Acute troglitazone action in isolated perfused rat liver

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1 The thiazolidinedione compound, troglitazone, enhances insulin action and reduces plasma glucose concentrations when administered chronically to type 2 diabetic patients.

2 To analyse to what extent thiazolidinediones interfere with liver function, we examined the acute actions of troglitazone (0.61 and 3.15 μM) on hepatic glucose and lactate fluxes, bile secretion, and portal pressure under basal, insulin- and/or glucagon-stimulated conditions in isolated perfused rat livers.

3 During BSA-free perfusion, high dose troglitazone increased basal ($P < 0.01$), but inhibited glucagon-stimulated incremental glucose production by $\sim 75\%$ (10.0 ± 2.5 vs control: 40.0 ± 7.2 $\mu\text{mol g liver}^{-1}$, $P < 0.01$). In parallel, incremental lactate release rose ~ 6 fold (13.1 ± 5.9 vs control: 2.2 ± 0.8 mmol g liver^{-1} , $P < 0.05$), while bile secretion declined by $\sim 67\%$ [0.23 ± 0.02 vs control: 0.70 ± 0.05 $\text{mg g liver}^{-1} \text{ min}^{-1}$], $P < 0.001$]. Low dose troglitazone infusion did not enhance the inhibitory effect of insulin on glucagon-stimulated glucose production, but rapidly increased lactate release ($P < 0.0005$) and portal venous pressure ($+0.17 \pm 0.07$ vs $+0.54 \pm 0.07$ cm buffer height, $P < 0.0001$).

4 These results indicate that troglitazone exerts both insulin-like and non-insulin-like hepatic effects, which are blunted by addition of albumin, possibly due to troglitazone binding.

Keywords: Troglitazone; insulin; glucagon; glucose production; glycogenolysis; lactate; portal pressure; bile secretion; liver; rat

Abbreviations: ADP, adenosine-diphosphate; AMP, adenosine-monophosphate; BSA, bovine serum albumin; DMSO, dimethyl sulphoxide; PPAR γ , peroxisome proliferator activated receptor γ

Introduction

Thiazolidinedione derivatives represent a new class of insulin-sensitizers that improve insulin resistance, glycemia, and dyslipidemia in animal models of obesity and diabetes (Saltiel & Olefsky, 1996). One of these compounds, troglitazone (CS-045) also decreases hyperglycemia and ameliorates insulin sensitivity in human type 2 diabetes mellitus (Iwamoto *et al.*, 1991; Schwartz *et al.*, 1998; Inzucchi *et al.*, 1998). The improvement of hyperglycemia requires the presence of insulin and usually develops after several days to weeks of oral treatment. This chronic effect of troglitazone could be linked to activation of the nuclear peroxisome proliferator activated receptor γ (PPAR γ), which induces adipocyte differentiation in cell cultures (Lehmann *et al.*, 1995; Teboul *et al.*, 1995). However, PPAR γ is only rarely detectable in skeletal muscle or liver and no association between PPAR γ gene expression and insulin-resistant states like obesity and type 2 diabetes mellitus could be found (Aubouef *et al.*, 1997).

Thiazolidinediones also exert short-term effects on glucose metabolism, which cannot be explained by regulation of gene expression and/or transcription (Fujiwara *et al.*, 1988; Kreutter *et al.*, 1990; Lee & Olefsky, 1995; Fürnsinn *et al.*, 1997). In nondiabetic rats, troglitazone increases peripheral glucose disposal and decreases hepatic glucose production within 1 h (Lee & Olefsky, 1995). The acute stimulatory effect on muscle glucose metabolism is not insulin-dependent and

exhibits hypoxia-like features (Fürnsinn *et al.*, 1997). The reduction of hepatic glucose production could be due to an insulin-like action as suggested by inhibition of gluconeogenesis in isolated rat hepatocytes (Fulgencio *et al.*, 1996; Raman *et al.*, 1998). Alternatively, these effects could also result from unspecific troglitazone action. In this context it is of note that liver dysfunction or hepatocellular injury has occurred during troglitazone treatment in man (Ault, 1997; Imura, 1998; Watkins & Whitcomb, 1998).

To understand better detrimental hepatic troglitazone action this study analyses: (a) basal and stimulated net glucose production, (b) transhepatic lactate fluxes, simultaneously with the time-course of (c) bile secretion and (d) portal pressure in isolated perfused rat liver.

Methods

Animals

Male Sprague-Dawley rats (Him: OFA/SPF, Tierforschungsinstitut, Himberg, Austria) weighing from 220–300 g were kept at a constant 12 h day-night cycle with free access to standard laboratory rat chow and water. Experiments were started before 10.00 a.m. by intraperitoneal thiopental anaesthesia (80 mg g body weight $^{-1}$ intraperitoneally; Sanabo, Vienna, Austria) of the non-fasted rat to provide full hepatic glycogen stores (Burton & Ishida, 1965). All protocols were performed according to local legislation and to the principles of laboratory animal care.

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Isolated liver perfusion

The preparation and perfusion was performed as previously reported (Roden *et al.*, 1992, 1995; Graf & Peterlik, 1976) with the described modifications. Briefly, the portal vein and the vena cava were cannulated and the isolated liver was perfused in anterograde direction in a non-recirculating system (flow rate: $\sim 3.5 \text{ ml g liver}^{-1} \text{ min}^{-1}$). The perfusion was performed at 37°C with oxygenated (95% O_2 /5% CO_2) perfusion medium [Krebs-Henseleit buffer + 5 mM D(+)-glucose, pH 7.4] with or without added 0.2% (w/v) bovine serum albumin (BSA, fraction V; Boehringer Mannheim, Mannheim, Germany). A small flexible cannula was inserted into the bile duct in order to allow continuous monitoring of bile flow by using a drop counter connected to an IBM-compatible personal computer. Portal hydrostatic pressure was measured by using a Y-tubing located 15 cm before the liver and liver viability was checked during the equilibration period ($t = -40$ – 0 min) by uniform colour of the blood-free liver, constant lactate release, stable portal hydrostatic pressure ($< +5 \text{ cm}$ buffer height), and bile flow ($> 0.5 \text{ mg g liver}^{-1} \text{ min}^{-1}$) (Gores *et al.*, 1986; Roden *et al.*, 1995).

Drugs and hormones

Troglitazone was generously donated by Sankyo (Tokyo, Japan). The drug was dissolved and diluted in dimethyl sulphoxide (DMSO) and continuously infused into the portal tubing at final concentrations of 0.61 or $3.15 \mu\text{M}$. The DMSO concentration did not exceed 0.2% (v/v) and was identical in the control experiments in which DMSO was infused without added troglitazone. Human insulin (calculated final concentrations: 5 nM; Actrapid, Novo-Nordisk 40 U, Copenhagen, Denmark) and glucagon (final concentration: 0.1 or 1 nM; Lilly, Indianapolis, IN, U.S.A.) were diluted in Krebs-Henseleit buffer containing 2% BSA.

Experimental protocols

After the equilibration period, insulin and/or troglitazone or DMSO alone (control) were continuously admixed to the influent perfusate (0.5 ml min^{-1} ; Perfusor V, Braun Melsungen, Germany). During the second phase of the experiments, glucagon was infused to stimulate glycogen breakdown and to test the liver's hormonal responsiveness (Roden *et al.*, 1992). Perfusate samples were taken from the portal tubing (pre-hepatic: $t = 0, 30$, and 80 min) and from the vena cava (post-hepatic: every 5 min).

Three study protocols were performed to examine (I) acute troglitazone action, (II) effects of BSA on acute troglitazone action, and (III) effects of insulin on acute troglitazone action. During protocol (I) 0.6 or $3.15 \mu\text{M}$ troglitazone or DMSO alone were infused for 90 min with 1 nM glucagon being infused during the last 30 min. For protocol (II), $3.15 \mu\text{M}$ troglitazone or DMSO were infused for 90 min in the presence of 0.2% BSA with added glucagon (1 nM) during the last 30 min. During protocol (III), 5 nM insulin alone or with $0.61 \mu\text{M}$ troglitazone or DMSO only were infused for 60 min and an infusion of 0.1 nM glucagon was commenced for the last 60 min.

Analytical procedures

Glucose and lactate concentrations were determined enzymatically by the hexokinase (Glucose liquiUV; Human, Taunusstein, Germany) and the lactate dehydrogenase method (ACA,

Du Pont Company, Wilmington, DE, U.S.A.), respectively. Rates of glucose/lactate production were calculated by multiplying the transhepatic concentration difference (post-hepatic minus pre-hepatic concentration) times flow rate divided by liver wet weight and are expressed as $\mu\text{mol g liver}^{-1} \text{ min}^{-1}$. Incremental glucose or lactate production in $\mu\text{mol g liver}$ was assessed by the trapezoidal rule from the area under the time curve corrected for glucose or lactate production rates at zero time for the first period and at $t = 60 \text{ min}$ for the second (glucagon) period, respectively. Bile flow was calculated by multiplying single drop weight (8 mg per drop) times drop frequency per liver wet weight and is expressed in $\text{mg bile g liver}^{-1} \text{ min}^{-1}$. Portal pressure is given as the buffer height in cm with the liver support plate as the reference level.

Data analysis and statistical evaluation

Data are presented as means $\pm$ s.e.mean. Differences between groups were analysed with the unpaired, two-tail *t*-test. *P* values less than 0.05 were considered to indicate significant differences.

Results

Acute troglitazone action

Hepatic glucose production rates decreased slightly during infusion of low dose ($0.61 \mu\text{M}$) troglitazone or DMSO alone, but remained unchanged during the 60 min infusion of high dose ($3.15 \mu\text{M}$) troglitazone (Figure 1). Stimulation of

Figure 1 Acute troglitazone action. Time-course of glucose production rates and Δ lactate release during the basal period and during glucagon stimulation (1.0 nM) in isolated perfused rat liver. Effect of troglitazone infusion ($0.61 \mu\text{M}$: $n = 7$, $3.15 \mu\text{M}$: $n = 8$) compared with control (Krebs-Henseleit buffer + 0.2% DMSO: $n = 7$) under BSA-free conditions. Data are given as means $\pm$ s.e.-mean.

glycogenolysis by glucagon resulted within 10 min in a 4–5 fold rise of glucose production rates in both the low dose troglitazone and control groups. However, in the presence of high dose troglitazone, glucagon-stimulated glucose production rates increased by only ~1.7 fold and were ~36% lower than those elicited in the low dose troglitazone ($P<0.005$) or control ($P<0.05$) groups. Thus, glucagon-stimulated incremental glucose release was not affected by low dose troglitazone, but suppressed by high dose troglitazone to ~25% of control values (Table 1).

Lactate production rates (in $\text{mmol g liver}^{-1} \text{ min}^{-1}$) did not differ at zero time in the three treatment groups (high dose troglitazone: 1.25 ± 0.09 , low dose troglitazone: 1.48 ± 0.12 , control: 1.34 ± 0.03). Rates of hepatic lactate release corrected for zero-time lactate production (Δ lactate release) were similar during the basal period, while the glucagon-induced fall in lactate production rates was less pronounced in the presence of high dose troglitazone ($P<0.005$ vs low dose troglitazone and control; Figure 1).

Hepatic bile secretion remained constant during infusion of low dose troglitazone or vehicle; in both groups glucagon infusion induced a transient decrease followed by a small increment in bile secretion which then returned to control values (Figure 2). In the presence of high dose troglitazone, bile secretion rates rapidly declined in a nearly linear manner until the start of the glucagon infusion. At this time ($t=60$ min) bile secretion rates were decreased by ~70% compared with the low dose troglitazone and control groups ($P<0.00001$). Portal venous pressure was similar in all treatment groups prior to the glucagon infusion (Figure 2). Infusion of glucagon produced a gradual increase in mean portal pressure in livers exposed to high dose troglitazone did not differ significantly from those of the low dose troglitazone and control groups.

Effect of BSA on acute troglitazone action

In order to test whether the observed effects of high dose ($3.15 \mu\text{M}$) troglitazone could be prevented by albumin, the above described experiments were repeated in the presence of 0.2% BSA. Addition of BSA reduced hepatic glucose production rates in livers exposed to high dose troglitazone ($P<0.05$) to a level similar to that observed in control livers exposed to BSA (Figure 3). The rise in glucose production

induced by subsequent stimulation by glucagon was similar in livers treated with high dose troglitazone + BSA exposure or BSA alone but ~35% higher than in livers treated with high dose troglitazone alone ($P<0.05$). In the presence of BSA incremental glucose release was similar between the high dose troglitazone and control groups before and during glucagon stimulation (Table 1).

Lactate production rates (in $\text{mmol g liver}^{-1} \text{ min}^{-1}$) were again not different at zero time (high dose troglitazone without BSA: 1.25 ± 0.09 , high dose troglitazone + BSA: 1.10 ± 0.03 , control + BSA: 1.21 ± 0.08). Lactate production rates were

Figure 2 Acute troglitazone action. Time-course of bile flow and Δ portal pressure during the basal period and during glucagon stimulation (1.0 nM) in the isolated perfused rat liver. Effect of troglitazone infusion ($0.61 \mu\text{M}$: $n=6$, $3.15 \mu\text{M}$: $n=6$) compared with control (Krebs-Henseleit buffer + 0.2% DMSO; $n=6$) under BSA-free conditions. Data are given as means $\pm$ s.e.mean.

Table 1 Incremental basal and stimulated hepatic glucose release (means $\pm$ s.e.mean) during infusion of DMSO, troglitazone $\pm$ BSA and/or insulin

Troglitazone (μM)	BSA	Insulin	n	Incremental glucose production ($\mu\text{mol g liver}^{-1}$)	
				Basal	Glucagon-stimulated
<i>Acute troglitazone action</i>					
0	—	—	7	-15.4 ± 2.7^a	40.0 ± 7.2^b
0.61	—	—	7	-10.9 ± 3.4	45.4 ± 3.8^c
3.15	—	—	8	$-0.2 \pm 3.7^{a,d,e}$	$10.0 \pm 2.5^{b,c,f,g}$
<i>Effect of BSA on acute troglitazone action</i>					
0	+	—	6	-30.1 ± 9.9^e	37.3 ± 6.4^f
3.15	+	—	6	-25.8 ± 4.8^d	38.6 ± 4.0^g
<i>Effect of BSA on acute troglitazone action</i>					
0	—	—	6	-33.6 ± 4.4^h	60.8 ± 10.3^i
0	—	+	6	$-55.1 \pm 5.5^{h,i}$	29.2 ± 6.9^j
0.61	—	+	6	-39.5 ± 3.2^i	48.4 ± 5.7

Superscripts indicate significant differences between the groups compared. ^a $P<0.01$, ^b $P<0.01$, ^c $P<0.001$, ^d $P<0.001$, ^e $P<0.01$, ^f $P<0.05$, ^g $P<0.01$, ^h $P<0.05$, ⁱ $P<0.05$, ^j $P<0.05$.

lower in livers treated with high dose troglitazone + BSA than in those treated with troglitazone alone at 10–70 min ($P < 0.05$) but did not differ from the controls (Figure 3). In parallel, incremental lactate release (0–60 min: $13.1 \pm 0.6 \mu\text{mol g liver}^{-1}$) was markedly higher than in the absence of BSA ($-7.8 \pm 3.3 \text{ mmol g liver}^{-1}$, $P < 0.05$) and in the controls ($2.2 \pm 0.8 \text{ mg g liver}^{-1}$, $P < 0.05$).

Hepatic bile secretion also decreased within 25 min of high dose troglitazone + BSA exposure ($P < 0.01$), but the decline was less pronounced than that observed in BSA-free conditions (Figure 4). After 90 min, rates of bile secretion were about twice those found with high dose troglitazone without BSA ($P < 0.00001$), but still $\sim 37\%$ lower than in BSA-free control conditions ($P < 0.00001$). Portal pressure was similar in all three experimental groups (Figure 4).

Effect of insulin on acute troglitazone action

In order to compare the acute troglitazone effect on insulin action under both basal and glucagon-stimulated conditions, insulin (5 nM) $\pm$ troglitazone ($0.61 \mu\text{M}$) was infused during the basal period from zero time to 60 min and glucagon was added from $t = 60$ –120 min. Glucagon was used at a 10 fold lower concentration than in the previous studies in order to examine effects of insulin and troglitazone on stimulated glucose production. Insulin alone and with low troglitazone decreased ($P < 0.05$) hepatic glucose production rates during the basal period and also during glucagon stimulation (Figure 5). Similarly, incremental glucose release was lower with insulin alone than either control or insulin + low dose troglitazone during the basal period (Table 1). Under glucagon-stimulated

Figure 4 Effect of BSA on acute troglitazone action. Time-course of bile flow and Δ portal pressure during the basal period and during glucagon stimulation (1.0 nM) in the isolated perfused rat liver. Effect of troglitazone infusion ($3.15 \mu\text{M}$) with ($n = 6$) or without ($n = 6$, see also Figure 1) addition of 0.2% BSA compared with control (Krebs-Henseleit buffer + 0.2% DMSO + 0.2% BSA; $n = 6$). Data are given as means $\pm$ s.e.mean.

Figure 3 Effect of BSA on acute troglitazone action. Time-course of glucose production rates and Δ lactate release during the basal period and during glucagon stimulation (1.0 nM) in the isolated perfused rat liver. Effect of troglitazone infusion ($3.15 \mu\text{M}$) with ($n = 6$) or without ($n = 8$, see also Figure 1) addition of 0.2% BSA compared with control (Krebs-Henseleit buffer + 0.2% DMSO + 0.2% BSA; $n = 6$). Data are given as means $\pm$ s.e.mean.

Figure 5 Effect of insulin on acute troglitazone action. Time-course of glucose production rates and Δ lactate release during the basal period and during glucagon stimulation (0.1 nM) in the isolated perfused rat liver. Effect of infusion of insulin with or without troglitazone ($0.61 \mu\text{M}$) compared with control (Krebs-Henseleit buffer + 0.2% DMSO) under BSA-free conditions. Data are given as means $\pm$ s.e.mean of six experiments in each group.

Figure 6 Effect of insulin on acute troglitazone action. Time-course of bile flow and Δ portal pressure during the basal period and during glucagon stimulation (0.1 nM) in the isolated perfused rat liver. Effect of infusion of insulin with or without troglitazone (0.61 μ M) compared with control (Krebs-Henseleit buffer + 0.2% DMSO) under BSA-free conditions. Data are given as means $\pm$ s.e. mean of six experiments in each group.

conditions, only insulin alone resulted in a decrease of glucose release; the gradual fall in glucose release observed with low dose troglitazone did not reach significance. Insulin induced a small decrease ($P < 0.01$) in lactate production rates with rapid onset; this was not seen when insulin and low dose troglitazone were administered together (Figure 5).

Low dose troglitazone with or without insulin did not affect hepatic bile secretion (Figure 6). Portal pressure rapidly (in cm buffer height) increased within 15 min during infusion of low dose troglitazone + insulin (0.54 ± 0.07) and was higher than observed during infusion of insulin (0.17 ± 0.07 , $P < 0.005$) or vehicle alone (0.08 ± 0.06 , $P < 0.0001$; Figure 6).

Discussion

Acute troglitazone exposure did not decrease basal hepatic glucose production, but inhibited glucagon-stimulated glucose release. The effect was observed only at perfusate troglitazone concentrations of 3.15 μ M, which is ~ 6 fold higher than plasma concentrations measured in effectively treated rodents (Lee *et al.*, 1994; Khourshed *et al.*, 1995). It is of note that 3.15 μ M troglitazone was ineffective in the presence of albumin as reported for isolated rat muscle (Fürnsinn *et al.*, 1997). This is most likely due to $\sim 99.9\%$ binding of troglitazone to albumin solutions and plasma (Shibukawa *et al.*, 1995) making it difficult to compare *in vitro* concentrations with those measured in plasma during treatment.

The present study employed an experimental model of hepatic glycogenolysis, since the livers from fed rats are full of glycogen and perfusion with 5 mM glucose favours hepatic glucose release (Brunengraber *et al.*, 1973). The observed

partial inhibition of glucagon-stimulated glucose production is therefore primarily due to decreased glycogenolysis. The reduction by troglitazone of glucagon-stimulated glycogenolysis can be interpreted rather as an unspecific toxic effect than an acute insulin-like action as shown for other compounds (Roden *et al.*, 1995). Previous reports examined effects of thiazolidinediones on lactate-dependent gluconeogenesis in isolated rat hepatocytes. At high concentrations of 100–1000 μ M, pioglitazone and more effectively troglitazone inhibited free fatty acid oxidation, triglyceride synthesis, and glucose production from lactate and pyruvate (Fulgencio *et al.*, 1996). The decrease of free fatty acid oxidation could likely account for the observed reduction of gluconeogenesis. But although the authors argued that a generalized toxic effect is unlikely, since other metabolic actions like phospholipid synthesis or carnitine palmitoyl transferase-1 activity were not affected, an unspecific troglitazone effect cannot be excluded from those data. By using lower troglitazone concentrations (30 μ M) gluconeogenesis decreased by $\sim 23\%$ in hepatocytes obtained from fasted rats, but not in those from fed rats (Raman *et al.*, 1998). It has been suggested (Raman *et al.*, 1998; Nishimura *et al.*, 1997) that thiazolidinediones decrease gluconeogenesis by elevation of hepatocyte fructose-2,6-bisphosphate which inhibits the key enzyme of gluconeogenesis, fructose-1,6-bisphosphatase (Van Schaftingen & Hers, 1981), while it activates the glycolytic key enzyme 6-phosphofructo-1-kinase (Pilkis *et al.*, 1990). However, considering the high concentrations employed in these studies, the decrease of lactate-dependent gluconeogenesis may have also resulted from an unspecific thiazolidinedione effect which would favour the glycolytic flux and thereby reduce gluconeogenesis. Recently it was reported that troglitazone at a comparable concentration of 3.25 μ M acutely induces an intracellular shift of glucosyl units into glycolysis and rather mimicks hypoxic than insulin-like action in isolated rat muscle (Fürnsinn *et al.*, 1997).

This study also found that 3.15 μ M troglitazone transiently increased hepatic lactate production. During simultaneous insulin exposure, lactate production rates were elevated even in the presence of 0.61 μ M troglitazone as observed in isolated rat muscle tissue (Fürnsinn *et al.*, 1997). In liver, insulin does not directly affect glucose transport but directs the flux of intracellular glucose to glycogen synthesis so that lactate production will decrease. Thus, the observed lactate release by troglitazone cannot be attributed to an insulin-like effect but rather indicates augmentation of anaerobic glycolysis in the liver cell, which is in keeping with a hypoxia-like mechanism for acute troglitazone action. It is of note that troglitazone must have led only to moderate hypoxia in the present study, since severe hypoxia or anoxia would have rapidly increased intracellular adenosine-monophosphate (AMP) or adenosine-diphosphate (ADP), which activate phosphorylase and thereby increase glycogenolysis (Hems & Whitton, 1980; Cheng *et al.*, 1985). During the not-stimulated perfusion period, no such increase in glycogenolysis was induced by troglitazone. Interestingly, higher troglitazone concentrations increase the ADP/ATP ratio, but not glucose production in hepatocytes obtained from fed rats (Fulgencio *et al.*, 1996).

Independently of the presence of BSA, 3.15 μ M troglitazone rapidly decreased bile flow during the basal period. Such acute reduction of bile flow has been described for classical hepatotoxic agents like carbon tetrachloride (Fracasso *et al.*, 1980) or phenothiazine (Eckhart *et al.*, 1963). The explanation for this action may be direct effects on the hepatocyte resulting in decreased bile formation and/or indirect effects resulting from redistribution of hepatic microcirculation (Tavoloni *et al.*

al., 1979; Krell & Dietze, 1989). The latter might in turn also increase portal pressure, which we noted to some extent in the present study. However, the effect of troglitazone on portal pressure was not as consistent as that on bile flow. In contrast, the concept of direct thiazolidinedione action on hepatocyte function is supported by pharmacokinetic studies demonstrating troglitazone uptake and metabolism by livers of rats, mice, and dogs and high biliary excretion rates of the resulting metabolites (Kawai *et al.*, 1997). This might explain the mild impairment of liver function, which was observed in ~2% of patients enrolled in clinical troglitazone trials, as well as the rare cases of severe liver failure requiring liver transplantation (Neuschwander-Tetri *et al.*, 1998).

Taken together, troglitazone given at concentrations generally used to study its metabolic actions decreased

glucagon-stimulated glucose production, but also increased lactate production, reduced bile flow, and slightly increased portal pressure. These data therefore provide evidence for an acute, non-insulin-like toxic effect of troglitazone in perfused liver.

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