Short Communication

Expression of *uncoupling protein-3* mRNA in rat skeletal muscle is acutely stimulated by thiazolidinediones: an exercise-like effect?

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Abstract

Aims/hypothesis. We examined whether thiazolidinediones (TZDs) acutely affect *uncoupling protein-3* (*UCP-3*) expression in skeletal muscle and plasma NEFA in Sprague-Dawley rats.

Methods. Expression of *UCP-3* mRNA in hindlimb muscles and plasma NEFA were measured after a single intraperitoneal injection of TZDs in healthy male rats.

Results. Independent of which TZD was injected (50 $\mu\text{mol/kg}$), *UCP-3* expression in gastrocnemius muscle was distinctly increased after 6 h (increase vs vehicle-injected control: pioglitazone, 10.3 $\pm$ 3.2-fold, $p=0.03$; rosiglitazone, 8.7 $\pm$ 1.2-fold, $p=0.001$; RWJ241947, 9.5 $\pm$ 2.7-fold, $p=0.03$). This was accompanied by elevated plasma NEFA (control 158 $\pm$

13 $\mu\text{mol/l}$; pioglitazone, 281 $\pm$ 40 $\mu\text{mol/l}$, $p=0.03$; rosiglitazone, 276 $\pm$ 27 $\mu\text{mol/l}$, $p=0.005$; RWJ241947, 398 $\pm$ 51 $\mu\text{mol/l}$, $p=0.004$). The increase in plasma NEFA could in part have mediated TZD-induced *UCP-3* expression, but increased *UCP-3* mRNA was also found in isolated muscle after 2 h of TZD exposure in vitro (25 $\mu\text{mol/l}$ pioglitazone, 1.7 $\pm$ 0.3-fold, $p=0.046$), suggesting that TZDs act directly and independently of NEFA on skeletal muscle.

Conclusions/interpretation. In healthy rats, a single dose of TZDs rapidly increases *UCP-3* mRNA in skeletal muscle and plasma NEFA. This effect resembles the acute response to a bout of exercise.

Keywords Non-esterified fatty acid · Pioglitazone · Rosiglitazone · Skeletal muscle · Thiazolidinedione · Uncoupling protein-3

Introduction

The glucose-lowering action of thiazolidinediones (TZDs) is predominantly due to improved insulin-stimulated glucose disposal into skeletal muscle. Since the

primary molecular target of TZDs, peroxisome proliferator-activated receptor- γ (PPAR γ), is most abundantly expressed in adipose tissue, effects on skeletal muscle have been attributed mainly to indirect action via changes in signal release from adipocytes. At variance to this, however, TZDs have been shown to impair cell respiration, reduce the cellular energy charge (ATP : AMP ratio), and activate AMP-activated protein kinase (AMPK) independently of PPAR γ , by direct action on isolated rat muscle or muscle cells in vitro [1, 2, 3]. Since acute cellular energy depletion and AMPK activation are believed to be crucial for the antidiabetic effects of exercise, we hypothesised that a PPAR γ -independent and exercise-like mechanism of action could contribute to TZD-induced insulin sensitisation (hypothesis outlined in [2]).

However, the antidiabetic effects of TZDs are delayed and there is little evidence that TZDs exert rapid

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Abbreviations: AMPK, AMP-activated protein kinase · PPAR, peroxisome proliferator-activated receptor · TZD, thiazolidinedione · UCP-3, *uncoupling protein-3*

hypoxia- or exercise-like effects in vivo. A characteristic response to contractions and hypoxia is an immediate increase in the expression of *uncoupling protein-3* (*UCP-3*) in skeletal muscle [4], which has also been observed only 6 h after the injection of troglitazone in mice [5]. Though interesting, the latter observation cannot be extrapolated uncritically to the whole class of TZDs, because troglitazone, which was used at a high dose [5], is superior to other TZDs as an inhibitor of cell respiration in vitro [1].

We therefore examined the rapid effects of TZDs on the expression of *UCP-3* mRNA in skeletal muscle in order to find evidence for or against rapid hypoxia- and exercise-like effects in vivo. Changes in the concentrations of plasma NEFA were also recorded, because NEFA increase during exercise and also stimulate *UCP-3* expression [6, 7].

Materials and methods

Rats. Healthy male Sprague-Dawley rats from the breeding facilities of the Medical University of Vienna (Himberg, Austria) were kept at an artificial 12-h light/12-h dark cycle at constant room temperature. Unless stated otherwise, they had free access to conventional laboratory diet and tap water. All experiments were according to local law and the principles of good laboratory animal care.

Thiazolidinedione injection in vivo. Food was withdrawn at 7.00 hours and two hours later rats (300–350 g body weight) were injected i.p. with a single dose of the TZDs pioglitazone, troglitazone (Sankyo, Tokyo, Japan), rosiglitazone, or RWJ947241 (also known as MCC-555; generously provided by Johnson & Johnson, Raritan, N.J., USA). The TZDs were dissolved in a mixture of equal volumes of DMSO and saline (6 ml/kg body weight). Control rats were injected only with vehicle. After 2 h or 6 h, rats were anaesthetised with isoflurane (Abbott, Abbott Park, Ill., USA) and killed by cervical dislocation. Blood was immediately collected by heart puncture for the later measurement of plasma NEFA (commercial kit from Roche, Indianapolis, Ind., USA). Specimens of gastrocnemius muscle (predominantly white type IIa fibres), tibialis anterior muscle (predominantly white type IIb fibres) and/or soleus muscle (predominantly red type I fibres) were excised for northern blot analysis.

Thiazolidinedione exposure in vitro. Overnight fasted rats (140–190 g body weight) were killed by cervical dislocation and a longitudinal strip of tibialis anterior muscle was prepared from each leg. Muscle specimens were put into Cell Culture Medium 199 (37 °C, pH 7.35; Sigma, St. Louis, Mo., USA; Cat. No. M-4530) and incubated without insulin and without NEFA under conditions already described [1, 2]. Whereas one muscle strip was incubated in the presence of 25 µmol/l pioglitazone, the strip from the other leg was incubated with vehicle only (0.1% DMSO; intra-individual control). After 2 h, muscles were quickly removed from the medium for northern blot analysis. The incubation procedure itself did not significantly affect *UCP-3* mRNA (data not shown).

Northern blot analysis. To stabilise RNA, muscle samples were immediately put into RNAlater solution (Ambion, Austin, Tex., USA). RNA isolation from homogenised muscle was

carried out using RNazol solution (Tel-Test, Friendswood, Tex., USA). Total RNA was size-fractionated on 6% formaldehyde/1% agarose gels, transferred to Hybond-N membranes (Amersham-Pharmacia Biotech, Uppsala, Sweden) by capillary transfer using 10X standard sodium chloride/sodium citrate buffer (SSC; Sigma) as the liquid phase. After fixing RNA to the membrane by UV cross-linking, prehybridisation was carried out at 42 °C for ≥4 h in NaH₂PO₄ buffer (pH 6.5) with 50% (vol/vol) formamide, 5× SSC, 8.4% Denhardt's solution (Sigma), 0.84% glycine solution (10%), and 84 µg/ml salmon sperm DNA (Stratagene, Cedar Creek, Tex., USA), all in 0.1% diethyl pyrocarbonate. The membrane was hybridised by overnight incubation at 42 °C in ULTRAhyb hybridisation buffer (Ambion) with a ³²P-dCTP Kleenow-labelled random primed probe specific for rat *UCP-3* (a gift from C. Zhang, Beth Israel Deaconess Medical Center and Harvard Medical School Boston, Mass., USA), employing the DECAPRIME II DNA labelling system from Ambion and the *GAPDH* probe to correct for loading inequalities. After overnight hybridisation, blots were washed using NorthernMax Low and High Stringency wash solutions according to instructions of the manufacturer (Ambion). Membranes were then subjected to autoradiography (Kodak XAR5-Omat films; Sigma) at –80 °C and quantified by densitometry (image analyser, MWG Biotech, Ebersberg, Germany). The effects of TZDs on *UCP-3* mRNA expression are given as fold change vs an intra-individual control muscle (in vitro experiments), or as fold change vs the mean of samples from vehicle-injected control rats as analysed on the same blot (in vivo experiments).

Statistical analysis. Results are given as means ± SEM. Differences vs control were analysed by paired or unpaired two-tailed Student's *t* test as appropriate, with a *p* value of less than 0.05 considered as significant.

Results and discussion

In agreement with previous findings on female mice [5], expression of *UCP-3* mRNA was distinctly elevated in gastrocnemius muscle of male rats after 24 h of starvation as well as 6 h after a single i.p. injection of 225 µmol/kg troglitazone (Fig. 1a). In addition, stimulation of *UCP-3* expression in skeletal muscle had the following properties: (i) it occurred very rapidly within only 2 h (Fig. 1a); (ii) it was a TZD class effect that was also induced by pioglitazone, rosiglitazone and RWJ241947 (Figs. 1a and b); (iii) it was less pronounced in red than white muscle (Fig. 1b and c); and (iv) it was triggered by a dose of pioglitazone (25 µmol/kg = 9 mg/kg) below the daily doses conventionally used to induce antidiabetic action in obese rats (10–30 mg·kg^{–1}·day^{–1}; Fig. 1c). These findings obviously challenge the idea that the metabolic actions of TZD treatment in vivo are entirely due to PPARγ activation and a typically delayed genomic mode of action.

Due to its high content of DMSO, the vehicle caused a transient increase in plasma NEFA, but independently of the vehicle effect and at variance with the study on mice [5], increased *UCP-3* expression was associated with elevated plasma NEFA (Table 1), which contrasts with the NEFA-lowering effect of

Fig. 1. Effect (a) of starvation or a single i.p. injection of troglitazone or pioglitazone (225 $\mu\text{mol/kg}$) on *UCP-3* mRNA expression in rat gastrocnemius muscle as measured after 2, 6, or 24 h ($n=5$ or 6 each; * $p<0.03$, † $p<0.01$, vs control). **b.** Effect of a single i.p. injection of pioglitazone, rosiglitazone, or RWJ241947 (50 $\mu\text{mol/kg}$ each) on *UCP-3* mRNA expression after 6 h in rat gastrocnemius and soleus muscle ($n=6$ each; except pioglitazone/soleus: $n=3$; * $p<0.05$, † $p<0.03$, vs control).

c. Effect of a single i.p. injection of pioglitazone (25 and 75 $\mu\text{mol/kg}$) on *UCP-3* mRNA expression after 6 h in rat gastrocnemius, tibialis anterior and soleus muscle ($n=6$ each; * $p<0.05$, † $p<0.02$, ‡ $p<0.005$, vs absence of pioglitazone). **d.** Effect on *UCP-3* mRNA expression of 2 h exposure of isolated rat tibialis anterior muscle to pioglitazone (25 $\mu\text{mol/l}$) in vitro ($n=16$; * $p<0.05$ vs absence of pioglitazone). All results are given as fold-change vs controls (= 1 per definition)

Table 1. Thiazolidinediones rapidly increase plasma NEFA

	Dose ($\mu\text{mol/kg}$)	<i>n</i>	NEFA ($\mu\text{mol/l}$)	<i>p</i> (vs vehicle)
2-hour treatment				
Vehicle	—	9	399 $\pm$ 77	—
Saline	—	3	182 $\pm$ 9	0.0028
Pioglitazone	225	6	576 $\pm$ 76	0.13, NS
6-hour treatment				
Vehicle	—	10	158 $\pm$ 13	—
Pioglitazone	25	6	263 $\pm$ 49	0.088, NS
Pioglitazone	50	6	281 $\pm$ 40	0.026
Pioglitazone	75	6	243 $\pm$ 34	0.054, NS
Pioglitazone	225	6	327 $\pm$ 50	0.013
Rosiglitazone	50	6	276 $\pm$ 27	0.0048
RWJ-241947	50	6	398 $\pm$ 51	0.0044
Troglitazone	225	6	273 $\pm$ 26	0.0067

Circulating plasma NEFA 2 h and 6 h after a single intraperitoneal injection of TZDs in rats. The vehicle was a mixture of equal volumes of saline and DMSO

chronic TZD administration. Since NEFA are known to induce *UCP-3* expression in skeletal muscle [7], their increase could have mediated the effect of TZDs. Our results, however, argue against *UCP-3* expression being entirely NEFA-dependent, because pioglitazone stimulated *UCP-3* expression also by direct action on

isolated muscle in vitro, i.e. without an increase in ambient NEFA (Fig. 1d). The direct effect in vitro occurred within 2 h and is likely to be independent of PPAR γ , because PPAR γ is weakly expressed in skeletal muscle and, unlike PPAR subtypes α and δ , does not directly regulate the *UCP-3* gene [7].

Since other studies have shown that hypoxia and exercise likewise induce immediate increases in both *UCP-3* mRNA and plasma NEFA [4, 6, 8], our findings are in striking agreement with the previously defined hypothesis that an exercise-like component of action, as triggered by inhibition of cell respiration and a reduced cellular energy charge, could contribute to TZD-induced insulin sensitisation (hypothesis outlined in [2]). This interpretation is corroborated in as far as a low cellular energy state induces *UCP-3* [4, 8], and also by the observed fibre type dependency, since mild inhibition of respiration could result in a more marked ATP deficiency in white than red muscle, according to their different oxidative potentials. Whereas there is no evidence that increased expression of *UCP-3* itself causes insulin sensitisation, exercise-induced *UCP-3* expression has been shown to be a component of mitochondrial biogenesis [9]. Given that reduced mitochondrial structures and enzyme capacities are strongly related to insulin resistance [10], it is possible that mitochondrial biogenesis could be a causal link between the immediate loss of cellular energy charge and the delayed improvement of insulin sensitivity, both of which are common to exercise and TZD treatment.

Together, our findings show that a single dose of TZDs immediately increases *UCP-3* mRNA in muscle and plasma NEFA. Although only a single observation, this is first evidence that a mechanism of TZD action exists *in vivo*, which is both exercise-like and independent of PPAR γ .

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