Insulin-sparing effects of troglitazone in rat pancreatic islets

L C Bollheimer, S Troll, H Landauer, C E Wrede, J Schölmerich and R Buettner

Department of Internal Medicine I, University of Regensburg, D-93042 Regensburg, Germany

(Requests for offprints should be addressed to R Buettner; Email: roland.buettner@klinik.uni-regensburg.de)

Abstract

Thiazolidinediones (TZDs) have been suggested to act beneficially on pancreatic islet function and on beta-cell viability but data concerning direct effects on isolated islets are controversial. Therefore, we have examined parameters of pancreatic insulin and glucagon secretion and biosynthesis in TZD-exposed rat pancreatic islets under physiological glucose level conditions and under conditions of glucolipotoxicity.

Primary rat islets were incubated for 2.5 h with or without troglitazone (10 μ M) in 5.6 mM glucose (standard glucose levels) and 16.7 mM glucose (high glucose levels); a subgroup was additionally treated with oleate (200 μ M) to simulate acute glucolipotoxicity. Insulin and glucagon secretion, intracellular content and their respective mRNAs were quantified. Newly synthesized insulin was determined by pulse-labeling experiments.

Troglitazone reduced insulin secretion at standard and high glucose levels by about one-third ($P \leq 0.05$). Insulin content was decreased at 5.6 mM glucose but increased at 16.7 mM glucose by the presence of troglitazone ($P \leq 0.05$). Newly synthesized insulin mRNA and preproinsulin mRNA decreased by about 20% at standard glucose levels ($P \leq 0.05$). Glucagon secretion was augmented by troglitazone in islets under high glucose conditions by an additional 50% ($P \leq 0.05$). No clear beneficial troglitazone effects were observed under glucolipotoxic conditions.

The reduced insulin secretion and biosynthesis at standard glucose levels can be interpreted as an insulin-sparing effect. Troglitazone effects were less pronounced at high glucose alone or in combination with oleate. From a clinical point of view, these results indicate a greater benefit of troglitazone for beta-cell function in hyperinsulinemic, but normoglycemic patients with insulin resistance or early type 2 diabetes without major insulin secretion deficits and/or pronounced hyperglycemia.

Journal of Molecular Endocrinology (2003) **31**, 61–69

Introduction

Thiazolidinediones (TZDs) are widely used for the treatment of type 2 diabetes (Olefsky & Saltiel 2000, Parker 2002). As ligands of the nuclear peroxisome proliferator-activated receptor- γ (PPAR γ) they mainly affect cellular gene expression leading to an improved peripheral tissue sensitivity (Reginato & Lazar 1999). As PPAR γ is highly expressed in islets of Langerhans (Dubois *et al.* 2000), direct antidiabetic effects of TZDs on the pancreatic beta-cell are also under discussion. By using hyperglycemic and hyperlipidemic (*fa/fa*) Zucker (ZDF) rats, Unger and coworkers suggested a protective lipopenic effect of the TZD compound troglitazone against fatty acid-induced loss of

beta-cell function and cell death (Shimabukuro *et al.* 1998, Higa *et al.* 1999). While these anti-lipotoxic effects of troglitazone were supported by an *in vitro* study with fatty acid-exposed INS-1 cells (Kawai *et al.* 2002), the findings are challenged by controversial results employing isolated primary islets (Cnop *et al.* 2002). In clinical studies it has been shown that insulin levels decrease during TZD treatment (Prigeon *et al.* 1998, Buchanan *et al.* 2000), and the question remains whether this is due solely to the reduction of peripheral insulin resistance or at least partially caused by a specific TZD effect on pancreatic islet function. In rat pancreatic islets, Masuda *et al.* (1995) described a dose-dependent effect of troglitazone with a potentiation of insulin secretion at doses of

1–10 μM and a diminution at doses of 100 μM . In the pharmacologically relevant range of 5–10 μM troglitazone, we and others have observed only marginal changes in insulin release when using the clonal beta-cell lines HIT or INS-1 (Ohtani *et al.* 1998, Bollheimer *et al.* 2002, Kawai *et al.* 2002).

Taken together, data concerning immediate effects of TZDs (such as troglitazone) on rat pancreatic islets are still sparse and inconclusive. Although previous studies hint at a direct effect of TZDs on the pancreatic beta-cell of normal and free fatty acid (FFA)-exposed islets – which might partly contribute to their clinical action – this aspect of TZD pharmacodynamics has not been well elucidated. In the present study we characterize the beta-cell insulin secretion and biosynthesis as well as the alpha-cell glucagon metabolism in TZD-exposed rat pancreatic islets under standard glucose (5.6 mM) as well as high glucose (16.7 mM) and high oleate levels (200 μM pre-complexed to 0.2% w/v BSA) to simulate normoglycemic, hyperglycemic and glucolipotoxic – i.e. hyperglycemia combined with hyperlipidemia – conditions. We have chosen troglitazone as a model TZD because it was the first clinically approved drug of its class with the largest background of clinical, physiological and molecular pharmacodynamic data at hand (Parker 2002).

Materials and methods

Materials

RIAs for immunoreactive insulin (IRI) and C-peptide as well as pancreatic glucagon (IRG) were performed using respective kits from Linco Research Inc. (St Louis, MO, USA). L-[^{35}S]methionine (43.5 TBq/mmol) was from NEN (Boston, MA, USA). Pansorbin was purchased from Calbiochem (La Jolla, CA, USA). Fatty acid ultra-free BSA (fraction V), protease inhibitor cocktail (Complete), Collagenase P (1.5 U/mg), as well as all technical and chemical equipment for semiquantitative LightCycler PCR were from Roche Diagnostics (Mannheim, Germany). Reverse transcription was performed employing a kit from Promega (Madison, WI, USA). Cellulose membrane filters (MF-Millipore, 0.45 μm) and a sample manifold apparatus were from Millipore (Eschborn, Germany). All other analytical grade biochemicals were purchased

from Sigma-Aldrich (Taufkirchen, Germany) or from Merck Eurolab (Darmstadt, Germany). Troglitazone (5-(4-(6-hydroxy-2,5,7,8-tetra-methylchroman-2-ylmethoxy)benzyl)-2,4-thiazolidine-dione) was kindly provided by Sankyo (Tokyo, Japan).

Islet isolation and culture

Pancreatic islets were isolated from 200 g male Wistar rats by collagenase digestion and Histopaque–Ficoll density gradient centrifugation. The newly isolated islets were maintained for 16 h in RPMI-1640/11.2 mM glucose/10% w/v fetal bovine serum at 37 °C in a 5% CO_2 atmosphere (Bollheimer *et al.* 1998). Afterwards islets were employed for the experiments as described below. All experimental animals were housed, handled and used in accordance with the local animal rights committee's guidelines.

Concentration of troglitazone and oleate

Troglitazone was used in a standard concentration of 10 μM with dimethylsulfoxide (DMSO) as vehicle for solubilization ($c_{\text{final}}[\text{DMSO}] = 0.01\%$ v/v) (Bollheimer *et al.* 2002, Cnop *et al.* 2002, Kawai *et al.* 2002). The latter was added at the same concentration to the non-troglitazone-containing samples. Oleic acid (*cis* Δ -9 octadecenoic acid) was used in a total concentration of 200 μM pre-complexed to 0.2% w/v fatty acid ultra-free BSA (fraction V), as previously described (Wrede *et al.* 2002).

Analysis of secretion and content of IRI, C-peptide and glucagon

Groups of 35 islets were washed and transferred into 300 μl Krebs–Ringer bicarbonate buffer/16 mM Hepes (pH 7.4) at 5.6 mM glucose. After a 2 h pre-incubation islets were incubated for further 150 min at either 5.6 or 16.7 mM glucose in the presence or absence of 10 μM troglitazone. The troglitazone exposure at 16.7 mM glucose was also performed in the additional presence of 200 μM oleate. At the end, the supernatants (300 μl) were collected, centrifuged and kept frozen pending analysis by RIA for IRI, C-peptide and glucagon. The islets themselves were washed in PBS and then lysed in 300 μl detergent-containing buffer (50 mM Hepes (pH 8.0), 0.1% v/v Triton X-100 plus

protease inhibitor cocktail). After sonication and centrifugation at 10 000 *g* for 2 min to remove debris (Bollheimer *et al.* 1998), aliquots were taken for assessment of (i) lysate protein (bicinchoninic method) and (ii) the corresponding contents of IRI, C-peptide and glucagon.

Analysis of total protein and proinsulin biosynthesis

To each sample, L-[³⁵S]-methionine (0.2 nmol/1.85 MBq) was added 30 min prior to the end of exposure. To measure the rate of total protein biosynthesis, aliquots of the insular lysates were treated with 10% w/v trichloroacetic acid (TCA) to precipitate cellular protein. The precipitates were then trapped onto MF-Millipore membranes and extensively washed in 5% w/v TCA by flow through on a sample manifold apparatus prior to analysis for incorporated ³⁵S by a standard scintillation counting procedure (Itoh & Okamoto 1980). To assess the rate of proinsulin biosynthesis, aliquots of the insular lysates were subjected to immunoprecipitation against IRI (Skelly *et al.* 1998). After additional chemical precipitation by TCA, IRI was spotted onto MF-Millipore membranes and analyzed for the amount of incorporated ³⁵S.

LightCycler analysis of preproinsulin (PPI) mRNA and preproglucagon (PPG) mRNA content

Batches of ten islets were incubated for 150 min under the same conditions as described above. Afterwards cells were lysed and cytoplasmic RNA was prepared according to the method of Gough (1988). First-strand complementary DNA was synthesized by priming with arbitrary hexamers. Subsequent PCR amplification and analysis were achieved in 20 µl glass capillaries using the Roche LightCycler system with the respective software (version 3.5) and a master mix containing Taq DNA polymerase, SYBR-green I and the deoxy-ribonucleotides (Lekanne Deprez *et al.* 2002). The following primer pairs were employed in a final concentration of 1 µM for (i) analysis of PPI mRNA: 5'-TGTGGTTCTCACTTGGTGGGA-3' (sense, 91–110) and 5'-ATGCTGGTGCAGCAC TGAT-3' (antisense, 278–296); (ii) analysis of PPG mRNA: 5'-TCGTGGCTGGATTGTTTGTA-3' (sense, 17–36) and 5'-CAATGTTGTTCCGGT

TCCTC-3' (antisense, 246–265); (iii) analysis of glyceraldehyde-3-phosphate-dehydrogenase mRNA (GAPDH): 5'-TTAGCACCCCTGGCCAAGC-3' (sense, 495–511) and 5'-CTTACTCCTTGGAGGCCATG-3' (antisense, 1014–1033). For each primer combination optimal MgCl₂ concentration and annealing temperature were experimentally determined. For PPI and PPG a MgCl₂ concentration of 5 mM and an annealing temperature of 57 °C resulted in efficiencies (*E*) of 1.89 and 1.97 respectively. For GAPDH optimal conditions were given by 3 mM MgCl₂ and 56 °C annealing temperature (*E*_{GAPDH} = 1.96). The first-strand DNA-samples were amplified for 40 cycles of denaturation (95 °C for 5 s), annealing (56 or 57 °C for 5 s) and extension (72 °C for 22 s). The initial amount of the cDNA content in each sample (*N*_{sample}) was then semiquantitatively assessed by comparing the experimentally determined crossing point (*CP*_{sample}) with crossing points from subsequent dilutions of a standardized pooled islet cDNA (*CP*_{standard}) and the respective concentrations (*N*_{standard}) according to: (*N*_{sample} ÷ *N*_{standard}) = (*E*^{*CP*_{standard}} ÷ *E*^{*CP*_{sample}}).

After completion of PCR amplification, melting curve analysis (Ririe *et al.* 1997) was routinely performed for each sample to confirm primer specificity.

Statistical analysis

Data are presented as means ± s.e. of at least five independent experiments. After verification of normal distribution by the Kolmogorov–Smirnov test, statistically significant differences were analyzed using unpaired Student's *t*-test where *P* ≤ 0.05 was considered significant.

Results

Troglitazone effects on the secretory output and cellular content of IRI mainly at physiological glucose levels

Pancreatic rat islets were cultured for 2.5 h with or without 10 µM troglitazone at standard glucose (5.6 mM glucose) and high glucose (16.7 mM glucose) levels. Cumulative release of IRI was determined from the cellular supernatants, whereas the final cellular IRI content was measured from the islet lysates. The results are given in detail in

Table 1 Effects of troglitazone on insulin and glucagon secretion and content. Rat pancreatic islets were incubated with or without troglitazone (10 μ M) at standard glucose (5.6 mM, ST^-/ST^+) and high glucose (16.7 mM, HT^-/HT^+) conditions for 2.5 h. Immunoreactive insulin and pancreatic glucagon were measured in supernatants and islet lysates by specific RIA. The results are corrected for equal amounts of islet lysate protein and are depicted as means $\pm$ S.E. of six or seven independent experiments

	ST^-	ST^+	HT^-	HT^+
Insulin				
Cumulative secretion (pmol/mg protein)	68 $\pm$ 18	42 $\pm$ 6*	138 $\pm$ 22	98 $\pm$ 17*
Content (nmol/mg protein)	12.0 $\pm$ 0.8	10.2 $\pm$ 1.0*	9.9 $\pm$ 1.2	12.6 $\pm$ 1.3*
Secretion:content (%)	0.53 $\pm$ 0.13	0.46 $\pm$ 0.09	1.44 $\pm$ 0.17	0.79 $\pm$ 0.15*
Glucagon				
Cumulative secretion (pmol/mg protein)	5 $\pm$ 2	4 $\pm$ 1	3 $\pm$ 1	5 $\pm$ 1
Content (nmol/mg protein)	0.7 $\pm$ 0.1	0.611 $\pm$ 0.1	0.6 $\pm$ 0.1	0.7 $\pm$ 0.2
Secretion:content (%)	0.63 $\pm$ 0.10	0.77 $\pm$ 0.13	0.6 $\pm$ 0.10	0.69 $\pm$ 0.10

* $P < 0.05$ compared with untreated controls at equal glucose levels.

Table 1. Untreated islets maintained at standard glucose levels (ST^-) released 68 $\pm$ 18 pmol insulin/mg lysate protein into the supernatant. Addition of troglitazone (ST^+) significantly reduced insulin secretion by 38 $\pm$ 9% ($P \leq 0.01$). Incubation of islets at high glucose conditions without troglitazone (HT^-) doubled insulin secretion when compared with islets at standard glucose ($P \leq 0.01$). In the presence of troglitazone (HT^+), insulin secretion was blunted also under high glucose conditions, although this effect was not as pronounced as at standard glucose levels (29 $\pm$ 12% reduction compared with control, $P \leq 0.05$). The stimulating effect of glucose on insulin secretion was not influenced by troglitazone treatment (IRI secretion of HT^- = 202 $\pm$ 32% of ST^- , IRI secretion of HT^+ = 233 $\pm$ 40% of ST^+ , $P = 0.29$, when comparing the IRI increments with and without troglitazone addition).

In ST^- islets, islet content of IRI was 12 $\pm$ 1 nmol/mg lysate protein (Table 1). Addition of troglitazone decreased islet insulin content by 15 $\pm$ 8% ($P \leq 0.05$). High glucose levels *per se* led to an average decline of 20% in intracellular IRI when compared with ST^- ($P \leq 0.05$). Addition of troglitazone at high glucose conditions elevated the final IRI content by 27 $\pm$ 13% when compared with HT^- ($P \leq 0.05$).

The conversion rate of proinsulin to insulin was assessed by relating corresponding C-peptide and IRI levels in the cellular supernatants as previously described (Bollheimer *et al.* 2001). The ratio between C-peptide and IRI did not differ

significantly between ST^- (0.39 $\pm$ 0.05) and ST^+ (0.47 $\pm$ 0.07, $P = 0.30$), nor between HT^- (0.66 $\pm$ 0.11) and HT^+ (0.46 $\pm$ 0.08, $P = 0.12$). Thus, we found no clear evidence for troglitazone-induced changes of posttranslational proinsulin processing at both standard and high glucose conditions.

Troglitazone effects on the secretory output and cellular content of pancreatic glucagon

The same islets used for the experiments described above were also analyzed for pancreatic glucagon. The results are included in Table 1. ST^- islets released 5 $\pm$ 2 pmol glucagon/mg lysate protein and contained 0.7 $\pm$ 0.1 nmol glucagon/mg lysate protein. Troglitazone exposure at 5.6 mM glucose did not significantly alter the examined parameters of islet alpha-cell function. Looking at high glucose conditions, glucagon secretion in HT^- islets was reduced by 32 $\pm$ 16% ($P \leq 0.05$) compared with the standard glucose ST^- controls without major changes in glucagon content. Here, the presence of troglitazone (HT^+) led to an augmented release of glucagon by an additional 50% ($P \leq 0.05$) without affecting intracellular glucagon content.

Molar ratios of insulin to glucagon

Assessing glucagon and insulin secretion in terms of molar quantities, we saw about a 15-fold higher secretion of insulin over glucagon in the ST^- group. Due to the decrease in insulin secretion after

Table 2 Effects of troglitazone on insulin biosynthesis. Rat pancreatic islets were incubated with or without troglitazone (10 μ M) at standard glucose (5.6 mM, ST^-/ST^+) and high glucose (16.7 mM, HT^-/HT^+) conditions together with [35 S]methionine. Newly synthesized protein was calculated from the incorporation of radioactivity into TCA precipitates, newly synthesized proinsulin from incorporation into insulin immunoprecipitates. The results are corrected for total lysate protein and shown as the mean change from control (ST^-) as percent $\pm$ S.E. of six independent experiments

	ST^-	ST^+	HT^-	HT^+
Newly synthesized total protein	100 $\pm$ 0	91 $\pm$ 15	136 $\pm$ 17*	131 $\pm$ 20
Newly synthesized insulin	100 $\pm$ 0	62 $\pm$ 9*	95 $\pm$ 13	92 $\pm$ 11
Specific insulin biosynthesis	100 $\pm$ 0	80 $\pm$ 12*	83 $\pm$ 8*	77 $\pm$ 8*

* $P < 0.05$ compared with control.

troglitazone incubation this ratio was lowered by about one-third in the ST^+ group, but this result did not reach statistical significance due to inter-experimental variability. In HT^- islets the insulin-to-glucagon ratio was approximately 3-fold higher than in ST^- islets (HT^- 49 $\pm$ 12:1 vs ST^- 15 $\pm$ 4:1, $P \leq 0.05$). Additional troglitazone treatment (HT^+) halved this ratio ($P \leq 0.05$).

Looking at molar quantities of intracellular IRI and IRG content, we found about 20 times more insulin than glucagon in cellular lysates of ST^- islets. In this regard there were no significant changes in ST^+ , HT^- and HT^+ .

Newly synthesized total protein and IRI in troglitazone-treated rat islets

35 S incorporation during the last 30 min of the 2.5 h incubation period served as a measure of total protein biosynthesis (Table 2). Compared with ST^- , troglitazone exposure at 5.6 mM glucose (ST^+) did not lead to major changes in protein synthesis. At high glucose levels (HT^-) the protein synthesis rate was increased by an additional 30% in comparison with ST^- . The presence of troglitazone (HT^+) generated no additional effect over HT^- .

By correcting 35 S incorporation into IRI immunoprecipitates with 35 S incorporation into total lysate protein, it is possible to bring out specific effects on the *de novo* biosynthesis of proinsulin that are above those on general protein biosynthesis (Itoh & Okamoto 1980, Bollheimer *et al.* 1998). Specific IRI biosynthesis decreased by about one-fifth after additional troglitazone exposure at standard glucose levels ($P \leq 0.05$). After a 2.5 h period at high glucose levels, specific IRI

synthesis in troglitazone-treated HT^+ islets was about one-third lower than in ST^- islets ($P \leq 0.05$), but no distinct effect of troglitazone could be observed when compared with untreated islets (HT^-) at high glucose levels.

Cellular content of PPI mRNA and PPG mRNA

For further investigation of biosynthetic effects, semiquantitative assessment for PPI mRNA, PPG mRNA and GAPDH mRNA as housekeeping gene were performed using a real-time PCR technique (Briaud *et al.* 2001). The results are detailed in Fig. 1. Compared with the ST^- group, islets which

Figure 1 Effects of troglitazone on PPI mRNA and PPG mRNA. Rat pancreatic islets were incubated with or without troglitazone (10 μ M) at standard glucose (5.6 mM) and high glucose (16.7 mM) levels. Intracellular mRNA content was quantified by real-time PCR analysis and standardized for GAPDH mRNA content as housekeeping gene. The results are shown as the mean change from control at equal glucose levels as percent $\pm$ S.E. of five or six independent experiments. * $P \leq 0.05$ compared with control.

Table 3 Effects of troglitazone on parameters of islet function under conditions of glucolipotoxicity. After preconditioning at 11.2 mM glucose, pancreatic islets were incubated at 16.7 mM glucose and 200 μ M oleate to simulate glucolipotoxic conditions. The effect of 10 μ M troglitazone on protein content, islet insulin and glucagon content, secretion and mRNA expression as well as specific insulin biosynthesis are shown as relative change (%) from the control (i.e. glucose 16.7 mM, no addition of oleate or troglitazone). The data represent means $\pm$ S.E. of six or seven independent experiments

	Troglitazone (μ M)	
	0	10
Insulin		
Content	92 $\pm$ 7	114 $\pm$ 13*
Secretion	96 $\pm$ 32	76 $\pm$ 27
mRNA content	90 $\pm$ 14	107 $\pm$ 29
Glucagon		
Content	118 $\pm$ 19	117 $\pm$ 13
Secretion	155 $\pm$ 38	161 $\pm$ 37
mRNA content	103 $\pm$ 18	115 $\pm$ 24

* $P < 0.05$ compared with islets not treated with troglitazone.

had been additionally exposed to troglitazone at standard glucose levels showed a decrease in their cellular PPI mRNA content by approximately one-fifth ($P \leq 0.05$). The PPI mRNA content at high glucose conditions (HT^-) tended to decline when compared with ST^- (0.85 ± 0.2 -fold of control, $P = 0.08$). Under high glucose conditions there was no clear independent effect of troglitazone on the cellular PPI mRNA content. Cellular PPG mRNA content of ST^+ and HT^+ did not significantly differ from that of their respective controls ($P = 0.07$ when comparing ST^+ and ST^- , $P = 0.28$ when comparing HT^+ and HT^-).

Effects of troglitazone under conditions of glucolipotoxicity

Elevated glucose and FFA levels are hallmarks of adipogenic type 2 diabetes. To elucidate whether FFA exposure changed the effects of troglitazone in a high glucose setting (so-called 'glucolipotoxicity'), we exposed islets to troglitazone and oleate at 16.7 mM glucose and measured the effects on insulin and glucagon content, secretion and mRNA content as well as the *de novo* insulin biosynthesis as described above. As shown in Table 3, troglitazone tended to impair insulin secretion, similar to the

results described above. However, due to high inter-experiment variability, the resulting 20% decrease was not statistically significant. Other specific troglitazone effects on the examined parameters of beta-cell function were not detected. With respect to glucagon, we did not find any troglitazone-induced changes of glucagon secretion, content or PPG mRNA content under the condition of glucolipotoxicity.

Discussion

TZD derivatives such as troglitazone modulate cell function in a pleiotropic manner mainly through PPARs (Reginato & Lazar 1999). Their anti-diabetic potency is thereby generally attributed to an improvement of peripheral insulin sensitivity. Nonetheless, TZDs may also directly influence the endocrine pancreas since PPAR γ is expressed both in rodent and in human pancreatic islets (Dubois *et al.* 2000). We therefore carried out an in-depth characterization of the effects of the TZD prototype troglitazone on parameters of rat islet beta- and alpha-cell function under standard and high glucose (5.6/16.7 mM) as well as glucolipotoxic (16.7 mM glucose and 200 μ M oleate pre-complexed to 0.2% BSA) conditions.

Islet hormone secretion

We observed a drop in insulin secretion of about one-third after a 2.5 h incubation of rat pancreatic islets with 10 μ M troglitazone at both standard and high glucose levels. Notably, the relative stimulation of insulin secretion by high glucose was not influenced by troglitazone treatment.

Such an insulin-sparing effect of troglitazone on beta-cell insulin output has not been described before. In a previous *in vitro* study looking at troglitazone effects on normal primary rat islets, Masuda *et al.* (1995) reported an increased insulin secretion as a response to troglitazone (1–10 μ M) after 30 min of incubation at 11.2 mM glucose, which reflects a first phase secretory response of the pancreatic beta-cell. It has to be noted that the same authors reported a troglitazone-induced decrease in rapid insulin secretion in islets at higher troglitazone concentrations ($c_{\text{troglitazone}} = 100 \mu\text{M}$) as well as in the clonal beta-cell line HIT-T15 ($c_{\text{troglitazone}} = 10\text{--}100 \mu\text{M}$) (Masuda *et al.* 1995).

In agreement with our results, Shimabukuro *et al.* (1998) report a moderate, albeit not statistically significant reduction of insulin secretion after a 48 h incubation with 10 μ M troglitazone in lean ZDF (+/+) rats. In the clonal beta-cell line INS-1, a stable to a decreasing insulin release has been reported after prolonged (4 h) as well as chronic (48 h) exposure to 10 μ M troglitazone (Bollheimer *et al.* 2002, Kawai *et al.* 2002).

The studies cited above illuminate the controversy about troglitazone-induced changes of insulin secretion *in vitro*. Clinical studies with animals and humans chronically treated with troglitazone consistently show a decrease in plasma insulin levels (Prigeon *et al.* 1998, Buchanan *et al.* 2000). This effect is commonly attributed to an increased peripheral insulin sensitivity, but it also might be partly accounted for by the reduction of insulin secretion we describe here. The fact that glucagon secretion was elevated in troglitazone-treated islets at high glucose levels rules out a general lowering, i.e. toxic, effect of troglitazone on islet hormone output at the concentration we employed. This stimulation of glucagon release rather strengthens the notion that troglitazone might acutely act in a contra-insular fashion.

Islet hormone synthesis

To discern whether the decrease in insulin secretion was accompanied by changes in islet hormone biosynthesis, we next examined the intracellular content of IRI and IRG and their corresponding mRNAs as well as the amount of newly synthesized PPI. To our knowledge, these aspects of troglitazone action on primary pancreatic islets have not been analyzed in detail before, thereby limiting the possibility of comparing our results with other studies (Bollheimer *et al.* 2002, Schinner *et al.* 2002).

Concerning insulin, we found a parallel decrease of both intracellular IRI content and intracellular PPI mRNA at standard glucose levels. This points to troglitazone-induced changes in PPI mRNA transcription and/or stability. Further evidence for a specific inhibition of PPI biosynthesis by troglitazone at standard glucose levels was provided from the pulse-labeling experiments showing a specific decrease of newly synthesized IRI. Non-specific causes of this impairment in insulin biosynthesis do not seem likely as the total amount

of newly synthesized protein did not differ between troglitazone-treated islets and controls.

Under high glucose conditions, IRI content was elevated by troglitazone. This could be due to the aforementioned sparing effect in insulin secretion, particularly as there was no significant change in pulse label-quantified PPI biosynthesis or mRNA content in comparison with the corresponding controls. This is in accord with our previous study with INS-1 cells, where troglitazone *per se* did not affect parameters of PPI transcription and translation at high glucose levels ($c_{\text{glucose}} = 11.2$ mM) (Bollheimer *et al.* 2002). Principally, we cannot exclude other mechanisms, e.g. a diminished intracellular insulin degradation which might further contribute to the increased intracellular insulin content after troglitazone exposure under high glucose conditions (Halban & Wollheim 1980, Bollheimer *et al.* 2001).

Concerning glucagon, neither troglitazone nor high glucose induced significant changes in the intracellular content. PPG mRNA levels tended to decrease after troglitazone treatment at both glucose levels employed. A recent study by Schinner *et al.* (2002) has confirmed a similar impairment of PPG mRNA synthesis in primary islets after TZD treatment, although a strict comparison with our results is not possible due to (i) the TZD employed, namely rosiglitazone, which can activate PPAR γ more effectively than troglitazone (Camp *et al.* 2000), (ii) a longer incubation time of 48 h, and (iii) the fact that mouse and not rat islets were used.

How might troglitazone act on islet hormone secretion and biosynthesis?

TZDs can influence gene transcription via PPAR γ within 30 min of exposure (Burris *et al.* 1999). Although a functional peroxisomal proliferator response element (PPRE) has not yet been demonstrated in the PPI gene, PPAR γ actions on insulin secretion and biosynthesis might be mediated by PPREs in the glucokinase as well as the GLUT2 gene (Kim *et al.* 2000, 2002). Both of these proteins are elements of the beta-cell glucose sensor and thus are directly involved in glucose-stimulated insulin secretion and biosynthesis (Newgard & McGarry 1995). Nonetheless, it has to be noted that troglitazone need not necessarily act through PPAR γ (Bahr *et al.* 1996, Wang *et al.* 1999,

Brunmair *et al.* 2001, Davies *et al.* 2001). In that context, one must consider that we used the relatively high – albeit commonly employed in *in vitro* settings (Cnop *et al.* 2002, Kawai *et al.* 2002) – concentration of 10 μ M troglitazone. As activation of PPAR γ can be achieved with as little as 100 nM troglitazone (Adams *et al.* 1997), pharmacological effects of troglitazone which might surmount physiological effects of PPAR γ activation cannot be fully excluded.

Troglitazone effects under glucolipotoxic conditions

Our results provide no evidence for positive direct effects of troglitazone on fatty acid-treated islets at high glucose levels in the short-term. A recent study by Patane *et al.* (2002) indirectly supports our findings with regard to insulin secretion. Here, FFA-treated islets showed elevated PPAR γ levels, and insulin secretion was stimulated by PPAR γ antagonists. In contrast to these studies and to our present findings, Higa *et al.* (1999) have demonstrated the capability of troglitazone – applied *in vivo* before onset of glucose intolerance as ‘prophylaxis’ or afterwards as ‘treatment’ – to restore the function of ZDF (*fa/fa*) rat islets with chronic fatty acid overload and defects in insulin secretion and content. Our *in vitro* model looking at direct short-term effects on previously healthy islets is not suitable for the analysis of such putative triglyceride-depleting effects of troglitazone.

Conclusions

Taken together, we have observed a decrease in insulin secretion and insulin biosynthesis after an acute treatment of isolated primary rat islets with troglitazone. This direct effect on the pancreatic islet was most marked at standard glucose levels. No clear beneficial effect of troglitazone was seen at glucolipotoxic conditions with simultaneously elevated glucose and oleate levels. We conclude from our results that troglitazone has a direct, not necessarily PPAR γ -related, inhibiting effect on beta-cell insulin secretion and production predominantly at standard glucose levels. This ‘insulin-sparing’ effect might reduce the pathological basal hyperinsulinemia seen in insulin-resistant subjects and in early stages of adipogenic type 2 diabetes

and thus, working hand in hand with the peripheral insulin-sensitizing effects, explain part of the clinical benefit of this TZD.

From our results, one would therefore expect the insulin-sparing effects of troglitazone to be most useful in prediabetic states. Appropriately, clinical studies examining the possibility of diabetes prevention by TZDs have already been initiated (Buchanan *et al.* 2000). The effects of troglitazone on glucagon secretion point to an elevation of alpha-cell endocrine activity at high glucose levels. The clinical significance of this remains unclear at present and warrants further investigation.

Acknowledgements

The authors acknowledge the excellent technical assistance of Iris Ottinger and Joachim Schweimer. The work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG) (Bo 1379/2-1, Bo 1379/2-2).

References

- Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L, Digby JE, Sewter CP, Lazar MA, Chatterjee VK *et al.* 1997 Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. *Journal of Clinical Investigation* **100** 3149–3153.
- Bahr M, Spelleken M, Bock M, von Holtey M, Kiehn R & Eckel J 1996 Acute and chronic effects of troglitazone (CS-045) on isolated rat ventricular cardiomyocytes. *Diabetologia* **39** 766–774.
- Bollheimer LC, Skelly RH, Chester MW, McGarry JD & Rhodes CJ 1998 Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *Journal of Clinical Investigation* **101** 1094–1101.
- Bollheimer LC, Kestler TM, Michel J, Buettner R, Scholmerich J & Palitzsch K-D 2001 Intracellular depletion of insulin by oleate is due to an inhibited synthesis and not to an increased secretion. *Biochemical and Biophysical Research Communications* **287** 397–401.
- Bollheimer LC, Kagerbauer SM, Buettner R, Kemptner DM, Palitzsch K-D, Scholmerich J & Hügl S 2002 Synergistic effects of troglitazone and oleate on the translatability of preproinsulin mRNA from INS-1-cells. *Biochemical Pharmacology* **64** 1629–1636.
- Briaud I, Harmon JS, Kelpe CL, Segu VB & Poirout V 2001 Lipotoxicity of the pancreatic beta-cell is associated with glucose-dependent esterification of fatty acids into neutral lipids. *Diabetes* **50** 315–321.
- Brunmair B, Gras F, Neschen S, Roden M, Wagner L, Waldhausl W & Fornsinn C 2001 Direct thiazolidinedione action on isolated rat skeletal muscle fuel handling is independent of peroxisome proliferator-activated receptor-gamma-mediated changes in gene expression. *Diabetes* **50** 2309–2315.
- Buchanan TA, Xiang AH, Peters RK, Kjos SL, Berkowitz K, Marroquin A, Goico J, Ochoa C & Azen SP 2000 Response of

- pancreatic beta-cells to improved insulin sensitivity in women at high risk for type 2 diabetes. *Diabetes* **49** 782–788.
- Burris TP, Pelton PD, Zhou L, Osborne MC, Cryan E & Demarest KT 1999 A novel method for analysis of nuclear receptor function at natural promoters: peroxisome proliferator-activated receptor gamma agonist actions on aP2 gene expression detected using branched DNA messenger RNA quantitation. *Molecular Endocrinology* **13** 410–417.
- Camp HS, Li O, Wise SC, Hong YH, Frankowski CL, Shen X, Vanbogaen R & Leff T 2000 Differential activation of peroxisome proliferator-activated receptor-gamma by troglitazone and rosiglitazone. *Diabetes* **49** 539–547.
- Cnop M, Hannaert JC & Pipeleers DG 2002 Troglitazone does not protect rat pancreatic beta cells against free fatty acid-induced cytotoxicity. *Biochemical Pharmacology* **63** 1281–1285.
- Davies GF, Khandelwal RL, Wu L, Juurlink BH & Roesler WJ 2001 Inhibition of phosphoenolpyruvate carboxykinase (PEPCK) gene expression by troglitazone: a peroxisome proliferator-activated receptor-gamma (PPARgamma)-independent, antioxidant-related mechanism. *Biochemical Pharmacology* **62** 1071–1079.
- Dubois M, Pattou F, Kerr-Conte J, Gmyr V, Vandewalle B, Desreumaux P, Auwerx J, Schoonjans K & Lefebvre J 2000 Expression of peroxisome proliferator-activated receptor gamma (PPAR gamma) in normal human pancreatic islet cells. *Diabetologia* **43** 1165–1169.
- Gough NM 1988 Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. *Analytical Biochemistry* **173** 93–95.
- Halban PA & Wollheim CB 1980 Intracellular degradation of insulin stores by rat pancreatic islets *in vitro*. An alternative pathway for homeostasis of pancreatic insulin content. *Journal of Biological Chemistry* **255** 6003–6006.
- Higa M, Zhou YT, Ravazzola M, Baetens D, Orci L & Unger RH 1999 Troglitazone prevents mitochondrial alterations, beta cell destruction, and diabetes in obese prediabetic rats. *PNAS* **96** 11513–11518.
- Itoh N & Okamoto H 1980 Translational control of proinsulin synthesis by glucose. *Nature* **283** 100–102.
- Kawai T, Hirose H, Seto Y, Fujita H, Fujita H, Ukeda K & Saruta T 2002 Troglitazone ameliorates lipotoxicity in the beta cell line INS-1 expressing PPAR gamma. *Diabetes Research and Clinical Practice* **56** 83–92.
- Kim HI, Kim JW, Kim SH, Cha JY, Kim KS & Ahn YH 2000 Identification and functional characterization of the peroxisomal proliferator response element in rat GLUT2 promoter. *Diabetes* **49** 1517–1524.
- Kim HI, Cha JY, Kim SY, Kim JW, Roh KJ, Seong JK, Lee NT, Choi KY, Kim KS & Ahn YH 2002 Peroxisomal proliferator-activated receptor-gamma upregulates glucokinase gene expression in beta-cells. *Diabetes* **51** 676–685.
- Lekanne Deprez RH, Fijnvandraat AC, Ruijter JM & Moorman AFM 2002 Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Analytical Biochemistry* **307** 63–69.
- Masuda K, Okamoto Y, Tsuura Y, Kato S, Miura T, Tsuda K, Horikoshi H, Ishida H & Seino Y 1995 Effects of troglitazone (CS-045) on insulin secretion in isolated rat pancreatic islets and HIT cells: an insulinotropic mechanism distinct from glibenclamide. *Diabetologia* **38** 24–30.
- Newgard CB & McGarry JD 1995 Metabolic coupling factors in pancreatic beta-cell signal transduction. *Annual Review of Biochemistry* **64** 689–719.
- Ohtani KI, Shimizu H, Sato N & Mori M 1998 Troglitazone (CS-045) inhibits beta-cell proliferation rate following stimulation of insulin secretion in HIT-T 15 cells. *Endocrinology* **139** 172–178.
- Olefsky JM & Saltiel AR 2000 PPARgamma and the treatment of insulin resistance. *Trends in Endocrinology and Metabolism* **11** 362–368.
- Parker JC 2002 Troglitazone: the discovery and development of a novel therapy for the treatment of type 2 diabetes mellitus. *Advanced Drug Delivery Reviews* **54** 1173–1197.
- Patane G, Anello M, Piro S, Vigneri R, Purrello F & Rabuazzo AM 2002 Role of ATP production and uncoupling protein-2 in the insulin secretory defect induced by chronic exposure to high glucose or free fatty acids and effects of peroxisome proliferator-activated receptor-gamma inhibition. *Diabetes* **51** 2749–2756.
- Prigeon RL, Kahn SE & Porte D Jr 1998 Effect of troglitazone on B cell function, insulin sensitivity, and glycemic control in subjects with type 2 diabetes mellitus. *Journal of Clinical Endocrinology and Metabolism* **83** 819–823.
- Reginato MJ & Lazar MA 1999 Mechanisms by which thiazolidinediones enhance insulin action. *Trends in Endocrinology and Metabolism* **10** 9–13.
- Ririe KM, Rasmussen RP & Wittwer CT 1997 Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry* **245** 154–160.
- Schinner S, Dellas C, Schroder M, Heinlein CA, Chang C, Fischer J & Knebel W 2002 Repression of glucagon gene transcription by peroxisome proliferator-activated receptor gamma through inhibition of Pax6 transcriptional activity. *Journal of Biological Chemistry* **277** 1941–1948.
- Shimabukuro M, Zhou YT, Lee Y & Unger RH 1998 Troglitazone lowers islet fat and restores beta cell function of Zucker diabetic fatty rats. *Journal of Biological Chemistry* **273** 3547–3550.
- Skelly RH, Bollheimer LC, Wicksteed BL, Corkey BE & Rhodes CJ 1998 A distinct difference in the metabolic stimulus-response coupling pathways for regulating proinsulin biosynthesis and insulin secretion that lies at the level of a requirement for fatty acyl moieties. *Biochemical Journal* **331** 553–561.
- Wang M, Wise SC, Leff T & Su TZ 1999 Troglitazone, an antidiabetic agent, inhibits cholesterol biosynthesis through a mechanism independent of peroxisome proliferator-activated receptor-gamma. *Diabetes* **48** 254–260.
- Wrede CE, Dickson LM, Lingohr MK, Briaud I & Rhodes CJ 2002 Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). *Journal of Biological Chemistry* **277** 49676–49684.

Received in final form 4 March 2003

Accepted 17 March 2003