

Differences in troglitazone action on glucose metabolism in freshly isolated *vs* long-term incubated rat skeletal muscle

¹Florian Gras, ¹Barbara Brunmair, ¹Michael Roden, ¹Werner Waldhäusl & ^{*,1}Clemens Fürnsinn

¹Department of Medicine III, Division of Endocrinology & Metabolism, University of Vienna, Austria

1 Exposure of isolated skeletal muscle to troglitazone has resulted in inconsistent findings ranging from inhibition to stimulation of fuel oxidation and the glycolytic pathway. To better understand such variation in outcome, the present study used isolated rat soleus muscle strips to examine the interdependent influences of prolonged maintenance *in vitro* and of troglitazone exposure.

2 If freshly isolated muscle strips were exposed to troglitazone ($1\ \mu\text{mol l}^{-1}$) for 24 h, glucose oxidation was markedly reduced ($-26\pm 1\%$, $P<0.0001$), whereas glycogen synthesis remained unaffected ($+9\pm 7\%$, n.s.).

3 In contrast, extended exposure to troglitazone for 72 h increased both glucose oxidation ($+65\pm 28\%$, $P<0.05$) and glycogen synthesis ($+46\pm 11\%$, $P<0.005$), and a similar stimulatory effect was also observed in muscles exposed to troglitazone only during the last 24 h of their 72 h preincubation period (glucose oxidation: $+61\pm 15\%$, $P<0.001$; glycogen synthesis: $+43\pm 15\%$, $P<0.01$).

4 Troglitazone thus stimulated glucose utilization in long-term incubated muscle independent of the duration of exposure (24 or 72 h), whereas it inhibited glucose utilization in freshly isolated muscle.

5 The observed differences in troglitazone action on freshly isolated *vs* long-term incubated muscle suggest that findings on muscle tissue subject to prolonged maintenance *in vitro* cannot be extrapolated to native muscle *in vivo*.

British Journal of Pharmacology (2003) **138**, 1140–1146. doi:10.1038/sj.bjp.705162

Keywords: Thiazolidinedione; troglitazone; skeletal muscle; glycogen storage; glucose oxidation; *in vitro*

Abbreviations: TZD, thiazolidinedione; PPAR γ , peroxisome proliferator-activated receptor; SD rats, Sprague–Dawley rats; DMSO, dimethyl sulfoxide

Introduction

Thiazolidinediones (TZDs) are potent agonists of the nuclear peroxisome proliferator-activated receptor γ (PPAR γ), which is predominantly expressed in adipose tissue and mediates adipocyte differentiation and adipose tissue remodelling (Spiegelman, 1998; Olefsky, 2000; Rosen & Spiegelman, 2001). The associated antihyperglycemic effect of TZDs, however, is mainly attributed to improved insulin-induced glucose disposal into skeletal muscle (Inzucchi *et al.*, 1998; Olefsky, 2000; Rosen & Spiegelman, 2001). It is as yet unclear, whether the observed improvement in muscle glucose disposal is due to direct TZD action on skeletal muscle or due to indirect action via substrate or hormone signals from adipose tissue (Spiegelman, 1998; Olefsky, 2000; Rosen & Spiegelman, 2001).

To separate direct from indirect effects, the interaction of TZDs with isolated specimens of skeletal muscle has been repeatedly examined *in vitro*. A bulk of studies showed that TZDs stimulate glucose transport into muscle cells independently of concomitant insulin stimulation (El-Kebbi *et al.*, 1994; Ciaraldi *et al.*, 1995; Arakawa *et al.*, 1998; Park *et al.*, 1998; Fürnsinn *et al.*, 1999, 2000; Brunmair *et al.*, 2001; Cha *et al.*, 2001; Kausch *et al.*, 2001; Yonemitsu *et al.*, 2001). In contrast to the effects on glucose uptake, the observed TZD-induced changes in intracellular glucose fluxes were rather inconsistent. Whereas prolonged exposure (≥ 48 h) to

troglitazone increased glycogen synthase activity, glycogen synthesis, and mitochondrial palmitate oxidation in human muscle cell cultures (Park *et al.*, 1998; Cha *et al.*, 2001; Kausch *et al.*, 2001), short-term exposure (≤ 24 h) to identical troglitazone concentrations inhibited mitochondrial fuel oxidation and glycogen storage in native rat soleus muscle strips (Fürnsinn *et al.*, 2000; Brunmair *et al.*, 2001). Although the latter short-term effects were apparently independent of PPAR γ (Brunmair *et al.*, 2001), it cannot be excluded that this receptor could mediate delayed actions on skeletal muscle (Zierath *et al.*, 1998; Loviscach *et al.*, 2000).

The present study was to unravel the cause of controversial troglitazone effects on glucose utilization *in vitro*. It is shown that the metabolic characteristics and the response to troglitazone of freshly isolated native muscle deviate strongly from those of long-term incubated muscle. From this, it appears that findings on muscle tissue subject to prolonged maintenance *in vitro* are of limited value for our understanding of TZD action on native muscle *in vivo*.

Methods

Rats

Where not stated otherwise, male Sprague – Dawley (SD) rats (approximately 5 weeks old, 140 g body weight) from the

*Author for correspondence; E-mail: clemens.fuernsinn@akh-wien.ac.at

breeding facilities of the University of Vienna (Himberg, Austria) were employed. In some experiments, male genetically obese Zucker rats (fa/fa; approximately 5 months old, 650 g body weight) from Harlan-Winkelmann (Borchen, Germany) were used. Rats were kept at an artificial 12-h-light/12-h-dark cycle at constant room temperature. They were provided with conventional laboratory diet and tap water *ad libitum*. Food, but not water, was withdrawn overnight before rats were killed by cervical dislocation between 8:30 and 9:30 a.m. All experiments were performed according to local law and according to the principles of good laboratory animal care.

Preincubation period

Immediately after killing, two (SD rats) or three (Zucker rats) longitudinal soleus muscle strips per leg (i.e. four or six strips per rat) were prepared, weighed (approximately 25 mg strip⁻¹), and tied under tension on stainless-steel clips as described previously (Crettaz *et al.*, 1980). According to procedures employed earlier (Stace *et al.*, 1990; Brunmair *et al.*, 2001), muscles were immediately put into 50-ml-Erlenmeyer flasks coated with BlueSlick solution (Serva, Heidelberg, Germany) and were placed into a shaking waterbath (six strips per flask; 37°C; 130 cycles min⁻¹). Each flask contained 20-ml cell culture medium 199 (pH 7.35, 5.5 mmol l⁻¹ glucose; Sigma, St Louis, MO, U.S.A. Cat. No. M-4530) with additions of 0.3% (w v⁻¹) fatty acid-free bovine serum albumin (BSA), 5 mmol l⁻¹ HEPES, 25,000 U l⁻¹ penicillin G, 25 mg l⁻¹ streptomycin, and 0.2 mg l⁻¹ ciprofloxacin. Palmitate was dissolved in ethanol and added to the medium to give final concentrations of 300 µmol l⁻¹ palmitate and 0.25% ethanol (v v⁻¹). Troglitazone (generously provided by Sankyo, Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO, USA) and was added to give 0.1% (v v⁻¹) DMSO and the indicated concentrations of troglitazone. The same concentration of DMSO was present in control media without troglitazone. All preincubations were in the absence of insulin and an atmosphere of 95% O₂:5% CO₂ was continuously provided within the flasks. Experimental protocols differed with regard to the periods of muscle preincubation and of troglitazone exposure (details described in the Results section and outlined in the respective figures). In experiments with preincubation periods of 48 or 72 h, fresh medium was provided every 24 h.

Measurement period

Immediately after preincubation, muscles were transferred for 1 h into 25-ml flasks (one strip per flask) containing 3 ml of medium additionally supplemented with trace amounts of D-[¹⁴C]glucose (Amersham, Amersham, U.K.). If not stated otherwise, measurements were performed under stimulation with 100 nmol l⁻¹ human insulin (Actrapid, Novo, Bagsvaerd, Denmark). At the end of the 1-h-measurement period, muscles were quickly removed from the flasks, blotted, and frozen in liquid nitrogen.

Analytical procedures

Frozen muscle strips were lysed in 1 mol l⁻¹ KOH at 70°C. The net rate of glucose incorporation into glycogen is referred to as glycogen synthesis and was calculated from the conversion of

[¹⁴C]glucose into [¹⁴C]glycogen as described previously (Crettaz *et al.*, 1980). Rates of CO₂ production from glucose are referred to as glucose oxidation and were calculated from the conversion of [¹⁴C] glucose into ¹⁴CO₂ which was trapped with a solution containing methanol and phenethylamine (1:1) (Fürnsinn *et al.*, 1995). The applied methods give the amount of extracellular glucose converted into glycogen and CO₂ but do not provide any information on glycogen cycling or on the oxidation of glucosyl units derived from the initially unlabelled glycogen pool.

For measurement of muscle glycogen content, glycogen in the muscle lysate was degraded to glucose with amyloglucosidase (Fürnsinn *et al.*, 1995) and glucose was then measured enzymatically by a commercial kit (Human, Taunusstein, Germany).

Statistics

All results are given as means ± s.e.m. Two-tailed paired or unpaired Student's *t*-test was used as appropriate, and a value of *P* < 0.05 was considered as significant. The influence of troglitazone was always evaluated by paired comparison of muscle strips from the same rat that were incubated for the same time period in the presence *vs* the absence of troglitazone.

Results

Effects of the incubation per se (Figure 1)

Muscle specimens were preincubated under control conditions (i.e. without troglitazone) immediately after preparation for 0.5, 24, 48, or 72 h. Subsequently, the rates of insulin-stimulated glucose metabolism were measured (Figure 1a). Figures 1b–f show the time-dependent influence of the incubation procedure *per se* on glucose metabolism. After 24 h of maintenance *in vitro*, glucose incorporation into glycogen (Figure 1b) and glycogen content (Figure 1c) were significantly diminished. The oxidation of glucose derived from the ambient medium was increased about four-fold (Figure 1d), obviously because the considerable reduction in glycogenic flux caused more radiolabelled glucose from the extracellular medium to directly enter glycolysis. Consequently, the ratio of net glucose flux into glycogenesis to glucose flux into oxidation was very markedly reduced from 10.6:1 to 1.2:1 (Figure 1f) and the sum of glucose converted into glycogen and CO₂ decreased during the first day of incubation (Figure 1e).

Maintenance of muscle strips *in vitro* for more than 24 h was associated with progressive reductions in glycogen synthesis, glucose oxidation, and glycogen content, whereas the ratio of glycogenesis/oxidation remained constant between 24 h and 72 h. Taken together, the incubation procedure *per se* caused a rapid and distinct reduction of glycogenic activity within 24 h, as well as a progressive reduction of the sum of glucose conversion into glycogen plus CO₂.

Time-dependent effects of troglitazone (Figure 2)

If troglitazone (1 µmol l⁻¹) was added under the same experimental conditions (Figure 2a), insulin-stimulated glucose

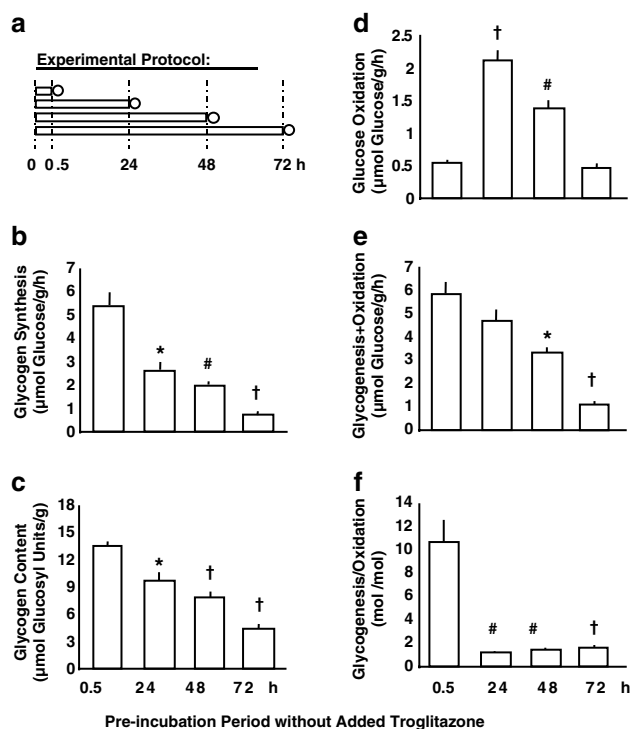


Figure 1 Effects of incubation *per se* (no troglitazone added). Experimental protocol (a): immediately after preparation, soleus muscle strips from Sprague–Dawley rats were preincubated for 0.5, 24, 48, or 72 h in the absence of insulin (preincubation period; bars), which was followed by 1 h under insulin stimulation (measurement period; circles). During the measurement period, rates of conversion of glucose into glycogen (glycogen synthesis; (b)) and CO₂ (glucose oxidation; (d)) were determined. Glycogen content as prevailing at the end of the experiments (c), as well as the sum and the quotient of glycogenesis plus/per glucose oxidation ((e) and (f), respectively) are also shown. Means \pm s.e.m.; $n = 6-12$ each; * $P < 0.005$, # $P < 0.001$, † $P < 0.0001$ vs preincubation for 0.5 h.

oxidation was markedly blunted *vs* the controls after 24 h (Figure 2d). At this time, glycogen storage was not significantly affected by troglitazone (Figures 2b and c). With more prolonged incubation, the troglitazone-induced reduction in glucose oxidation faded (48 h) and then turned into a troglitazone-dependent increase (72 h). After 72 h, insulin-stimulated glycogen synthesis, glycogen content, and glucose oxidation were higher in troglitazone-exposed *vs* control muscles. Troglitazone thus acted both as an inhibitor and as a stimulator of glucose utilization, depending on the duration of the preincubation period. Inhibitory action predominated during the initial 24 h and was progressively overcome by stimulatory action during more prolonged incubation.

Effects of 72 h troglitazone: dose- and insulin-dependency (Figure 3)

In muscle strips exposed to troglitazone for 72 h (Figure 3a), increases in insulin-stimulated net glycogen synthesis and glycogen content were observed at 1 and 3 $\mu\text{mol l}^{-1}$ troglitazone (Figures 3b and c). At 9 $\mu\text{mol l}^{-1}$ troglitazone, insulin-stimulated glycogenesis and glycogen content were back to control values. Furthermore, troglitazone dose-dependently stimulated glucose oxidation which, in contrast to glycogen storage, increased further at 9 $\mu\text{mol l}^{-1}$ troglitazone (Figure 3d).

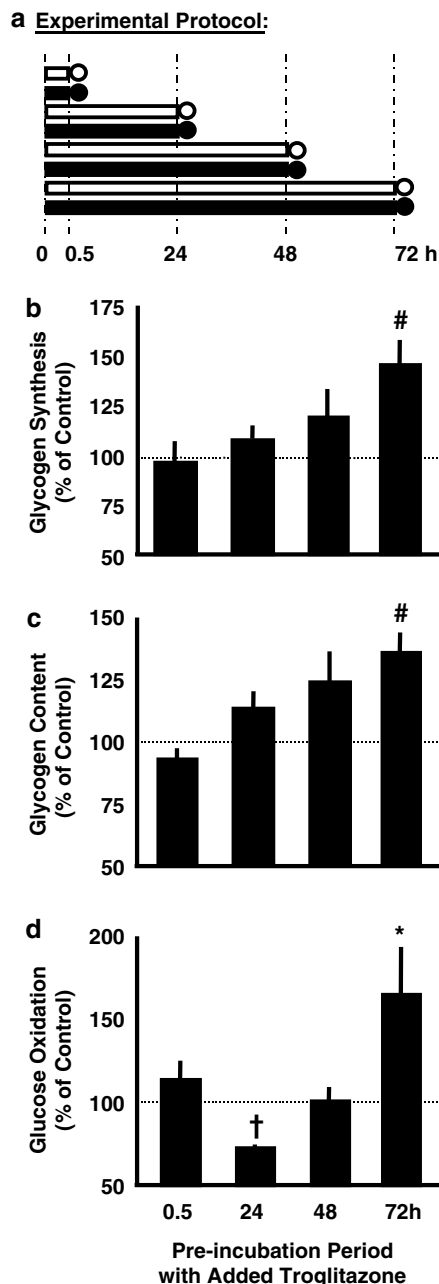


Figure 2 Time-dependent effects of troglitazone. Experimental protocol (a): soleus muscle strips from Sprague–Dawley rats were preincubated without insulin in the absence (open symbols) or presence (full symbols) of 1 $\mu\text{mol l}^{-1}$ troglitazone for 0.5, 24, 48, or 72 h (preincubation period; bars), which was followed by 1 h under insulin stimulation (measurement period; circles). During the measurement period, rates of conversion of glucose into glycogen (glycogen synthesis; (b)) and CO₂ (glucose oxidation; (d)) were determined. Glycogen content is shown as prevailing at the end of the respective experiment (c). Data are given as a percentage of an intraindividual control muscle incubated in the absence of troglitazone (absolute rates under control conditions are given in Figure 1). Means \pm s.e.m.; $n = 6-12$ each; * $P < 0.05$, # $P < 0.005$, † $P < 0.0001$ vs control without troglitazone (= 100%).

Basal and insulin-stimulated glucose oxidation increased to the same extent in response to incubation with 1 $\mu\text{mol l}^{-1}$ troglitazone for 72 h (Figure 3g). Basal glycogen synthesis prevailing during the last hour of the experiment (measurement

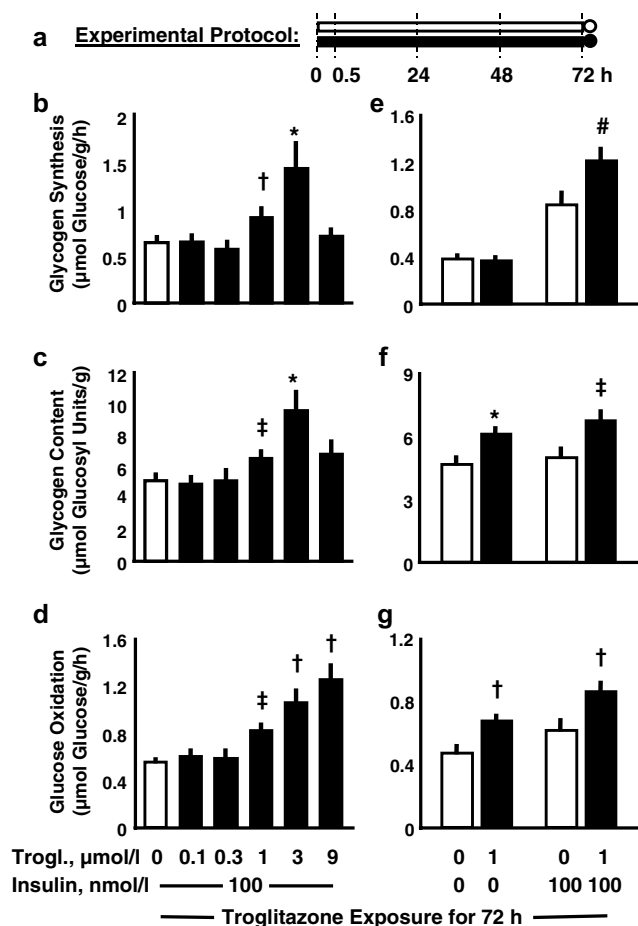


Figure 3 Effects of 72 h troglitazone; dose-dependency and insulin-dependency. Experimental protocol (a): soleus muscle strips from Sprague–Dawley rats were preincubated without insulin in the absence (open symbols) or presence (full symbols) of various concentrations of troglitazone for 72 h (preincubation period; bars), which was followed by 1 h under basal conditions or under insulin stimulation (measurement period; circles). During the measurement period, rates of conversion of glucose into glycogen (glycogen synthesis; (b), (e)) and CO₂ (glucose oxidation; (d), (g)) were determined. Glycogen content is shown as prevailing at the end of the respective experiment ((c), (f)). Means \pm s.e.m.; $n=6-18$ each; * $P<0.02$, # $P<0.01$, † $P<0.005$, ‡ $P<0.001$ vs control without troglitazone.

period) was the same in control and troglitazone-exposed muscle (Figure 3e), whereas glycogen content, which reflects net glycogen storage during the whole experiment (including the preincubation period), was elevated in troglitazone-exposed *vs* control muscles (Figure 3f). Troglitazone thus affected glucose oxidation as well as glycogen storage independent of insulin.

Effects of 72 h troglitazone: insulin-resistant muscle (Figure 4)

Specimens of soleus muscle from obese Zucker rats, which exhibit marked insulin resistance (Crettaz *et al.*, 1980), were continuously exposed to troglitazone for 72 h (Figure 4a). At $1 \mu\text{mol l}^{-1}$ troglitazone, basal and insulin-stimulated glucose oxidation were increased to a similar extent (Figure 4d). Basal and insulin-stimulated glycogen synthesis were increased by approximately 25 and 45%, respectively, but the latter effect

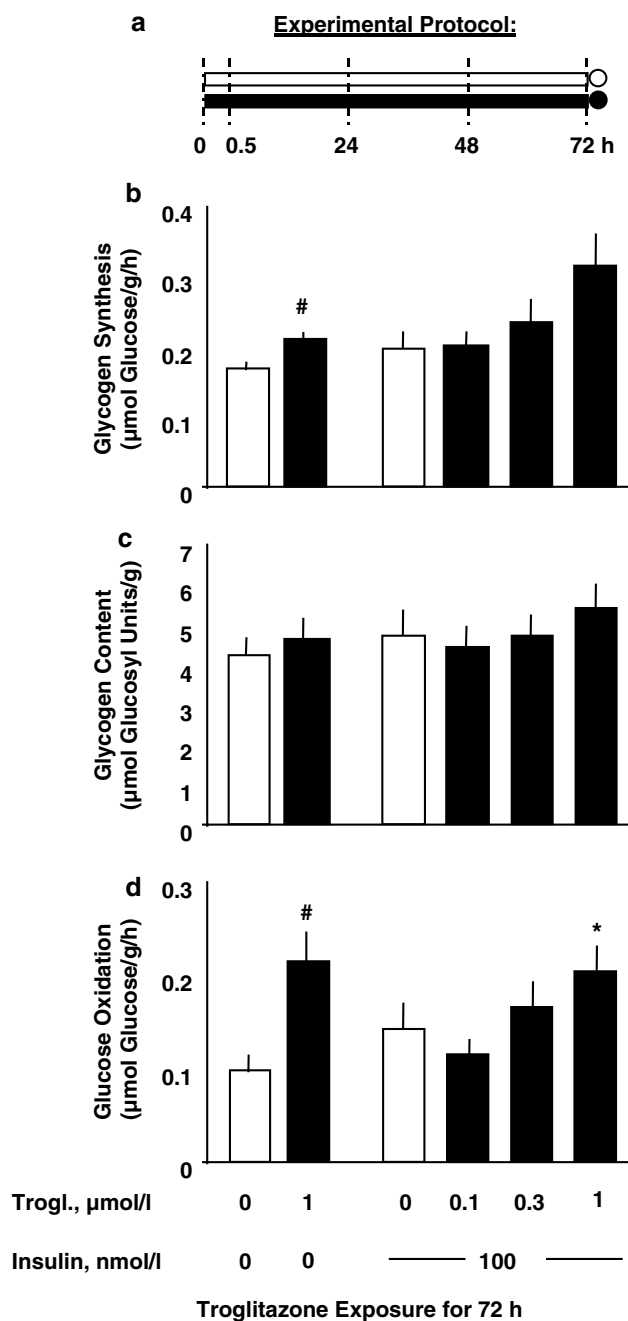


Figure 4 Effects of 72 h troglitazone; insulin resistant muscle. Experimental protocol (a): soleus muscle strips from obese Zucker rats (fa/fa) were preincubated without insulin in the absence (open symbols) or presence (full symbols) of various concentrations of troglitazone for 72 h (preincubation period; bars), which was followed by 1 h under basal conditions or under insulin stimulation (measurement period; circles). During the measurement period, rates of conversion of glucose into glycogen (glycogen synthesis; (b)) and CO₂ (glucose oxidation; (d)) were determined. Glycogen content is shown as prevailing at the end of the respective experiment (c). Means \pm s.e.m.; $n=9-12$ each; * $P<0.05$, # $P<0.01$ vs control without troglitazone.

was not significant ($P=0.06$; Figure 4b). Glycogen content was not significantly affected (Figure 4c). Hence, incubation with $1 \mu\text{mol l}^{-1}$ troglitazone for 72 h caused similar increases in glucose utilization by healthy and insulin-resistant muscle strips (Figures 3 and 4, respectively).

Effects of duration of troglitazone exposure on muscle strips incubated for 72 h (Figure 5)

The effects of 24, 48, and 72 h of troglitazone exposure were compared in muscle strips kept *in vitro* for the same total period of 72 h (Figure 5a). Addition of $1 \mu\text{mol l}^{-1}$ troglitazone during the last 24 h of the 72 h preincubation period resulted in increased rates of insulin-stimulated glycogen synthesis and glucose oxidation (Figures 5b and d). Thus, a dose and duration of troglitazone exposure ($1 \mu\text{mol l}^{-1}$ for 24 h), which distinctly inhibited glucose oxidation in freshly isolated skeletal muscle (Figure 2d), exerted stimulatory action in muscle strips preadapted to the *in vitro* environment for 48 h (Figure 5d).

Acute effects of troglitazone after different periods of preincubation (Figure 6)

Muscles were preincubated for different time periods, before the acute effects of $20 \mu\text{mol l}^{-1}$ troglitazone were determined during the last hour of the experiment (= measurement period; Figure 6a). In muscle strips allowed to adapt to the *in vitro* environment for the preceding 24 or 48 h, acute treatment with troglitazone blunted both insulin-stimulated glycogen synthesis and glucose oxidation. In muscle strips subjected to prolonged preincubation for 72 h, however, troglitazone failed to elicit a comparable response (Figures 6b and d). Glycogen content was not affected within 1 h of exposure to $20 \mu\text{mol l}^{-1}$ troglitazone independent of the preincubation period (Figure 6c). Thus, responsiveness to the acute inhibitory action of troglitazone was lost during prolonged maintenance *in vitro*.

Discussion and conclusions

The results confirm the finding of 24-h exposure to $1 \mu\text{mol l}^{-1}$ troglitazone to distinctly inhibit mitochondrial fuel oxidation but not glycogen synthesis in freshly isolated soleus muscle strips obtained from SD rats (Fürnsinn *et al.*, 2000). Higher concentrations of the TZD ($\geq 2.5 \mu\text{mol l}^{-1}$) are known to reduce glycogen synthesis in parallel with glucose oxidation (Fürnsinn *et al.*, 2000; Brunmair *et al.*, 2001). It is of note, however, that inhibitory action of short-term troglitazone exposure was reversed to stimulatory action during prolonged incubation exceeding 24 h. After 72 h, both glycogen synthesis and glucose oxidation were markedly increased in troglitazone-treated vs control muscles. Low glycogen content, a potent stimulator of glycogen synthesis in skeletal muscle

(Nielsen *et al.*, 2000), cannot explain our findings because glycogen content was elevated after 72 h troglitazone. Such delayed increase in glucose utilization is in line with reports on muscle-derived cell cultures that exhibit increased glycogen

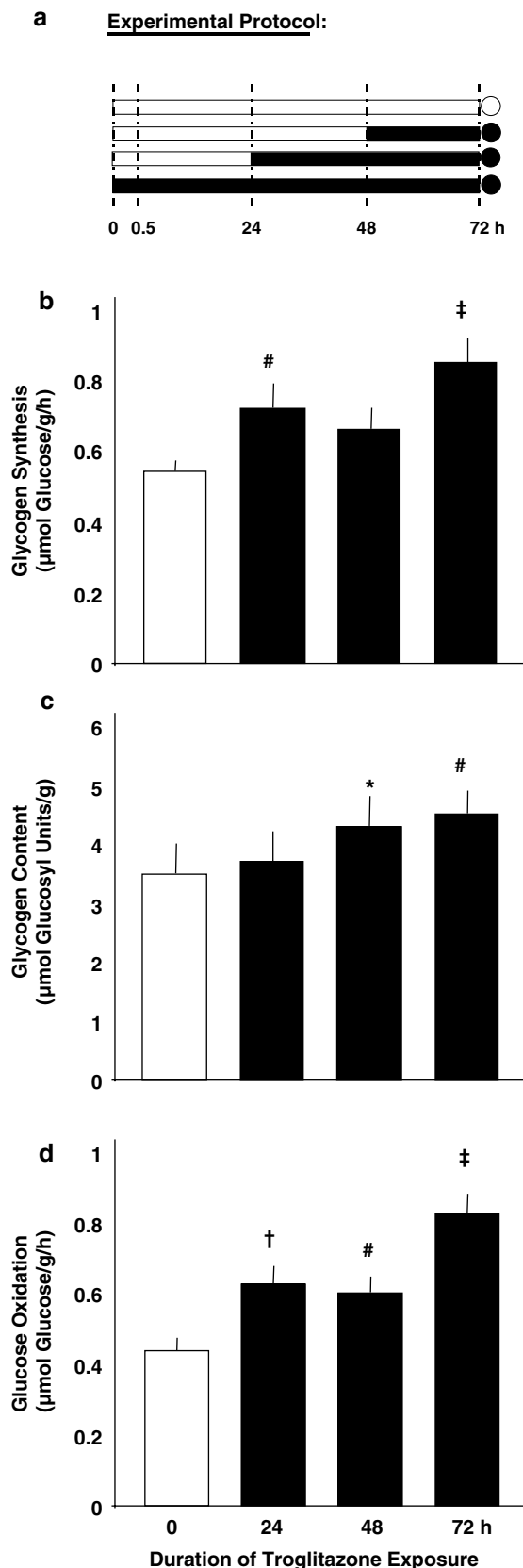


Figure 5 Effects of duration of troglitazone exposure on muscle strips incubated for 72 h. Experimental protocol (a): soleus muscle strips from Sprague–Dawley rats were preincubated without insulin for a total period of 72 h (preincubation period, bars) without troglitazone or with $1 \mu\text{mol l}^{-1}$ troglitazone added during the last 24 h, the last 48 h, or the whole preincubation period (absence of troglitazone: open symbols; presence of troglitazone: full symbols), which was followed by 1 h under insulin stimulation (measurement period; circles). During the measurement period, rates of conversion of glucose into glycogen (glycogen synthesis; (b) and CO_2 (glucose oxidation; (d)) were determined. Glycogen content is shown as prevailing at the end of the respective experiment (c). Mean \pm s.e.m.; $n=23$ or 24 each; $*P<0.05$, $^{\#}P<0.03$, $^{\dagger}P<0.002$, $^{\ddagger}P<0.001$ vs control without troglitazone.

synthase activity, glycogen synthesis, and mitochondrial palmitate oxidation after long-term troglitazone exposure (Park *et al.*, 1998; Cha *et al.*, 2001; Kausch *et al.*, 2001).

The delayed stimulatory effects on glucose metabolism seen after 72 h of troglitazone exposure of rat soleus muscle *in vitro* exhibit several parallels with changes in glucose metabolism as

associated with oral TZD treatment *in vivo*. They include that (i) glucose lowering action *in vivo* and increased utilization *in vitro* are delayed responses; (ii) the effects *in vivo* and *in vitro* are seen at troglitazone concentrations in the low micromolar range (prevailing in the incubation medium and in circulating plasma, respectively; Plosker & Faulds, 1999); and (iii) in rat soleus muscle exposed to troglitazone for 72 h *in vitro* as well as in soleus muscle from orally TZD-treated obese rodents, glucose oxidation is elevated independent of concomitant insulin stimulation, whereas glycogen synthesis is increased in an insulin-dependent manner (Stevenson *et al.*, 1990; Fürsinn *et al.*, 1999; Sreenan *et al.*, 1999).

On the first glimpse, these parallels in troglitazone action *in vitro* and *in vivo* make it tempting to speculate that the same mechanism(s) could be responsible for the delayed actions of troglitazone under both conditions, and that isolated muscle could be ideal to investigate the mechanisms of antidiabetic TZD action. However, the presented data suggest a very different conclusion.

We show that the simple maintenance of isolated skeletal muscle *in vitro* causes very distinct changes in its glucose handling. The most salient effect of the employed incubation procedure was a considerable reduction in glycolytic activity within only 24 h, which could be caused by the absence of regular cycles of glycogen depletion and restorage under *in vitro* conditions. Prolonged exposure of skeletal muscle to its artificial environment within the test flask also caused major changes in how the tissue specimens responded to troglitazone. Exposure to $1 \mu\text{mol l}^{-1}$ troglitazone for 24 h markedly inhibited glucose oxidation and failed to affect glycogen synthesis in freshly isolated muscle, but the same troglitazone concentration and the same duration of exposure exerted stimulatory action in muscle strips that were already adapted to the *in vitro* situation for a prolonged period of time. Furthermore, acute inhibition of glucose oxidation by $20 \mu\text{mol l}^{-1}$ troglitazone within 1 h is seen in freshly prepared muscles (Brunmair *et al.*, 2001) and in muscle strips kept *in vitro* since 24 or 48 h (this study), but is absent after more prolonged maintenance *in vitro* for 72 h. Hence, the delay in troglitazone-induced stimulation of glucose utilization is obviously not because such response to troglitazone requires prolonged exposure to the agent, but rather because such response characterizes long-term incubated muscle and cultured muscle cells, but not freshly isolated native muscle.

As insulin resistance has been reported to develop in denervated skeletal muscle (Burant *et al.*, 1984), it could be speculated that the different responses to troglitazone by fresh vs long-term incubated muscle are related to prevailing insulin sensitivity. This, however, is excluded by our previous

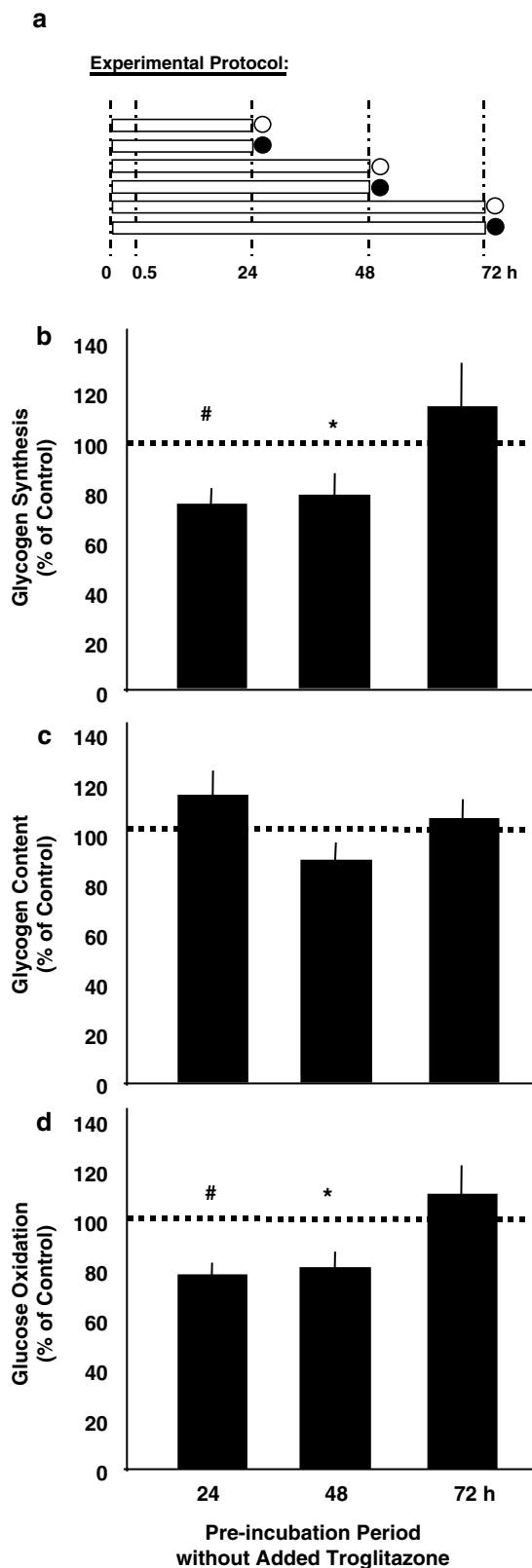


Figure 6 Acute effects of troglitazone after different periods of pre-incubation. Experimental protocol (**a**): soleus muscle strips from Sprague–Dawley rats were preincubated in the absence of insulin and troglitazone for 24, 48, or 72 h (preincubation period; bars), which was followed by 1 h of insulin stimulation in the absence or presence of $20 \mu\text{mol l}^{-1}$ troglitazone (measurement period; absence of troglitazone: open circles; presence of troglitazone: full circles). During the measurement period, rates of conversion of glucose into glycogen (glycogen synthesis; **b**) and CO_2 (glucose oxidation; **d**) were determined. Glycogen content is shown as prevailing at the end of the respective experiment (**c**). Data are given as a percentage of an intraindividual control muscle that was incubated in the absence of troglitazone. Means \pm s.e.m.; $n = 12-14$ each; $^*P < 0.03$, $^{\#}P < 0.003$ vs control (= 100%).

observation that 24 h of incubation with troglitazone inhibits glucose oxidation not only in normal but also in insulin resistant muscle from obese Zucker rats (Brunmair *et al.*, 2001), which opposes stimulatory action on long-term incubated muscle. As troglitazone's stimulatory effect after 72 h of incubation is also seen in both normal and insulin-resistant muscle, the response to troglitazone is obviously determined by the period of maintenance *in vitro* independently of prevailing insulin sensitivity.

Taken together, we show that glucose handling by isolated muscle progressively changes in response to its artificial environment, which necessarily implies that extrapolations from *in vitro* to *in vivo* become increasingly doubtful the longer the employed muscle specimens or muscle cells are separated from their physiologic environment. Adaptions to the *in vitro* conditions include that of troglitazone, which acts as an inhibitor of glucose utilization in freshly isolated native muscle, stimulates glucose utilization in long-term incubated

or cultured muscle. It is therefore doubtful whether the demonstration of direct stimulatory action of troglitazone on fuel oxidation and glycogenic activity in long-term incubated muscle or cultured muscle cells (Park *et al.*, 1998; Cha *et al.*, 2001; Kausch *et al.*, 2001; and this study) is appropriate evidence for such actions *in vivo*. Although it cannot be principally excluded that the mechanism responsible for troglitazone-induced stimulation of glucose utilization in long-term incubated muscle is relevant for the agent's antidiabetic action *in vivo*, our findings clearly define the limits in drawing conclusions from long-term incubated skeletal muscle or muscle-derived cell lines.

We thank Sankyo (Tokyo, Japan) for generously providing troglitazone. We thank the staff at the Biomedical Research Centre, University of Vienna, for taking care of the rats. This work was supported by the Austrian Science Fund (Grant No. P13049-MED).

References

- ARAKAWA, K., ISHIHARA, T., AOTO, M., INAMUSA, M., SAITO, A. & IKEZAWA, K. (1998). Actions of novel antidiabetic thiazolidinedione, T-174, in animal models of non-insulin-dependent diabetes mellitus (NIDDM) and in cultured muscle cells. *Br. J. Pharmacol.*, **125**, 429–436.
- BRUNMAIR, B., GRAS, F., NESCHEN, S., RODEN, M., WAGNER, L., WALDHÄUSL, W. & FÜRNSINN, C. (2001). Direct thiazolidinedione action on isolated rat skeletal muscle fuel handling is independent of peroxisome proliferator-activated receptor- γ -mediated changes in gene expression. *Diabetes*, **50**, 2309–2315.
- BURANT, C.F., LEMMON, S.K., TREUTELAAR, M.K. & BUSE, M.G. (1984). Insulin resistance of denervated rat muscle: a model for impaired receptor – function coupling. *Am. J. Physiol.*, **247**, E657–E666.
- CHA, B.S., CIARALDI, T.P., CARTER, L., NIKOULINA, S.E., MUDALIAR, S., MUKHERJEE, R., PATERNITI, J.R. & HENRY, R.R. (2001). Peroxisome proliferator-activated receptor (PPAR) γ and retinoid X receptor (RXR) agonists have complementary effects on glucose and lipid metabolism in human skeletal muscle. *Diabetologia*, **44**, 444–452.
- CIARALDI, T.P., HUBER-KNUDSEN, K., HICKMAN, M. & OLEFSKY, J.M. (1995). Regulation of glucose transport in cultured muscle cells by novel hypoglycemic agents. *Metabolism*, **44**, 976–982.
- CRETTEAZ, M., PRENTKI, M., ZANIETTI, D. & JEANRENAUD, B. (1980). Insulin resistance in soleus muscle from obese Zucker rats. Involvement of several defective sites. *Biochem. J.*, **186**, 525–534.
- EL-KEBBI, I.M., ROSER, S. & POLLET, R.J. (1994). Regulation of glucose transport by pioglitazone in cultured muscle cells. *Metabolism*, **43**, 953–958.
- FÜRNSINN, C., BRUNMAIR, B., MEYER, M., NESCHEN, S., FURTMÜLLER, R., RODEN, M., KÜHNLE, H.F., NOWOTNY, P., SCHNEIDER, B. & WALDHÄUSL, W. (1999). Chronic and acute effects of thiazolidinediones BM13.1258 and BM15.2054 on rat skeletal muscle glucose metabolism. *Br. J. Pharmacol.*, **128**, 1141–1148.
- FÜRNSINN, C., BRUNMAIR, B., NESCHEN, S., RODEN, M. & WALDHÄUSL, W. (2000). Troglitazone directly inhibits CO₂ production from glucose and palmitate in isolated rat skeletal muscle. *J. Pharmacol. Exp. Ther.*, **293**, 487–493.
- FÜRNSINN, C., EBNER, K. & WALDHÄUSL, W. (1995). Failure of GLP-1(7-36)amide to affect glycogenesis in rat skeletal muscle. *Diabetologia*, **38**, 864–867.
- INZUCCHI, S.E., MAGGS, D.G., SPOLLETT, G.R., PAGE, S.L., RIFE, F.S., WALTON, V. & SHULMAN, G.I. (1998). Efficacy and metabolic effects of metformin and troglitazone in type II diabetes mellitus. *N. Engl. J. Med.*, **338**, 867–872.
- KAUSCH, C., KRÜTZFELDT, J., WITKE, A., RETTIG, A., BACHMANN, O., RETT, K., MATTHAEI, S., MACHICAO, F., HÄRING, H.-U. & STUMVOLL, M. (2001). Effects of troglitazone on cellular differentiation, insulin signaling, and muscle glucose metabolism in cultured human skeletal muscle cells. *Biochem. Biophys. Res. Commun.*, **280**, 664–674.
- LOVISCACH, M., REHMANN, N., CARTER, L., MUDALIAR, S., MOHADEEN, P., CIARALDI, T.P., VEERKAMP, J.H. & HENRY, R.R. (2000). Distribution of peroxisome proliferator-activated receptors (PPARs) in human skeletal muscle and adipose tissue: relation to insulin action. *Diabetologia*, **43**, 304–311.
- NIELSEN, J.N., DERAIVE, W., KRISTIANSEN, S., RALSTON, E., PLOUG, T. & RICHTER, E.A. (2000). Glycogen synthase localization and activity in rat skeletal muscle is strongly dependent on glycogen content. *J. Physiol.*, **531**, 757–769.
- OLEFSKY, J.M. (2000). Treatment of insulin resistance with peroxisome proliferator-activated receptor γ agonists. *J. Clin. Invest.*, **106**, 467–472.
- PARK, K.S., CIARALDI, T.P., ABRAMS-CARTER, L., MUDALIAR, S., NIKOULINA, S.E. & HENRY, R.R. (1998). Troglitazone regulation of glucose metabolism in human skeletal muscle cultures from obese type II diabetic subjects. *J. Clin. Endocrinol. Metab.*, **83**, 1636–1643.
- PLOSKER, G.L. & FAULDS, D. (1999). Troglitazone. A review of its use in the management of type 2 diabetes mellitus. *Drugs*, **57**, 279–462.
- ROSEN, E.D. & SPIEGELMAN, B.M. (2001). PPAR- γ : a nuclear regulator of metabolism, differentiation, and cell growth. *J. Biol. Chem.*, **276**, 37731–37734.
- SPIEGELMAN, B.M. (1998). PPAR- γ : adipogenic regulator and thiazolidinedione receptor. *Diabetes*, **47**, 507–514.
- SREENAN, S., KECK, S., FULLER, T., COCKBURN, B. & BURANT, C.F. (1999). Effects of troglitazone on substrate storage and utilization in insulin-resistant rats. *Am. J. Physiol.*, **276**, E1119–E1129.
- STACE, P.B., MARCHINGTON, D.R., KERBEY, A.L. & RANDLE, P.J. (1990). Long term culture of rat soleus muscle *in vitro*. Its effects on glucose utilization and insulin sensitivity. *FEBS Lett.*, **273**, 91–94.
- STEVENSON, R.W., HUTSON, N., KRUPP, M.N., VOLKMAN, R.A., HOLLAND, G.F., EGGLER, J.F., CLARK, D.A., MCPHERSON, R.K., HALL, K.L., DANBURY, B.H., GIBBS, E.M. & KREUTER, D.K. (1990). Actions of novel antidiabetic agent englitazone in hyperglycemic hyperinsulinemic *ob/ob* mice. *Diabetes*, **39**, 1218–1227.
- YONEMITSU, S., NISHIMURA, H., SHINTANI, M., INOUE, R., YAMAMOTO, Y., MASUZAKI, H., OGAWA, Y., HOSODA, K., INOUE, G., HAYASHI, T. & NAKAO, K. (2001). Troglitazone induces GLUT4 translocation in L6 myotubes. *Diabetes*, **50**, 1093–1101.
- ZIERATH, J.R., RYDER, J.W., DOEBBER, T., WOODS, J., WU, M., VENTRE, J., LI, Z., MCCRARY, C., BERGER, J., ZHANG, B. & MOLLER, D.E. (1998). Role of skeletal muscle in thiazolidinedione insulin sensitizer (PPAR γ agonist) action. *Endocrinology*, **139**, 5034–5041.

(Received September 18, 2002

Revised November 22, 2002

Accepted December 20, 2002)