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Acute non-insulin-like stimulation of rat muscle glucose metabolism by troglitazone *in vitro*

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1 The direct short-term effects of troglitazone on parameters of glucose metabolism were investigated in rat soleus muscle strips.

2 In muscle strips from Sprague-Dawley rats, troglitazone (3.25 μmol l^{-1}) increased basal and insulin-stimulated glucose transport by 24% and 41%, respectively ($P<0.01$ each).

3 In the presence of 5 nmol l^{-1} insulin, stimulation of glucose transport by 3.25 μmol l^{-1} troglitazone was accompanied by a 36% decrease in glycogen synthesis, while glycolysis was increased (112% increase in lactate production) suggesting a catabolic response of intracellular glucose handling.

4 Whereas insulin retained its stimulant effect on [^{3}H]-2-deoxy-glucose transport in hypoxia-stimulated muscle (by 44%; c.p.m. mg^{-1} h^{-1}: 852 ± 77 vs 1229 ± 75, $P<0.01$), 3.25 μmol l^{-1} troglitazone failed to increase glucose transport under hypoxic conditions (789 ± 40 vs 815 ± 28, NS) suggesting that hypoxia and troglitazone address a similar, non-insulin-like mechanism.

5 No differences between troglitazone and hypoxia were identified in respective interactions with insulin.

6 Troglitazone acutely stimulated muscle glucose metabolism in a hypoxia/contraction-like manner, but it remains to be elucidated whether this contributes to the long-term antidiabetic and insulin enhancing potential *in vivo* or is to be regarded as an independent pharmacological effect.

Keywords: Troglitazone; insulin sensitivity; glucose transport; glycogen synthesis; glycolysis; lactate; skeletal muscle

Introduction

Insulin resistance is a wide-spread feature of common metabolic diseases characterized by derangement of glucose homeostasis, which include obesity and type 2 diabetes (DeFronzo, 1988; Olefsky *et al*., 1982). With respect to glucose uptake, skeletal muscle represents the quantitatively most important target tissue of insulin (Baron *et al*., 1988; DeFronzo, 1988), and, hence, improvement of muscle insulin sensitivity is regarded a priority aim of potential therapeutic intervention.

Troglitazone (CS-045, (±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy) benzyl]-2,4-thiazolidinedione) belongs to a new class of antidiabetic agents, the thiazolidinediones, which ameliorate insulin resistance and therefore are referred to as insulin sensitizers. Chronic oral administration markedly improves insulin sensitivity as well as glucose and lipid metabolism in various animal models of obesity and type 2 diabetes (Fujiwara *et al*., 1988; 1991; 1995; Lee *et al*., 1994). A beneficial action of chronic troglitazone treatment has also been demonstrated in man (Iwamoto *et al*., 1991; Nolan *et al*., 1994) making the compound an important new alternative for the treatment of insulin resistance and type 2 diabetes.

The precise mechanism via which thiazolidine derivatives influence glucose metabolism is still poorly understood. Thiazolidinediones are known to bind to and activate nuclear peroxisome proliferator-activated receptor γ (PPARγ), which regulates the expression of genes involved in adipocyte differentiation (Forman *et al*., 1995; Lehmann *et al*., 1995; De Vos *et al*., 1996), but the extent to which PPARγ-dependent modulation of gene expression contributes to the antidiabetic action of these compounds has not yet been established. In addition to their chronic insulin-sensitizing action, thiazolidinediones can affect glucose metabolism more rapidly than might be expected via gene transcription (Fujiwara *et al*., 1988; Kreutter *et al*., 1990; Lee & Olefsky, 1995; Okuno *et al*., 1997).

The present study investigates the direct and acute effects of troglitazone on glucose handling by skeletal muscle, an important target tissue of insulin under physiological circumstances. Dose-dependent effects of acute troglitazone exposure on glucose uptake, glycogen synthesis and glycolysis were measured in the absence and in the presence of insulin in rat isolated soleus muscle strips displaying either normal or impaired insulin sensitivity. Furthermore, experiments were performed to distinguish whether stimulation by troglitazone of soleus muscle glucose metabolism rather resembles the action of insulin or hypoxia, both of which stimulate glucose transport although via different biochemical mechanisms (Cartee *et al*., 1991; Fürnsinn *et al*., 1996).

Methods

Rats

Male Sprague-Dawley (SD) rats were purchased from the breeding facilities of the University of Vienna (Himberg, Austria), lean (Fa/−) and genetically obese (fa/fa) Zucker rats were from Harlan Olac (Bicester, U.K.). Experiments were performed according to local law and to the principles of laboratory animal care. Rats were kept at an artificial 12 h/12 h light-dark cycle at constant room temperature. Conventional laboratory diet and tap water were provided *ad libitum* until the evening before killing, when only food was withdrawn. Rats were used at fasted body weights of approximately 140 g (5 week-old SD rats), 250 g (11 week-old lean Zucker rats), and 350 g (11 week-old obese Zucker rats), respectively. They were killed by cervical dislocation between 8 h 30 min and 9 h 30 min.

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Incubation procedures

Immediately after killing, two (SD rats) or three (Zucker rats) longitudinal soleus muscle strips per leg were prepared, weighed (approximately 25 mg), and tied under tension on stainless steel clips as previously described (Crettaz *et al*., 1980).

Preincubation Muscle strips were immediately put into 25 ml-Erlenmeyer flasks coated with BlueSlick solution (Serva, Heidelberg, Germany) and placed into a shaking water bath (1 strip/flask; 37°C; 130 cycles min^{-1}). Each flask contained 3 ml Krebs-Ringer buffer solution (KRB, pH 7.35) supplemented with 0%, 0.1%, or 1% (w/v) bovine serum albumin (BSA) and, if not stated otherwise, with 5.5 mmol l^{-1} glucose. During preincubation, which lasted for 30 min, an atmosphere of 95% O_2:5% CO_2 was continuously provided within the flasks.

Incubation After preincubation, muscles were immediately transferred into another set of flasks and incubated in 3 ml of identical buffer solution additionally containing tracer amounts of (+)-[U-^{14}C]-glucose or, alternatively, 2-deoxy-(+)-[2,6-^{3}H]-glucose plus [U^{14}C]-sucrose (all from Amersham, Amersham, U.K.). Except for some experiments investigating the effects of hypoxia (see below), the incubation period lasted for 60 min at 37°C, after which muscle strips were quickly removed, blotted, and frozen in liquid nitrogen. Later muscle strips were lyzed in 1 mol l^{-1} KOH at 70°C, the lysate was then employed for further analytical procedures described below.

Troglitazone Troglitazone was generously provided by Sankyo (Tokyo, Japan). The drug was dissolved and diluted in dimethyl sulphoxide (DMSO) and added to KRB to provide troglitazone concentrations ranging from 5 to 3250 nmol l^{-1} (2.2 to 1435.1 ng ml^{-1}) during preincubation as well as during the incubation period. DMSO concentration in KRB was not more than 0.2% (v/v) and was identical in media containing troglitazone and in respective control solutions. All experiments on the effects of troglitazone were performed in the absence of BSA, except when troglitazone-BSA interaction was examined.

High glucose pretreatment In some experiments, 50 mmol l^{-1} glucose was present in KRB during 30 min preincubation only to provide a model of hyperglycaemia-induced insulin resistance. During the subsequent 60 min incubation period, parameters of glucose metabolism were measured at 5.5 mmol l^{-1} glucose.

Insulin To investigate muscle glucose metabolism under insulin-stimulated conditions, human insulin (Actrapid, Novo, Bagsvaerd, Denmark) was added to the KRB during the incubation period only. The concentrations of insulin used ranged from 0.1 to 100 nmol l^{-1} as indicated in the tables and figures.

Hypoxia After preincubation in the presence of O_2, muscle strips were transferred to incubation flasks continuously gassed with N_2. For hypoxia-exposed and respective control muscles, incubation time was reduced to 20 min (vs 60 min in all other experiments), since prolonged incubation in the absence of O_2 would have caused loss of tissue viability.

Glycogen synthase activity Preincubation and incubation preceding the measurement of glycogen synthase activity were as described above except that no radioactive compounds were added to the incubation medium and the incubation period was reduced to 30 min (vs 60 min in other experiments). After incubation, muscle strips were immediately homogenized for 10–15 s in 600 μl buffer solution containing 500 mmol l^{-1} imidazole, 250 mmol l^{-1} KF, and 200 mmol l^{-1} EDTA (pH 7.0) by use of a Polytron homogenizer (Kinematica, Luzern, Switzerland); 25 μl of the homogenate was then transferred to 45 μl of assay solution containing 0.94% (w/v) glycogen (free of any glucose-6-phosphate), 1.05% (w/v) UDP-glucose, 11.2% (v/v) of the above described buffer, 0.01 mmol l^{-1} glucose-6-phosphate, and a trace amount of [^{14}C]-UDP-glucose. This assay solution was incubated for 10 min at 32°C, after which 50 μl was pipetted onto a piece of chromatography paper (Whatman, Maidstone, England, U.K.), which after 1 min was dropped into a shaking bath containing 750 ml of 66% (v/v) cold ethanol. Thereby, the reaction was stopped and the glycogen with incorporated ^{14}C-glucosyl units was precipitated to the chromatography paper. After 105 min of continuous washing in 66% cold ethanol, which was renewed several times, chromatography papers were dropped into acetone for 2 min, dried, and covered with scintillation fluid. ^{14}C-content was counted in a β-counter (Packard Instrument Company, Meriden, CT).

Analytical procedures

Net uptake of 2-deoxy-D-[2,6-^{3}H]-glucose, a glucose analogue which does not enter glycolysis, was determined by employing [^{14}C]-sucrose as an extracellular space marker by methods described previously (Fürnsinn *et al*., 1995). Under the applied experimental conditions, insulin-stimulated [^{3}H]-2-deoxy-glucose uptake does not reach saturation within the incubation period of 60 min (data not shown). Net glucose incorporation into glycogen are referred to as glycogen synthesis, and was determined by measuring conversion of [^{14}C]-glucose to [^{14}C]-glycogen by methods described previously (Crettaz *et al*., 1980). Aerobic glycolysis, i.e. CO_2 release, was calculated from conversion of [^{14}C]-glucose into $^{14}CO_2$. To this end, the flask was sealed during the last 45 min of muscle incubation, after which the muscle strip was quickly removed and the flask was immediately resealed with a stopper carrying a hang-in container provided with 200 μl CO_2-trapping solution (phenethylamine:methanol, 1 : 1). By use of a syringe, 200 μl of 3 mmol l^{-1} perchloric acid was injected into the incubation buffer within the flask to quantitatively release CO_2 from the medium. After incubation for at least 1 h at room temperature, the trapping solution was brought into scintillation fluid, which was vigorously shaken and counted for ^{14}C-content. Anaerobic glycolysis, i.e. lactate release, was calculated from KRB lactate concentration measured enzymatically by the lactate dehydrogenase method (Engel & Jones, 1978). For determination of muscle glycogen content, glycogen in the muscle lysate was completely degraded to glucose with amyloglucosidase (Dimitriadis *et al*., 1988). Glucose was then measured enzymatically by a commercial kit from Human (Taunusstein, Germany).

Calculations and statistics

All data are presented as means $\pm$ s.e.mean and $P<0.05$ was considered significant. For comparison of two groups, *P*-values were calculated by two-tailed paired or unpaired Student's *t* test as appropriate. Multiple comparisons with a control were performed according to the method of Dunnett (Dunnett, 1964), and where stated, linear contrast analysis was employed to test for dose-dependency of effects.

Results

High glucose pretreatment

As shown for an insulin dose-response curve ranging from 0.1 to 100 nmol l^{-1} insulin, 30 min of high glucose preincubation (50 mmol l^{-1}) significantly reduced insulin-stimulated 2-deoxy-glucose transport and glucose incorporation into glycogen in rat isolated soleus muscle. However, at the same time anaerobic glycolysis was increased and muscle glycogen content determined at the end of the experiment was moderately elevated by high glucose preexposure (Table 1).

Acute troglitazone action on muscles from SD rats

Independent of glucose concentration during preincubation and independent of the presence of 5 nmol l^{-1} insulin, 3250 nmol l^{-1} troglitazone moderately, but significantly increased glucose uptake in muscle strips from SD rats (Figure 1A). In the presence but not in the absence of added insulin, stimulation of glucose transport was associated with a distinct decrease in glucose incorporation into glycogen (Figure 1B). Significant inhibition of insulin-stimulated glycogen synthesis

Table 1 Effect of high glucose pretreatment on insulin-stimulated glucose metabolism in rat isolated muscle

Insulin (nmol l^{-1})	*0.1*		*1*		*10*		*100*	
Glucose during preincubation (mmol l^{-1})	*5.5*	*50*	*5.5*	*50*	*5.5*	*50*	*5.5*	*50*
Glucose transport	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
(c.p.m. mg^{-1} h^{-1})	272±12	194†±11	370±16	246†±9	550±43	490±63	551±41	463*±35
Glycogen synthesis	(6)	(6)	(5)	(5)	(5)	(5)	(6)	(6)
(μmol g^{-1} h^{-1})	2.00±0.17	1.54*±0.05	3.12±0.51	2.13*±0.29	6.03±0.30	4.39*±0.39	5.33±0.63	4.01*±0.37
CO_2 release	(6)	(6)	(5)	(5)	(6)	(6)	(6)	(6)
(μmol glucose g^{-1} h^{-1})	0.44±0.08	0.38±0.04	0.34±0.03	0.46±0.06	0.48±0.05	0.49±0.02	0.53±0.08	0.48±0.06
Lactate release	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
(μmol g^{-1} h^{-1})	6.2±0.3	8.7*±0.6	6.1±0.4	9.5*±0.7	7.9±1.0	9.6*±0.7	7.9±0.7	10.3*±1.0
Glycogen content	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
(μmol glucosyl units g^{-1})	12.4±0.8	17.4†±0.3	14.4±0.7	16.3±0.6	20.4±1.3	21.5±0.9	20.2±0.7	21.2±1.4

Data shown are means±s.e.mean of n (number in parentheses). Isolated soleus muscle strips from SD rats were preincubated for 30 min in KRB (1% BSA) containing 5.5 or 50 mmol l^{-1} glucose. During the following 60 min, parameters of glucose metabolism were determined at 5.5 mmol l^{-1} glucose in the presence of 0.1, 1, 10, or 100 nmol l^{-1} insulin. *$P<0.05$, †$P<0.01$ vs preincubation with 5.5 mmol l^{-1} glucose.

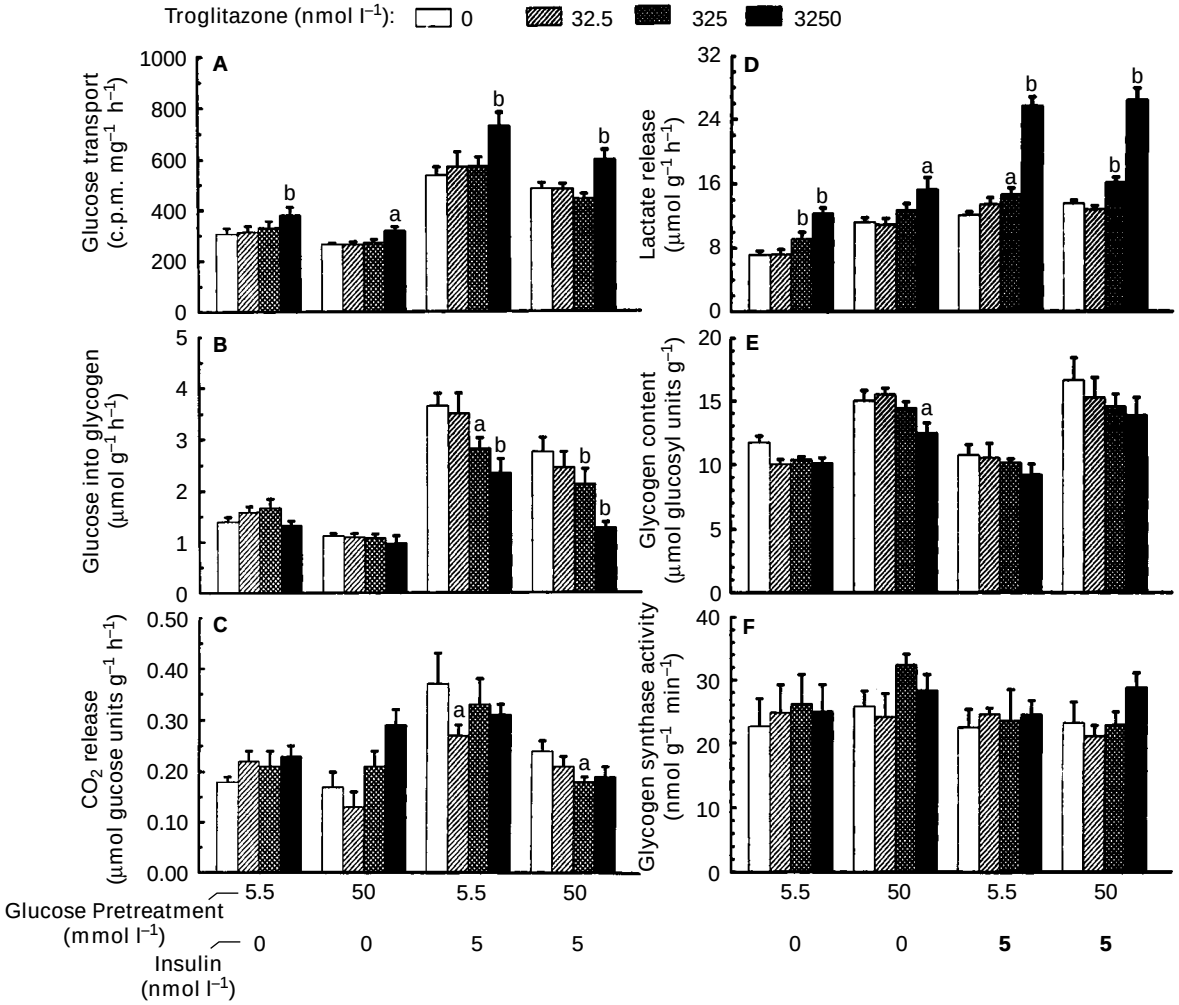

Figure 1 Effect of troglitazone on glucose metabolism in rat isolated muscle. Isolated soleus muscle strips from Sprague-Dawley rats were preincubated in KRB (without BSA) containing 5.5 mmol l^{-1} or 50 mmol l^{-1} glucose. Subsequently, [^{3}H]-2-deoxy-glucose transport (A), glucose incorporation into glycogen (B), aerobic glycolysis (CO_2 release; C), and anaerobic glycolysis (lactate release; D) were determined in the absence or presence of 5 nmol l^{-1} insulin. Glycogen content (E) and glycogen synthase activity (F) are given as determined after incubation; troglitazone concentrations were as indicated. Means±s.e.mean; $n=5-12$; [a]$P<0.05$, [b]$P<0.01$ vs absence of troglitazone.

occurred with 325 nmol l^{-1} troglitazone, a concentration which did not affect glucose uptake. Glycogen synthase activity remained unchanged by troglitazone (Figure 1F). Aerobic glycolysis as reflected by CO_2 release lacked a marked response to troglitazone treatment (Figure 1C), while anaerobic glycolysis, i.e. lactate release, increased in the presence of troglitazone with very distinct troglitazone-effects revealed under insulin-stimulated conditions (Figure 1D). Glycogen content as measured after incubation fell moderately but only in part significantly with troglitazone exposure (Figure 1E).

Modulation of acute troglitazone action by BSA

To analyse the effect of protein binding, troglitazone action on glycogen synthesis in insulin-stimulated muscle from SD rats was determined in the presence of BSA. While 325 nmol l^{-1} troglitazone significantly inhibited muscle glycogen synthesis in the absence of BSA (Figure 1B), 3250 nmol l^{-1} of the drug were required to elicit a significant effect in the presence of 0.1% BSA and failed to affect glycogenesis in the presence of 1% BSA (Table 2). Such shift to the right of the troglitazone dose-response curve suggests its strong binding to BSA.

Acute troglitazone action on muscles from Zucker rats

Genetically obese rats were markedly overweight at an age of approximately 11 weeks (obese rats, 352 ± 5 g vs lean littermates, 250 ± 1 g; $P < 0.01$). Compared with tissue from lean littermates, muscles from genetically obese Zucker rats exhibited insulin resistance as evident from decreased insulin-stimulated (5 nmol l^{-1}) glucose transport (c.p.m. [^{3}H]-2-deoxyglucose mg^{-1} h^{-1}: lean, 894 ± 72, vs obese, 494 ± 37; $P < 0.01$), glycogen synthesis (μmol glucose incorporated into glycogen g^{-1} h^{-1}: lean, 4.05 ± 0.56 vs obese, 2.37 ± 0.33; $P < 0.05$), and aerobic as well as anaerobic glycolysis (CO_2 release from μmol glucose g^{-1} h^{-1}: lean, 1.54 ± 0.17, vs obese, 0.32 ± 0.05, $P < 0.01$; lactate release, μmol g^{-1} h^{-1}: lean, 16.7 ± 1.5, vs obese, 11.7 ± 0.9, $P < 0.05$), but higher glycogen content (μmol glucosyl units g^{-1}: lean, 16.2 ± 0.9 vs obese, 20.6 ± 1.5; $P < 0.05$). In line with results obtained for SD rat muscle, troglitazone dose-dependently reduced insulin-stimulated glycogen synthesis in lean and obese rats with significant effects found at concentrations of at least 163 nmol l^{-1} and 325 nmol l^{-1}, respectively (Figure 2). Troglitazone significantly stimulated CO_2 release (Table 3) and reduced glycogen

Table 2 Effects of troglitazone on glycogen synthesis: modulation by BSA

Troglitazone (nmol l^{-1})	*0*	*325*	*1000*	*3250*	n	*BSA* (%)
Glycogen synthesis (μmol g^{-1} h^{-1})	3.61 ± 0.20	3.44 ± 0.34	3.19 ± 0.33	$2.69^* \pm 0.37$	5	0.1
Glycogen synthesis (μmol g^{-1} h^{-1})	4.88 ± 0.41	4.51 ± 0.32	4.71 ± 0.30	4.38 ± 0.23	6	1

Data shown are means $\pm$ s.e.mean. Isolated soleus muscle strips from SD rats were incubated in KRB containing 0.1% or 1% BSA (w/v) and 5.5 mmol l^{-1} glucose. Glucose incorporation into glycogen was determined in the presence of 5 nmol l^{-1} insulin. Troglitazone concentrations were as indicated. $^*P < 0.05$ vs absence of troglitazone.

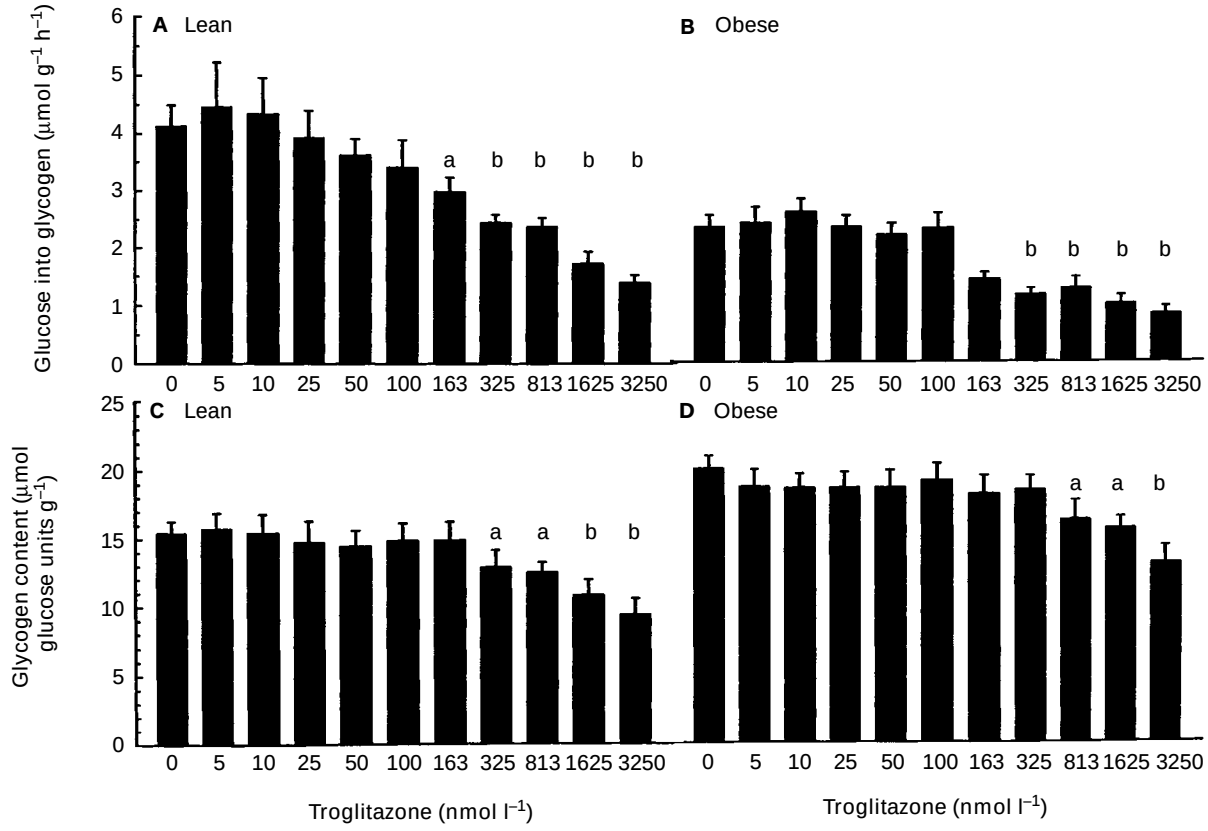

Figure 2 Effect of troglitazone on (A, B) glycogen synthesis and (C, D) glycogen content in isolated muscle from lean and obese Zucker rats. Isolated soleus muscle strips from (A, C) lean (Fa/−) and (B, D) genetically obese (fa/fa) Zucker rats were incubated in KRB (without BSA) and glucose incorporation into glycogen was determined in the presence of 5 nmol l^{-1} insulin. Glycogen content is given as determined after incubation. Troglitazone concentrations were as indicated. Means $\pm$ s.e.mean; $n = 5-8$; $^aP < 0.05$, $^bP < 0.01$ vs absence of troglitazone.

Table 3 Effect of troglitazone on glucose transport and glycolysis in isolated muscle from lean and obese Zucker rats

Lean rats (Fa/-) *Troglitazone* (nmol l^{-1})	*0*	*163*	*325*	*813*	*1625*	*3250*	n
Glucose transport (c.p.m. mg^{-1} h^{-1})	894±72	862±67	860±34	905±51	848±41	846±66	8
CO_2 release (μmol glucose g^{-1} h^{-1})	1.54±0.17	1.58±0.18	1.63±0.23	2.04±0.26	2.39*±0.22	2.12±0.26	5
Lactate release (μmol g^{-1} h^{-1})	16.7±1.5	15.7±0.8	16.6±1.4	19.0±1.7	19.8±1.3	17.8±1.5	8
Obese rats (fa/fa) *Troglitazone* (nmol l^{-1})	*0*	*163*	*325*	*813*	*1625*	*3250*	n
Glucose transport (c.p.m. mg^{-1} h^{-1})	494±37	438±37	440±20	436±33	424±46	457±37	8
CO_2 release (μmol glucose g^{-1} h^{-1})	0.32±0.05	0.31±0.02	0.33±0.03	0.55†±0.04	0.74†±0.08	0.74†±0.09	7
Lactate release (μmol g^{-1} h^{-1})	11.7±0.9	10.8±1.1	11.2±0.8	11.2±0.5	14.1±1.0	15.4*±0.8	8

Data shown are means±s.e.mean. Isolated soleus muscle strips from lean (Fa/-) and genetically obese (fa/fa) Zucker rats were incubated in KRB (without BSA) and parameters of glucose metabolism were measured in the presence of 5 nmol l^{-1} insulin. Troglitazone concentrations were as indicated. *$P<0.05$, †$P<0.01$ vs absence of troglitazone.

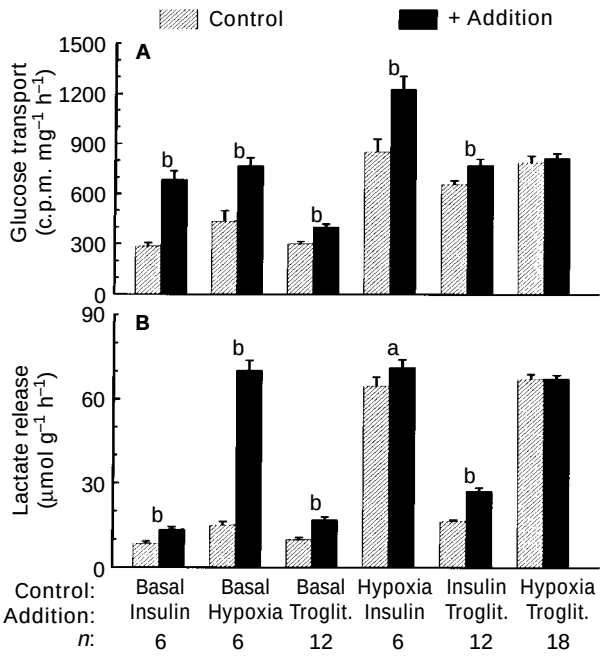

Figure 3 Additivity of insulin, hypoxia, and troglitazone action on glucose metabolism in rat isolated muscle. Isolated soleus muscle strips from SD rats were incubated in KRB (without BSA) and interactions of insulin (100 nmol l^{-1}), hypoxia (N_2-atmosphere), and troglitazone (Troglit.; 3250 nmol l^{-1}) on [^{3}H]-2-deoxy-glucose transport and lactate release were determined.Means±s.e.mean; [a]$P<0.05$, [b]$P<0.01$ vs respective control.

stores (Figure 2) in muscles from Zucker rats, but glucose transport was not significantly affected.

Additivity of the actions of troglitazone, insulin and hypoxia

In soleus muscle from SD rats insulin, hypoxia and troglitazone significantly increased basal glucose transport and lactate release. To examine whether these stimulant effects are triggered via identical or different mechanisms, potential additive interaction was evaluated. Insulin retained its full stimulant action under hypoxic conditions, and so did troglitazone in the presence of insulin (Figure 3). In contrast, troglitazone did not significantly increase glucose transport and lactate release in hypoxic muscle strips (Figure 3).

Synergy with insulin of the actions of troglitazone and hypoxia

To test for synergism of the interaction with insulin, the respective incremental effects induced by 3250 nmol l^{-1} troglitazone and hypoxia were measured at insulin concentrations of 0, 1, 5 and 25 nmol l^{-1}. Intra-individual increments were used to test for insulin-dose-dependent effects by linear contrast analysis. Results from one muscle were disregarded, because an exaggerated increase in glucose transport and lactate release in the presence of troglitazone suggested insufficient oxygen supply due to a technical problem (>6 s.d. above the means of others; significant outlier by Grubbs-test). The troglitazone-induced increase in lactate release and decrease in glucose incorporation into glycogen became progressively greater in an insulin dose-dependent manner ($P<0.0001$ each, by linear contrast analysis). Although 3.2 fold more pronounced troglitazone stimulation of glucose transport was found in the presence versus the absence of 5 nmol l^{-1} insulin, the applied statistical approach failed to provide significant evidence for insulin-dose-dependency ($P=0.069$). The stimulating effect of hypoxia on glucose transport and lactate release increased progressively in an insulin-dependent fashion suggesting a synergistic interaction of hypoxia with insulin ($P<0.02$ each) (Table 4).

Discussion

Short-term stimulation by troglitazone of glucose uptake into rat isolated soleus muscle is in line with results on troglitazone-perfused rat hindlimb (Okuno *et al*., 1997) and on muscle and fat cells exposed to other thiazolidinediones (Kreutter *et al*., 1990; Slieker *et al*., 1994; Ciaraldi *et al*., 1995). The troglitazone-induced increase in glucose transport remained moderate in amplitude and was only seen in SD, but not in Zucker rats. While stimulation of glucose uptake was only observed at 3250 nmol l^{-1} troglitazone, a 10 fold lower concentration significantly augmented lactate release and inhibited insulin-stimulated glycogenesis in muscle from SD rats. The minimal troglitazone concentration in the incubation medium required to affect significantly glucose metabolism of muscle isolated from lean Zucker rats was 163 nmol l^{-1}, which is approximately a third of effective levels circulating in blood of chronically treated animals (Khourshed *et al*., 1995; Lee *et al*.,

Table 4 Synergistic action with insulin of hypoxia and troglitazone on glucose metabolism in rat isolated muscle

Insulin (nmol l^{-1})		*0*	*1*	*5*	*25*
Glucose transport	*n*	8	9	8	9
(c.p.m. mg^{-1} h^{-1})	−Troglitazone	358±23	427±30	532±22	749±37
	+Troglitazone	410±25	484±23	697±36	843±54
	Intra-individual Δ	+52±14*	+57±38	+166±29*	+94±23*
Dose-dependence of Δ			$P=0.069$		
Glycogen synthesis	*n*	9	9	9	9
(μmol g^{-1} h^{-1})	−Troglitazone	1.45±0.15	1.94±0.16	4.05±0.41	4.71±0.33
	+Troglitazone	1.19±0.14	1.90±0.23	2.67±0.21	3.20±0.19
	Intra-individual Δ	−0.25±0.18	−0.04±0.18	−1.38±0.31*	−1.51±0.31*
Dose-dependence of Δ			$P<0.0001$		
Lactate release	*n*	8	9	8	9
(μmol g^{-1} h^{-1})	−Troglitazone	9.2±0.7	9.7±0.9	11.6±0.5	13.5±1.1
	+Troglitazone	14.0±1.0	16.4±0.9	20.6±1.4	24.9±1.8
	Intra-individual Δ	+4.8±0.9*	+6.8±1.1*	+9.0±1.2*	+11.4±1.0*
Dose-dependence of Δ			$P<0.0001$		
Glucose transport	*n*	12	10	12	12
(c.p.m. mg^{-1} h^{-1})	−Hypoxia	425±15	431±18	678±31	859±42
	+Hypoxia	952±48	1000±35	1339±56	1549±77
	Intra-individual Δ	+527±46*	+570±38*	+661±49*	+690±67*
Dose-dependence of Δ			$P=0.015$		
Lactate release	*n*	11	11	12	11
(μmol g^{-1} h^{-1})	−Hypoxia	13.7±0.8	14.4±1.3	17.5±1.6	18.0±1.6
	+Hypoxia	74.6±3.5	79.0±2.7	85.6±2.6	89.6±3.6
	Intra-individual Δ	+60.9±3.0*	+64.5±2.5*	+68.1±2.4*	+71.6±3.8*
Dose-dependence of Δ			$P=0.011$		

Data shown are means±s.e.mean. Isolated soleus muscle strips from SD rats were incubated in KRB (without BSA) and parameters of glucose metabolism were measured at different prevailing insulin concentrations in the presence or absence of troglitazone (3250 nmol l^{-1}) or hypoxia (N_2 atmosphere). *P* values for insulin-dose-dependency by linear contrast analysis; *$P<0.01$ for intra-individual effect of troglitazone/hypoxia by paired Student's *t* test.

1994). However, conclusions drawn from *in vitro* for *in vivo* conditions are hampered by putative differences in tissue availability of the hydrophobic drug. In agreement with binding of more than 99% of troglitazone to protein in BSA solutions and plasma (Shibukawa *et al*., 1995), BSA-dependent loss of drug efficacy was observed in this study. Influences of other factors on troglitazone efficacy are conceivable and may include facilitation of drug availability by DMSO *in vitro* or by lipoprotein particles *in vivo*. Furthermore, troglitazone metabolites, which circulate at high concentrations in chronically treated rats, may significantly contribute to its long-term action *in vivo* (Ciaraldi *et al*., 1995; Khourshed *et al*., 1995).

Beside stimulation of glucose uptake *in vivo* and *in vitro* (Kreutter *et al*., 1990; Lee & Olefsky, 1995; Okuno *et al*., 1997), short-term exposure to troglitazone or other thiazolidine derivatives triggers a number of further insulin-like responses (Fujiwara *et al*., 1988; Zhang *et al*., 1994; Maegawa *et al*., 1995). However, with regard to isolated muscle glucose handling, insulin-induced glucose uptake is associated with an anabolic response characterized by distinct augmentation of glycogenesis, moderate stimulation of glycolysis and glycogen accumulation (Crettaz *et al*., 1980; Fürnsinn *et al*., 1995; 1996; and this study). In contrast to insulin, troglitazone-induced glucose transport was accompanied by distinct inhibition of glycogen synthesis and marked stimulation of glycolysis resulting in a reduction of glycogen stores. Troglitazone stimulation of glycogen synthase activity observed in other experimental setups (Ciaraldi *et al*., 1990; Inoue *et al*., 1995) has not been substantiated in this study. Thus, acute troglitazone exposure exerts non-insulin-like and non-insulin-sensitizing actions on intracellular routing of glucosyl units and, hence, beyond glucose transport resembles the catabolic effects of hypoxia (Fürnsinn *et al*., 1996). Stimulation of glycolysis secondary to inhibition of oxidative glucose metabolism was hypothesized for other thiazolidinedione compounds (Slieker *et al*., 1994), but with regard to troglitazone, increased O_2-consumption of perfused hindlimb (Okuno *et al*., 1997) and dose-dependent stimulation of glucose-derived CO_2 release from soleus muscles of Zucker rats argue against such an interpretation and suggest a parallel increase in both aerobic and anaerobic glycolysis.

In spite of the non-insulin-like and non-insulin-sensitizing actions on intracellular glucose routing, troglitazone may stimulate glucose transport via a mechanism also addressed by insulin. Extensive evidence has been provided for two different biochemical pathways mediating the stimulant effects on glucose transport exerted by insulin and hypoxia/contractions, respectively (DeFronzo *et al*., 1981; Cartee *et al*., 1991; Wheeler *et al*., 1994; Fürnsinn *et al*., 1996). To define better the biochemical pathway via which troglitazone stimulates glucose transport, interactions of insulin, hypoxia, and troglitazone were determined. In agreement with the concept of different underlying mechanisms, insulin retained its full stimulating potential on glucose transport under hypoxic conditions. Troglitazone was capable of inducing an increase in glucose transport and lactate release in the presence of insulin, but was ineffective in the presence of hypoxia. These findings suggest that in line with hypoxia-like effects on intracellular glucose handling, acute troglitazone stimulation of muscle glucose transport is mediated via a hypoxia-like but not an insulin-like mechanism. With regard to acute stimulation of glucose uptake, troglitazone may thus add to the stimulating effect of insulin rather than mimicking or supporting the insulin signal.

The latter experiments provide evidence for at least additive interaction of insulin with troglitazone and hypoxia, respectively. To test for more than additivity, i.e. synergy, we also compared the incremental effects induced by hypoxia or by 3250 nmol l^{-1} troglitazone at different ambient insulin concentrations. In parallel to described synergic stimulation of muscle glucose uptake by insulin and muscle contractions (DeFronzo *et al*., 1981; Vergauwen *et al*., 1994), our findings clearly indicate synergy of insulin and hypoxia, which goes beyond conclusions drawn from previous studies demonstrating only additivity (Cartee *et al*., 1991; Azevedo *et al*., 1995;

Fürnsinn *et al*., 1996). The troglitazone-dependent shift of intracellular glucose flux into glycolysis rather than glycogenesis was found to persist independently of prevailing insulin stimulation and a considerably higher troglitazone-induced increment in glucose transport was observed in the presence than in the absence of 5 nmol l^{-1} insulin ($P<0.005$ by Student's t test). However, since linear contrast analysis failed to provide significant evidence ($P=0.069$), the data obtained on glucose transport do not allow final conclusions regarding a potential synergistic troglitazone-insulin interaction. Nevertheless, it is obvious that the experiments failed to discriminate the acute effects of troglitazone from those of hypoxia, which is in agreement with hypothesized parallel mechanisms of action.

Taken together, our findings suggest that acute and direct troglitazone effects on skeletal muscle resemble the effects of hypoxia and contractions rather than those of insulin. This conclusion is supported by several lines of evidence, including that troglitazone (i) induces an intracellular shift of glucosyl units into glycolysis rather than glycogenesis (catabolic response), (ii) significantly increases glucose transport under insulin-stimulated, but not under hypoxic conditions, and (iii) does not differ from hypoxia with respect to interaction with insulin.

Our findings imply that the mode of acute troglitazone action differs considerably from insulin sensitization as observed in response to long-term oral treatment (Fujiwara *et al*., 1988; 1991; 1995; Iwamoto *et al*., 1991; Lee *et al*., 1994; Nolan *et al*., 1994). Acute troglitazone stimulation of glucose transport did not require concomitant addition of insulin to the incubation buffer; this contrasts with a lack of improvement of glucose homeostasis in insulin-deficient animals chronically treated with troglitazone *in vivo* (Fujiwara *et al*., 1988). Furthermore, glycogen depletion found with short-term exposure of isolated muscle (this study) and perfused rat hindlimb (Okuno *et al*., 1997) is in contradiction to the augmented glycogen content of skeletal muscle derived from long-term troglitazone-treated obese rats (Oshida *et al*., 1997). Such higher glycogen content may occur secondary to insulin sensitization and increased insulin-dependent glycogen synthesis as observed after chronic oral administration of other thiazolidinediones than troglitazone (Stevenson *et al*., 1990; Sugiyama *et al*., 1990).

The precise mechanisms and primary target tissues responsible for the antidiabetic and insulin-sensitizing action of troglitazone are still unknown and no conclusive evidence has as yet been provided that direct interaction of the drug with skeletal muscle is instrumental for its beneficial long-term effects. It also remains to be elucidated, whether the non-insulin-like acute catabolic action described *in vitro* (i) is a pharmacological effect that does not play a role during chronic treatment *in vivo*, (ii) is a secondary effect independent of the PPARγ-mediated insulin sensitization *in vivo*, or (iii) is an essential prerequisite for the well-documented antidiabetic potential *in vivo* (Fujiwara *et al*., 1988; 1991; 1995; Iwamoto *et al*., 1991; Lee *et al*., 1994; Nolan *et al*., 1994). With regard to the latter possibility, it might be speculated that during prolonged exposure, modulation of muscle glucose metabolism can shift from short-term catabolic stimulation towards long-term insulin-sensitization. Such improved insulin sensitivity following catabolic stimulation of skeletal muscle is also found after catabolic episodes associated with exercise (Richter *et al*., 1982; Devlin, 1992) or with adrenalin stimulation (Nolte *et al*., 1994) and parallels in underlying biochemical mechanisms may exist.

In conclusion, further investigations are required to examine if the described acute potential of troglitazone to stimulate muscle glucose metabolism in a hypoxia/contraction-like manner *in vitro* is of any relevance to its long-term antidiabetic action *in vivo* or rather must be regarded as an independent and previously unknown pharmacological effect.

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