr (Table 3).

In the present study, we could not confirm earlier reports on a protective effect of troglitazone against FFA-induced β cell death. On the contrary, we noticed a higher sensitivity

Table 2
Effect of troglitazone (10 μM) on free fatty acid-induced β cell apoptosis and necrosis after 8 days of culture

	% Apoptosis		% Necrosis	
Troglitazone (μM)	0	10	0	10
Oleate				
0.25	9 ± 3	21 ± 4$^{**,\#}$	12 ± 5	3 ± 2
0.50	23 ± 5**	32 ± 5***	15 ± 5	8 ± 4
Palmitate				
0.25	6 ± 2	25 ± 2$^{***,\#\#}$	7 ± 3	13 ± 4*
0.50	26 ± 5**	33 ± 4***	24 ± 6**	26 ± 5**
Oleate:palmitate (2:1)				
0.50	5 ± 2	16 ± 3$^{**,\#}$	6 ± 3	3 ± 2

FFA-induced β cell apoptosis and necrosis in presence or absence of troglitazone after 8 days of culture in Ham's F10 medium with 6 mM glucose and 1% BSA. Data represent mean ± SEM for six experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control (without FFA added); $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ vs. cells exposed to FFA in the absence of troglitazone (ANOVA, followed by Student's paired *t*-test with the Bonferroni correction).

of rat β cells for FFA-induced necrosis and apoptosis. It is conceivable that the use of purified β cells in our study does not mimic the processes that occur in isolated islets of Langerhans (as used in [13,20,21]), which are known to contain several other cell-types. The presence of other cell-types like macrophages or fat cells may have an influence on processes that are studied in isolated islets of Langerhans. The purpose of our work was not to investigate the possible contribution of these non-β cells but to examine the effects of FFA on β cells [14]. Other studies support the possible role of contaminating non-endocrine cells. PPARγ agonists have been shown to prevent cytokine-induced activation of intra-islet macrophages and subsequent NO-mediated β cell damage [22]; such protective effect was not found when troglitazone was examined in rat insulinoma Rin m5F cells or in normal rat β cells [23] using drug concentrations that are comparable to physiologic serum levels (≤10 μM) [24]. Because both FFA and troglitazone tightly bind to proteins, the unbound troglitazone and/or FFA concentrations in the culture medium may be higher in conditions where both were added. We have not performed the high performance frontal analysis to measure free troglitazone [25], so, we cannot exclude that the combination condition exhibits higher free drug concentrations.

Table 3
Effect of troglitazone (10 μM) on free fatty acid-induced β cell apoptosis and necrosis after 48 hr of culture

	% Apoptosis		% Necrosis	
Troglitazone (μM)	0	10	0	10
Oleate				
0.25	4 ± 2	4 ± 2	5 ± 2	5 ± 5
0.50	8 ± 3	9 ± 3	12 ± 5	12 ± 5
Palmitate				
0.25	4 ± 2	4 ± 2	9 ± 3	22 ± 4$^{*,\#}$
0.50	8 ± 2*	9 ± 3	32 ± 4**	36 ± 4**
Oleate:palmitate (2:1)				
0.50	4 ± 1	5 ± 2	12 ± 4	9 ± 3

FFA-induced β cell apoptosis and necrosis in presence or absence of troglitazone after 48 hr of culture in Ham's F10 medium with 6 mM glucose and 1% BSA. Data represent mean ± SEM for fuor experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (without FFA added); $^{\#}P < 0.05$ vs. cells exposed to FFA in the absence of troglitazone (ANOVA, followed by Student's paired *t*-test with the Bonferroni correction).

Our observation that troglitazone can promote apoptosis of β cells is consistent with earlier findings of its proapoptotic action through PPARγ activation in adipocytes [26], tumor cells [27,28], and HIT-T 15 cells, a Syrian hamster clonal β cell line [24]. The apoptotic effect in β cells was, however, not seen in lipid-free culture conditions (present observations). Rather, the combination of FFA plus troglitazone sensitized β cells to FFA cytotoxicity, which occurred *via* necrosis and apoptosis. PPARγ activation up regulates cellular uptake of FFA and could thus enhance FFA cytotoxicity. We have previously shown that the cytotoxicity of FFA in pancreatic islet cells is inversely related to their esterification. Thus, palmitate increased the triglyceride content of β cells to a lesser extent than oleate and resulted in higher cytotoxicity [14]. PPARγ activation leads to lipogenesis in adipocytes and in monocytes [29]. Overexpression of the nuclear factor in myoblasts and fibroblasts resulted in adipocyte transdifferentiation, activation of lipogenesis and storage of triglycerides [30,31]. However, PPARγ-stimulated triglyceride accumulation has not been demonstrated in other cell-types at physiological PPARγ levels. Rather, troglitazone decreased FFA metabolism in isolated hepatocytes [32] by inhibition of acylCoA synthetase and specific abolishment of triglyceride synthesis. FFA esterification also decreased in lean Zucker rat islets of Langerhans [13], but studies in isolated β cells are not yet available. A decrease in triglyceride formation in β cells by troglitazone could conceivably increase β cell death, *via* the same cellular mechanism as described for FFA alone [14]. Because troglitazone did not confer a protective effect against FFA, we did not carry out triglyceride measurements, which consume large numbers of primary β cells. Additional studies using other PPARγ ligands, such as rosiglitazone, will help elucidate whether this β cell sensitization to FFA by troglitazone is PPARγ mediated. Recent studies have implied UCP-2 in the function of pancreatic β cells [33–35]. The expression of this protein was induced by chronic exposure of INS-1 cells to FFA and resulted in impaired β cell function *via* depletion of cellular ATP [35]. Troglitazone has also been shown to increase UCP-2 expression in Zucker rat islets [36]. While most studies on pancreatic β cells deal with functional effects of UCP-2, its induction has also been associated with increased vulnerability to necrosis [37] and apoptosis [38,39] in other cell-types.

In conclusion, the present *in vitro* findings suggest that troglitazone does not protect β cells against FFA-induced cell damage. In contrast, it facilitates FFA-induced necrosis

and apoptosis in normal rat β cells. Our observations argue against a direct beneficial effect of this drug on pancreatic β cell lipotoxicity. Although restricted to *in vitro* conditions, they raise the possibility that troglitazone therapy in type 2 diabetic patients may increase FFA-induced toxicity at the level of the β cells in cases where FFA concentrations remain elevated.

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