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ARTICLE

Increased levels of peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) improve lipid utilisation, insulin signalling and glucose transport in skeletal muscle of lean and insulin-resistant obese Zucker rats

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Abstract

Aims/hypothesis Reductions in peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC-1α) levels have been associated with the skeletal muscle insulin resistance. However, in vivo, the therapeutic potential of PGC-1α has met with failure, as supra-physiological overexpression of PGC-1α induced insulin resistance, due to fatty acid translocase (FAT)-mediated lipid accumulation. Based on physiological and metabolic considerations, we hypothesised that a modest increase in PGC-1α levels would limit FAT upregulation and improve lipid metabolism and insulin sensitivity, although these effects may differ in lean and insulin-resistant muscle.

Methods *Pgc-1α* was transfected into lean and obese Zucker rat muscles. Two weeks later we examined mitochondrial biogenesis, intramuscular lipids (triacylglycerol, diacylglycerol, ceramide), GLUT4 and FAT levels, insulin-stimulated glucose transport and signalling protein phosphorylation (thymoma viral proto-oncogene 2 [Akt2], Akt substrate of 160 kDa [AS160]), and fatty acid oxidation in subsarcolemmal and intermyofibrillar mitochondria.

Results Electrotransfection yielded physiologically relevant increases in *Pgc-1α* (also known as *Ppargc1a*) mRNA and protein (~25%) in lean and obese muscle. This induced mitochondrial biogenesis, and increased FAT and GLUT4 levels, insulin-stimulated glucose transport, and Akt2 and AS160 phosphorylation in lean and obese animals, while bioactive intramuscular lipids were only reduced in obese muscle. Concurrently, PGC-1α increased palmitate oxidation in subsarcolemmal, but not in intermyofibrillar mitochondria, in both groups. In obese compared with lean animals, the PGC-1α-induced improvement in insulin-stimulated glucose transport was smaller, but intramuscular lipid reduction was greater.

Conclusions/interpretations Increases in PGC-1α levels, similar to those that can be induced by physiological stimuli, altered intramuscular lipids and improved fatty acid oxidation, insulin signalling and insulin-stimulated glucose transport, albeit to different extents in lean and insulin-resistant muscle. These positive effects are probably attributable to limiting the PGC-1α-induced increase in FAT, thereby preventing bioactive lipid accumulation as has occurred in transgenic PGC-1α animals.

Keywords Akt2 · AS160 · Ceramide · Diacylglycerol · FAT · Fatty acid oxidation · GLUT4 · Mitochondria · Muscle fibres · Triacylglycerol

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Abbreviations

Akt2	Thymoma viral proto-oncogene 2
AMPK	AMP-activated protein kinase

AS160	Akt substrate of 160 kDa
COXIV	Cytochrome *c* oxidase subunit IV
CPTI	Carnitine palmitoyltransferase 1
EDL	Extensor digitorum longus
FABP	Fatty acid binding protein
FAT	Fatty acid translocase
PGC-1α	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PI3 kinase	Phosphatidylinositol 3 kinase
PL	Plantaris
TA	Tibialis anterior
WG	White gastrocnemius

Introduction

Insulin resistance is a key feature of type 2 diabetes. The accumulation of lipids within skeletal muscle has been associated with the development of insulin resistance, as excess intramuscular lipid metabolites interfere with insulin signalling, leading to impaired removal of glucose from the circulation [1]. Increasing fatty acid oxidation while reducing intramuscular lipids is thought to be an effective means to improve insulin sensitivity, as has been shown with genetic studies and with exercise [2–4].

The peroxisome proliferator-activated receptor γ co-activator 1 alpha (PGC-1α) has been implicated in the regulation of skeletal muscle oxidative metabolism and mitochondrial biogenesis [5]. In muscle cell lines, increased levels of PGC-1α induced insulin-sensitising effects via the upregulation of selected genes involved in fatty acid β-oxidation, glucose transport and oxidative phosphorylation [6, 7], while in type 2 diabetes, muscle *PGC-1α* expression was reduced [8, 9]. Collectively, these studies suggest that PGC-1α may be a key factor regulating insulin sensitivity in mammalian muscle [5–9]. However, experimental studies in vivo have been disappointing. Transgenic *Pgc-1α* (also known as *Ppargc1a*) overexpression yielded an insulin-resistant phenotype [10, 11], calling into question the therapeutic potential of PGC-1α. Alternatively, a model of transgenic *Pgc-1α* overexpression may have obscured the positive metabolic effects of this co-activator.

In transgenic animals, *Pgc-1α* mRNA overexpression is very large (6- to 21-fold increase) [10–12], well beyond that which occurs physiologically [13]. Excessive PGC-1α production induces many undesirable consequences, including abnormal mitochondrial proliferation, disruption of myofibrillar architecture, displacement of nuclei [12], obesity, intramuscular lipid accumulation and insulin resistance [10, 11]. We [3, 13] have suggested that insulin resistance in PGC-1α transgenic mice [10, 11] may stem from a large, PGC-1α-induced increase in the fatty acid transporter, fatty acid translocase (FAT), which has been associated with increases in intramuscular lipids and insulin resistance in humans and in animals [14, 15]. Viewed in this light, PGC-1α-induced insulin resistance in transgenic mice is less of a paradox than has been suggested [11]. Recently, we showed that inducing a modest increase in PGC-1α protein, based on physiological and metabolic considerations, has an insulin-sensitising effect in healthy muscle [3]. Nevertheless, some very critical questions remain unanswered, e.g.: (1) whether modest PGC-1α overproduction also improves insulin action in severely insulin-resistant muscle; and (2) whether PGC-1α-induced metabolic effects are similar in lean and insulin-resistant muscle.

Skeletal muscle insulin resistance can be examined in a model of high fat feeding [4] or in a genetically predisposed model such as obese Zucker rats [14]. Since insulin resistance is much greater in obese Zucker rats, we selected this more severe model to ascertain whether PGC-1α overproduction within physiological limits increased mitochondrial biogenesis, and improved mitochondrial fatty acid oxidation and insulin-stimulated glucose transport and signalling. In addition, we determined whether PGC-1α induced similar effects in healthy and insulin-resistant muscles. We hypothesised that modest PGC-1α overproduction would improve insulin action in lean and insulin-resistant muscle.

Methods

Materials

The following were purchased: [1-^{14}C]palmitate and 3-*O*-methyl-[^{3}H]glucose (GE Health Care, Oakville, ON, Canada); insulin (Humulin-R; Eli-Lilly, Toronto, ON, Canada); and the following antibodies: PGC-1α (VWR Canlab, Mississauga, ON, Canada); thymoma viral proto-oncogene 2 (Akt2), anti-IRS-1, anti-AMP-activated protein kinase (AMPK)α_2, anti-phosphatidylinositol 3 (PI3)-kinase (Upstate, Lake Placid, NY, USA); anti-Akt phosphos473, anti-Akt phosphothr308, anti-Akt substrate of 160 kDa (AS160; Cell Signalling, Danvers, MA, USA); anti-Akt substrate of 160 kDa (AS160) phosphothr642 (Medicorp, Montreal, QC, Canada); anti-GLUT4 (Chemicon, Temecula, CA, USA); anti-cytochrome *c* oxidase subunit IV (COXIV; Molecular Probes, Eugene, OR, USA); and goat anti-rabbit and goat anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The monoclonal antibody MO25 [14] was used to detect FAT. All other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA).

Animals

Age-matched, 7-week-old, male lean (180–200 g) and obese (350 g) Zucker rats (Charles River, Baie d'Urfé, QC, Canada) were housed in controlled facilities (temperature 20°C, 40% humidity, 12 h light–dark cycle) with free access to water and chow. Principles of laboratory animal care (National Institutes of Health publication no. 85-23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) were followed, as well those of the Canadian Council on Animal Care. All procedures were approved by the University of Guelph Animal Care Committee.

Descriptive characteristics of lean and obese Zucker rats

Glucose, fatty acids and insulin Glucose was determined using the glucose oxidase method (Beckman glucose analyser; Beckman Coulter Canada Inc., Mississauga, ON, Canada). Commercial kits were used for fatty acids (Wako Diagnostics, Richmond, VA, USA) and insulin (Millipore, Danvers, MA, USA).

Tissue removal and muscle fibre composition Rats were anaesthetised with Somnotol (6 mg/100 g body weight, i.p.). Metabolically heterogeneous muscles (extensor digitorum longus [EDL], red tibialis anterior, white tibialis anterior [TA], plantaris [PL], soleus, red gastrocnemius and white gastrocnemius [WG]) were excised, freeze clamped (liquid nitrogen) and stored at −80°C until analysed. Muscle fibre composition of muscles in lean and obese animals was determined as described by us recently [3], by staining serial cross sections (10 µm) for myofibrillar ATPase and succinate dehydrogenase, from which we determined their physiological and metabolic characteristics [3].

Transfecting muscle with Pgc-1α The *Pgc-1α* expression construct (gift from B. Spiegelman, Harvard University, Boston, MA, USA) was produced by subcloning the *Pgc-1α* coding sequence into a mammalian expression vector (pcDNA 3.0; Invitrogen, Burlington, ON, Canada). The pcDNA3.0 vector was used for control experiments (Invitrogen, Burlington, ON, Canada). *Pgc-1α*-pcDNA and pcDNA3.0 plasmid stocks for electroporation were produced by large-scale plasmid isolation from transformed *Escherichia coli* cells (One-Shot; Invitrogen, San Diego, CA, USA) using commercially available kits (GIGA-prep kits; Invitrogen, Burlington, ON, Canada).

Electrotransfection of tibialis anterior muscle was performed as previously described in detail [3, 16, 17]. Briefly, animals were anaesthetised with isoflurane and the lower hindlimbs were shaved and sterilised (iodine and 70% ethanol [vol./vol.]). To increase transfection efficiency [18–20], hyaluronidase (100 µl, i.e. 0.15 U/µl in 50% [vol./vol.] saline) was injected through the skin and into the TA muscle. After 2 h, TA muscles were then either electrotransfected with *Pgc-1α*-pcDNA plasmid (500 µg *Pgc-1α* in 50% [vol./vol.] saline) or with empty pcDNA3.0 plasmid (500 µg pcDNA in 50% [vol./vol.] saline). Electroporation of the intact TA muscle was performed as previously reported by us [3, 16, 17, 21] using plate electrodes and nine electric pulses (electric field 180 V/cm, 1 Hz, 20 ms in duration; ECM 830 Square Wave Electroporator; BTX, San Diego, CA, USA). Animals were then provided with an analgesic (Temgesic) and allowed to recover for 2 weeks after transfection. This protocol results in transfection of 30% of the muscle fibres (data not shown). Transfection with higher voltages can improve transfection efficiency, but causes muscle damage (C. R. Benton and A. Bonen, unpublished observations; [20]).

Mitochondrial DNA, *Pgc-1α* mRNA, western blotting and enzyme activities

Mitochondrial DNA Mitochondrial DNA content was determined using real-time PCR, as described previously [3]. Total DNA was isolated using a kit (DNeasy Blood and Tissue Kit; Qiagen Inc. Canada, Burlington, ON, Canada). Real-time PCR was performed (7500 Real-Time PCR System; Applied Biosystems Canada, Streetsville, ON, Canada) using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). mtDNA primers were designed using the rat mitochondrial genome sequence (Genbank accession number NC_001665) within the NADH dehydrogenase subunit 5 gene: forward 5′-GCAGCCACAGGAAAATCCG-3′; reverse 5′-GTAGGGCAGAGACGGGAGTTG-3′. Primers measuring genomic content were designed within the solute carrier family 16, member1 gene sequence on chromosome 2 (Genbank accession number NC_005101): forward 5′-TAGCTGGATCCCTGATGCGA-3′; reverse 5′-GCATCAGACTTCCCAGCTTCC-3′. mtDNA content was calculated by the $\Delta\Delta C_t$ method using genomic DNA content as an internal standard with 7500 System SDS Software (version 1.2.1.22; Applied Biosystems Canada, Streetsville, ON, Canada).

Pgc-1α mRNA This was determined as we have described previously [3]. Total RNA was isolated from muscle using a combination of Trizol reagent (Invitrogen, Burlington, ON, Canada) and a kit (RNeasy Mini Kit; Qiagen Inc., Canada, Burlington, ON, Canada). Reverse transcription was carried out using a kit (First Strand cDNA Synthesis Kit for RT-PCR [AMV]; Roche, Mississauga, ON, Canada) using random primers. Real-Time PCR was performed using

7500 Real-Time PCR System (Applied Biosystems) using Platinum SYBR Green qPCR Supermix-UDG (Invitrogen). Relative *Pgc-1α* mRNA levels were calculated using the $\Delta\Delta C_t$ method using 7500 System SDS Software Version 1.2.1.22 (Applied Biosystems Canada, Streetsville, ON, Canada). 18S ribosomal RNA was used as an endogenous control. The following primer sets were used: *Pgc-1α* forward 5′-CGATGACCCTCCTCACACCA-3′, *Pgc-1α* reverse 5′-TTGGCTTGAGCATGTTGCG-3′; 18S forward 5′-GTTGGTTTTCGGAACTGAGGC-3′, 18S reverse 5′-GTCGGCATCGTTTATGGTCG-3′.

Protein isolation, activity and western blotting Muscles were homogenised and proteins separated using SDS-PAGE as previously described [3, 14]. Equal quantities of protein were loaded for control and transfected muscles (confirmed with Ponceau S staining). Signals were detected using enhanced chemiluminescence (Perkin Elmer Life Science, Boston, MA, USA) and quantified by densitometry (SynGene, ChemiGenius2; Perkin-Elmer, Woodbridge, ON, Canada). Citrate synthase activity was measured in muscle homogenate [14].

Basal and insulin-stimulated glucose transport, and Akt and AS160 phosphorylation

Basal and insulin-stimulated glucose transport We determined 3-*O*-methyl-[^{3}H]glucose transport under basal conditions and during a euglycaemic–hyperinsulinaemic clamp of perfused tibialis anterior muscles (18 ml/min, 95% O_2/5% CO_2 Krebs–Henseleit buffer, 2 mmol/l pyruvate, 4% BSA [wt/vol.]) as described previously [3]. After 30 min, control and transfected muscles were excised, blotted dry, digested and radioactivity counted. 3-*O*-Methyl-[^{3}H]glucose transport rates were determined using standard calculations.

Insulin-stimulated phosphorylation of Akt and AS160 Phosphorylation of Akt (thr308, ser473) and AS160 (thr642) was determined in control and P*GC-1α*-transfected muscles under basal conditions (saline injection, i.p.) and after injecting insulin (20 U/rat i.p. 10 min) [3]. Thereafter, muscles were frozen and stored (−80°C).

Intramuscular lipids and mitochondrial fatty acid oxidation

Intramuscular lipids Control and transfected muscles were analysed for triacylglycerol, diacylglycerol and ceramide content as reported previously [22, 23]. Briefly, lipids were extracted with a modified Folch procedure and thin-layer chromatography was used to separate lipids. Separated lipids were methylated and fatty acid methyl esters were extracted. Samples were analysed by gas–liquid chromatography (5890 Series II; Hewlett-Packard, Houston, TX, USA) and flame-ionisation detector (Agilent Technologies, Santa Clara, CA, USA).

Mitochondrial isolation and oxidation To obtain sufficient mitochondria, muscles from three rats were pooled for each of five independent experiments in lean and obese rats. Preparation of subsarcolemmal and intermyofibrillar mitochondria, and palmitate oxidation in these mitochondria were performed as described [3, 24].

Statistics

Correlations were performed using least squares linear regression. Two factor (lean vs obese) repeated measures (transfected vs control muscle) analyses of variance were used to compare the variables under investigation. All data are reported as mean ± SEM.

Results

Comparison of selected metabolic indices in lean and obese Zucker rats

Circulating fatty acid (lean 0.33±0.06 mmol/l, obese 0.81±0.12 mmol/l) and insulin (lean 1.8±0.5 ng/ml, obese 8.3±1.3 ng/ml) levels were greater in obese rats ($p<0.05$). Glucose concentrations were comparable in both groups (lean 10.5±1.2 mmol/l, obese 11.8±1.5 mmol/l).

The proportion of oxidative muscle fibres was greater in selected muscles (EDL, PL, WG) of obese rats than in lean animals (Table 1). In both groups, PGC-1α correlated highly ($r \geq 0.88$) with the proportion of oxidative fibres, glucose (GLUT4) and fatty acid transporters (FAT, fatty acid binding protein [FABP]pm), and COXIV (Fig. 1a–f), but not with AMPKα2 or selected insulin-signalling proteins (IRS1, PI3-kinase, Akt2), which were reduced by 20 to 45% in obese Zucker rats (Fig. 1a).

Modest PGC-1α overproduction increases mitochondrial density

Pgc-1α was transfected into the tibialis anterior muscle in one hindlimb, while the contralateral muscle served as control (empty vector). Transfection increased *Pgc-1α* mRNA (lean by 31%, obese by 37%; Fig. 2a) and protein (lean by 20%, obese by 27%; Fig. 2b). This modest PGC-1α increase induced mitochondrial biogenesis, as shown by the increases in mitochondrial DNA (lean 55%, obese 44% higher; Fig. 2c) and citrate synthase activity (lean 21%, obese 20% higher; Fig. 2d). PGC-β protein levels were not different in lean and obese animals, nor were they altered by *Pgc-1α* transfection (data not shown)

Table 1 Muscle fibre composition of hindlimb muscles in lean and obese Zucker rats

Muscle fibre type per group	Skeletal muscles						
	EDL	RTA	WTA	SOL	PL	RG	WG
Oxidative (%SO+%FOG)[a]							
Lean	42.3±3.9	68.6±1.4	25.0±4.4	100	42.2±5.5	93.3±2.9	7.1±2.3
Obese	58.5±3.6*	70.0±0.3	15.9±1.9*	100±1.9	72.3±2.3*	94.3±2.5	27.3±5.8*
SO (%)							
Lean	2.2±0.4	6.5±0.3	0	74.8±2.4	2.8±0.5	38.1±4.7	0
Obese	2.4±0.8	4.1±1.1	0	67.6±2.6	9.0±0.7*	31.4±4.7	0
FOG (%)							
Lean	40.1±3.5	62.1±1.6	25.0±4.4	25.5±2.6	39.3±5.4	55.1±2.4	7.1±5.1
Obese	56.1±2.9*	65.9±1.1	15.9±1.9	32.2±0.3	63.8±2.4*	62.9±2.3	27.3±5.8*
FG (%)							
Lean	57.7±3.9	31.2±1.6	75.0±4.4	0	57.8±5.5	6.7±2.8	93.1±2.2
Obese	43.5±5.2*	30.1±0.3	84.1±1.9*	0	27.3±2.2*	5.7±2.5	72.7±6.0*

Values are mean ± SEM, $n=5$ lean and $n=5$ obese Zucker rats

*$p<0.05$ for obese vs lean

FG, fast-twitch glycolytic muscle fibre content of each muscle; RG, red gastrocnemius; RTA, red tibialis anterior; SOL, soleus; WTA, white tibialis anterior

[a] Oxidative muscle fibre composition was based on summing the fast-twitch oxidative glycolytic (FOG) and slow-twitch oxidative (SO) muscle fibre content of each muscle

Glucose transport, GLUT4 and insulin signalling proteins

Lean and obese control muscles (empty vector) Insulin-stimulated, but not basal rates of glucose transport were lower in obese rats (Fig. 3a, b). Protein levels of GLUT4 (Fig. 3d) and AS160 (Fig. 3f) were similar in lean and obese Zucker rat muscles, but Akt2 protein was reduced in obese animals (−60%; Fig. 3e). Basal phosphorylation of Akt and AS160 did not differ in lean and obese animals (data not shown). However, insulin-stimulated phosphorylation of insulin signalling proteins was reduced in muscles of obese rats (Akt thr308 −82% [Fig. 3e] and ser473 –71% [data not shown]; AS160 thr642 –45% [Fig. 3f]; $p<0.05$).

Lean and obese Pgc-1α-transfected muscles Pgc-1α transfection increased the insulin-stimulated, but not basal (Fig. 3a) rates of glucose transport in lean (19%) and obese (21%) rat muscles ($p<0.05$; Fig. 3b). The absolute increase in *Pgc-1α*-transfected muscle was 60% smaller in the obese rats compared with lean rats (Fig. 3c).

PGC-1α also induced an increase in GLUT4 (lean 13%, obese 27%; Fig. 3d), but not in Akt2 and AS160 protein levels (Fig. 3e, f). However, *Pgc-1α* transfection did increase the insulin-stimulated phosphorylation of Akt thr308 (lean 47%, obese 44%; Fig. 3e) and ser473 (lean 29%, obese 24%, data not shown), although in obese animals Akt phosphorylation remained lower (Fig. 3f). Relatively greater improvements in insulin-stimulated AS160 phosphorylation (thr642) occurred in obese (45%) compared with lean (18%) animals. Hence, AS160 phosphorylation was restored to 80% of that in lean control muscle (Fig. 3f).

Intramuscular lipids, FAT and both subsarcolemmal and intermyofibrillar mitochondrial fatty acid oxidation

Lean and obese control muscles (empty vector) Intramuscular triacylglycerol, diacylglycerol and ceramide concentrations were greater in the obese animals (Fig. 4a–c). FAT protein levels did not differ between groups (Fig. 4d). In lean rats, the rate of palmitate oxidation by subsarcolemmal mitochondria was 27% lower than that by intermyofibrillar mitochondria. In obese rats, by contrast, subsarcolemmal mitochondrial palmitate oxidation was upregulated (by 56%) and was similar to the rate of intermyofibrillar mitochondrial palmitate oxidation (Fig. 4e, f).

Lean and obese Pgc-1α-transfected muscles Increases in PGC-1α protein decreased intramuscular triacylglycerol content in obese animals (−60%) but increased it in lean animals (+31%; Fig. 4a). Intramuscular diacylglycerol (−20%) and ceramide content (−28%) were reduced in obese Zucker rat muscle only (Fig. 4b, c). In both groups, PGC-1α protein increased FAT protein levels in muscle (lean +25%, obese +16%; Fig. 4d).

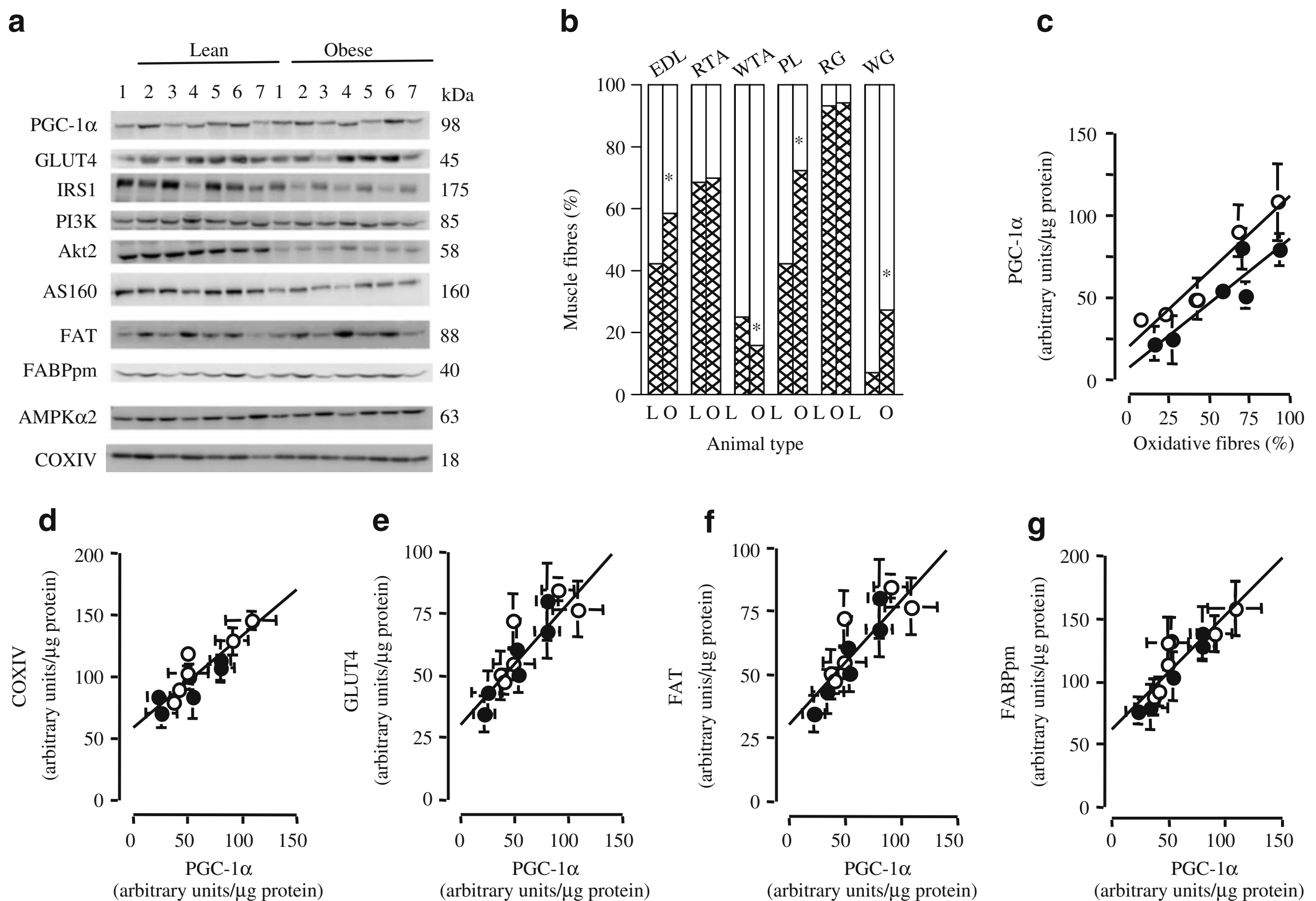

Fig. 1 Relationship of PGCα protein with muscle fibre composition and with selected metabolic proteins in metabolically heterogenous skeletal muscles in lean and obese Zucker rats. Oxidative muscle fibre composition was determined as previously reported [3], i.e. the sum of slow oxidative muscle+fast-twitch oxidative muscle fibres. **a** Western blots of PGC-1α and proteins involved in glucose transport, insulin signalling, fatty acid transport, fuel utilisation and an index of mitochondrial density. Lanes: *1*, EDL; *2*, red TA (RTA); *3*, white TA (WTA); *4*, soleus (SOL); *5*, plantaris (PL); *6*, red gastrocnemius (RG); *7*, white gastrocnemius (WG). **b** Muscle fibre composition in lean (L) and obese (O) rat muscles. White bars, fast-twitch glycolytic muscle fibres; hatched bars, oxidative muscle fibres (fast-twitch oxidative glycolytic fibers+slow-twitch oxidative fibers). **c** Relationship between muscle fibre composition and PGC-1α protein levels in lean (L) and obese (O) rats (r=0.96 in lean; r=0.92 in obese). The relationship of PGC-1α protein with (**d**) indices of mitochondrial density (COXIV) (r=0.88), (**e**) glucose transport (GLUT4) (r=0.87) and (**f**) fatty acid transport, i.e. FAT (r=0.96), and (**g**) FABPpm (r=0.89) White circles, lean; black circles, obese. Data are shown as mean ± SEM and are based on muscles from four to six animals

PGC-1α selectively targeted subsarcolemmal mitochondria, as palmitate oxidation was increased in this subgroup of mitochondria (lean +37% and obese +18%) relative to the contralateral control muscles (Fig. 4d). PGC-1α did not alter intermyofibrillar mitochondrial palmitate oxidation either in lean or in obese Zucker rat muscles (Fig. 4e).

Discussion

We have shown for the first time that a modest increase in PGC-1α levels increases mitochondrial biogenesis and improves both lipid metabolism and insulin-stimulated glucose transport in insulin-resistant muscle of obese Zucker rats in vivo. These changes occurred in concert with targeted improvement of fatty acid oxidation in subsarcolemmal mitochondria and increases in GLUT4 protein and in insulin-stimulated phosphorylation of insulin signalling proteins (Akt, AS160). The intramuscular lipid reduction induced by PGC-1α was greater in obese Zucker rats, while the increase in insulin-stimulated glucose transport that was induced by PGC-1α was greater in the lean animals.

Characterisation of lean and obese Zucker rats

The obese Zucker rats studied exhibited the expected characteristics, including: (1) increased circulating concentrations of fatty acids and insulin, with comparable levels of glucose; (2) impairment of insulin-stimulated glucose transport in muscle [25, 26]; (3) impaired Akt and AS160

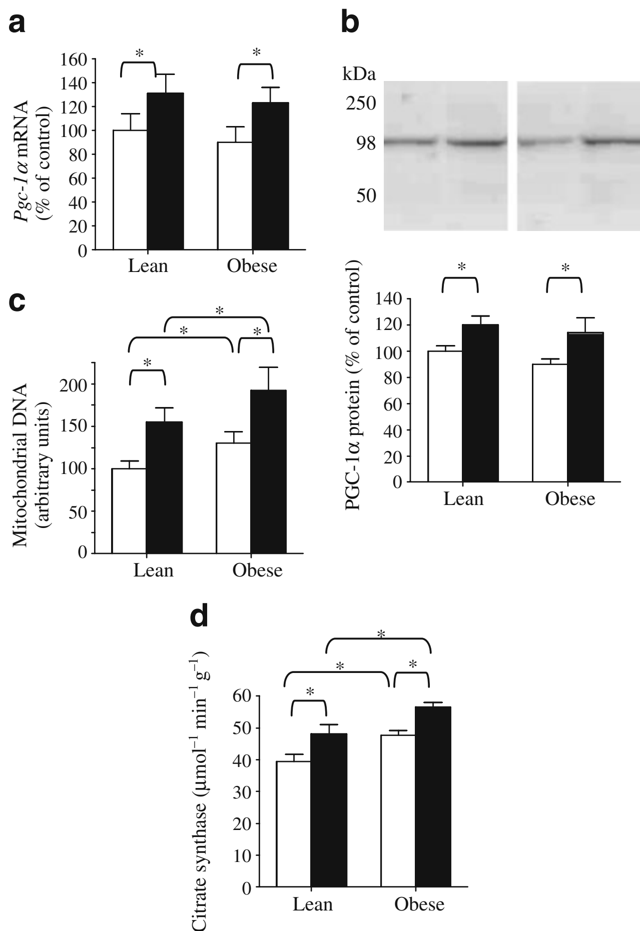

Fig. 2 Effects of *Pgc-1α* transfection on *Pgc-1α* mRNA and protein, and on indices of mitochondrial density in lean and obese Zucker rat muscle. **a** *Pgc-1α* mRNA, (**b**) PGC-1α protein as blot and bar graph, (**c**) mitochondrial DNA and (**d**) citrate synthase activity in *PGC-1α*-transfected (black bars) and contralateral control (white bars) muscles. **b** Equal protein concentrations were loaded for each muscle. Data are shown as mean ± SEM and were compared using paired analyses within each group and unpaired analyses between groups, n=4–5 for each muscle. *p<0.05 for comparisons indicated with the brackets

phosphorylation [27, 28]; and (4) increased concentration of intramuscular triacylglycerol [14, 25, 26]. The latter presumably reflects an attempt to limit intramuscular muscle lipotoxicity, as this lipid depot is increased in insulin-sensitive athletes (the athlete paradox) [2] and triacylglycerol provides a cytoprotective role in lipid overload states [29].

As in healthy animals [3, 30], we found that a smaller than twofold range of PGC-1α protein levels is highly related to the oxidative and substrate transport capacities among metabolically heterogeneous muscles in lean and obese Zucker rats. The correlations between PGC-1α and rodent muscle oxidative capacity, and between PGC-1α and both GLUT4 and FAT support the idea that in lean and obese muscles PGC-1α is central to a coordinated metabolic programme that upregulates a number of genes simultaneously to produce an oxidative muscle phenotype that relies extensively on blood-borne substrates for energy provision [3, 5, 31]. The small range of PGC-1α protein abundance across a range of heterogeneous muscles implies that small, physiologically induced changes in PGC-1α protein [6, 30, 32, 33] can have pronounced effects on muscle metabolism, as shown by the present study and others [3, 16].

Overexpression of *Pgc-1α* in skeletal muscle of lean and obese Zucker rats

To upregulate PGC-1α protein in muscle, we used an electrotransfection procedure, as we [3, 16, 17, 21, 34] and others [4, 20, 35] have done previously for different genes. Muscle transfection efficiencies are typically maintained within a 30% to 40% range, as was done in the present study and others [3, 4, 16, 17, 20, 34, 35]. A greater transfection efficiency requires higher electroporation voltages, which induce muscle damage (C. R. Benton and A. Bonen, unpublished data) [20]. Compared with transgenic *Pgc-1α* animals, transfection of a single muscle with *Pgc-1α* offers several advantages: (1) it is possible to examine PGC-1α-induced metabolic regulation in a controlled manner, in vivo; (2) this can be done without disturbing whole-body fuel homeostasis; (3) the animal serves as its own control; and (4) the resulting phenotype is not unusual, as is the case in *Pgc-1α* transgenic mice [10, 11, 36].

It is well known that only a select number of muscle fibres can ‘drive’ the metabolic changes observed within the whole muscle. Indeed, much of our understanding of the adaptive capacities in muscle (e.g. enzyme activities, glucose transport, gene expression and transcription rates, etc.) is based on studies in which only some of the muscle fibres have been affected during exercise (i.e. the motor unit size recruitment principle). Yet, large changes within only some muscle fibres, whether induced by exercise [37] or electrotransfection as in the present study and others [3, 4], permit us to observe meaningful metabolic adaptations within the whole muscle (i.e. an average effect across all muscle fibres). Based on our transfection efficiency, we estimate that PGC-1α is upregulated by 60 to 75% in the transfected muscle fibres, an effect that is within a physiologically meaningful range [30, 38]. Importantly, this yields metabolically meaningful changes in whole-muscle mitochondrial biogenesis, protein levels, insulin sensitivity and lipid metabolism, as seen in the present study and others [3, 16].

Modest PGC-1α protein increases improve insulin signalling and sensitivity

Modest PGC-1α protein increases induced improvements in the glucose transport system in lean and obese animals,

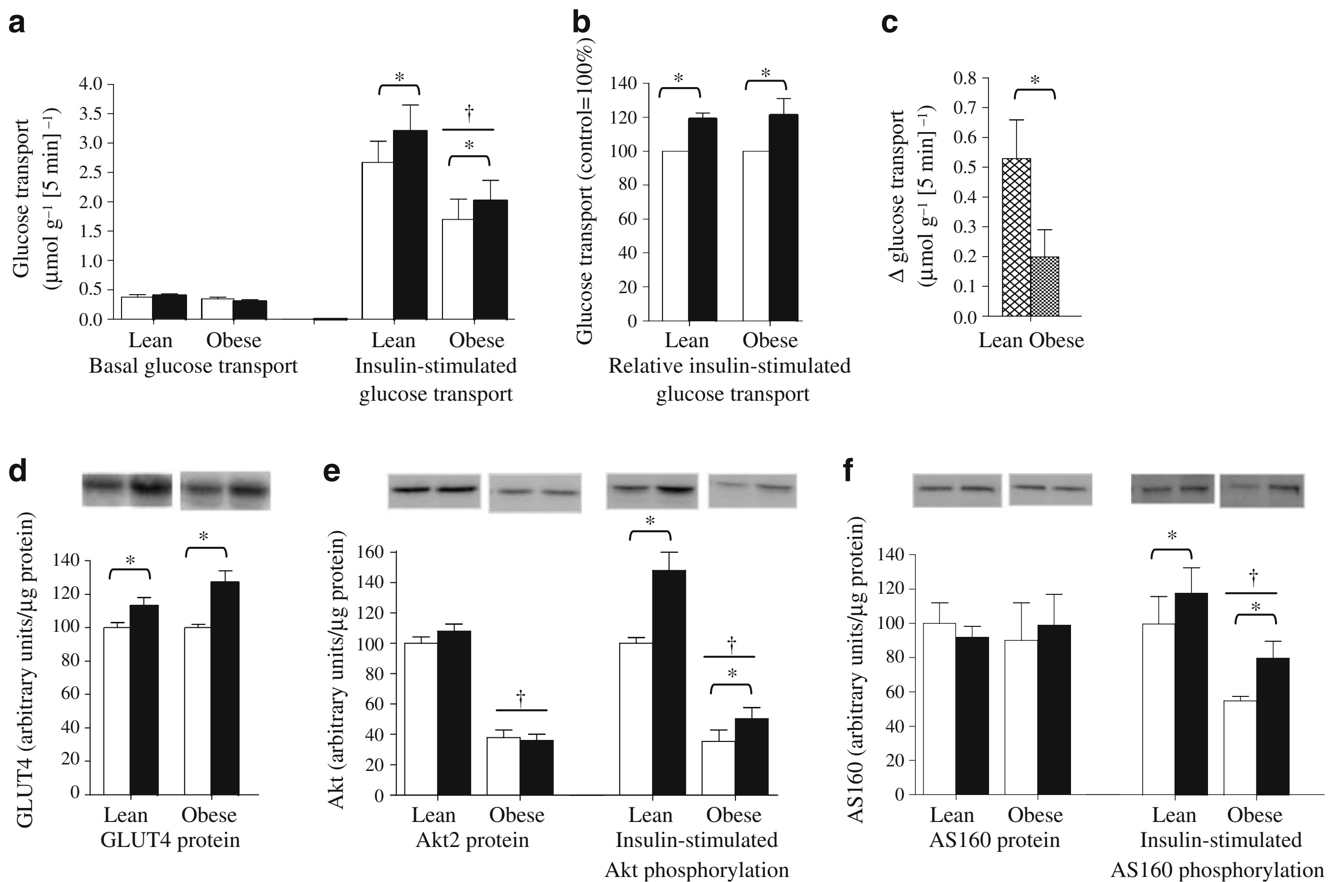

Fig. 3 Effects of a modest increase in PGC-1α protein on insulin sensitivity, the glucose transporter GLUT4 and insulin signalling proteins in lean and obese Zucker rat muscles. **a** Basal and insulin-stimulated glucose transport in perfused hindlimb muscles, as (**b**) relative changes (%=100×transfected/control) and (**c**) absolute changes in insulin-stimulated glucose transport in *Pgc-1α*-transfected muscles (Δ, transfected-control). **d** GLUT4 protein levels, (**e**) Akt2 levels and insulin-stimulated Akt thr308 phosphorylation, and (**f**) AS160 protein levels and insulin-stimulated AS160 thr642 phosphorylation. **d–f** Equal protein concentrations were loaded for each muscle. White bars, control (muscles transfected with empty vector); black bars, *Pgc-1α* transfected muscles. Data are shown as mean ± SEM and were compared using paired analyses within each group and unpaired analyses between groups, n=6–7 control and transfected muscles. *$p<0.05$ for transfected vs control; †$p<0.05$ for obese muscles (control and transfected) vs lean muscles (control and transfected)

including increases in GLUT4 levels and in insulin-stimulated Akt and AS160 phosphorylation, without any concurrent changes in Akt and AS160 protein levels. Given that insulin-stimulated AS160 phosphorylation was almost fully restored to normal in the *Pgc-1α*-transfected muscles, it was surprising that the extent of PGC-1α-induced improvement in insulin-stimulated glucose transport was smaller in insulin-resistant than in lean muscles. Other studies have shown that insulin-stimulated AS160 phosphorylation is highly correlated with muscle insulin sensitivity [39] and its restoration in insulin-resistant muscle [40]. Presumably, with *Pgc-1α* transfection, other GLUT4 signalling/trafficking processes were not sufficiently altered to fully normalise insulin-stimulated glucose transport in insulin-resistant muscles, despite almost full restoration of AS160 phosphorylation.

The basis for the PGC-1α-induced improvement in insulin-stimulated phosphorylation of signalling proteins in skeletal muscle in this and other studies [3, 41, 42] is unknown. It is tempting to speculate that these increases resulted from the reductions in intramuscular lipids [1]. Others have speculated that the unexpected increase in intramuscular lipids in muscles of *Pgc-1α* transgenic mice accounted for the resulting impairment of insulin-stimulated Akt2 activity and glucose transport [11]. However, some caution is required, as changes in intramuscular lipids are not always associated with changes in insulin-stimulated phosphorylation of signalling proteins [28, 40].

The improvements (~20%) in insulin-stimulated glucose transport in muscles of lean and insulin-resistant obese rats were within the range (%) observed with other therapeutic interventions, i.e. either exercise training in lean or obese animals [43], or rosiglitazone treatment [44, 45]. However, these changes were observed after many weeks of treatment, whereas *Pgc-1α* transfection induced changes within 2 weeks. Other muscle transfection studies have also observed

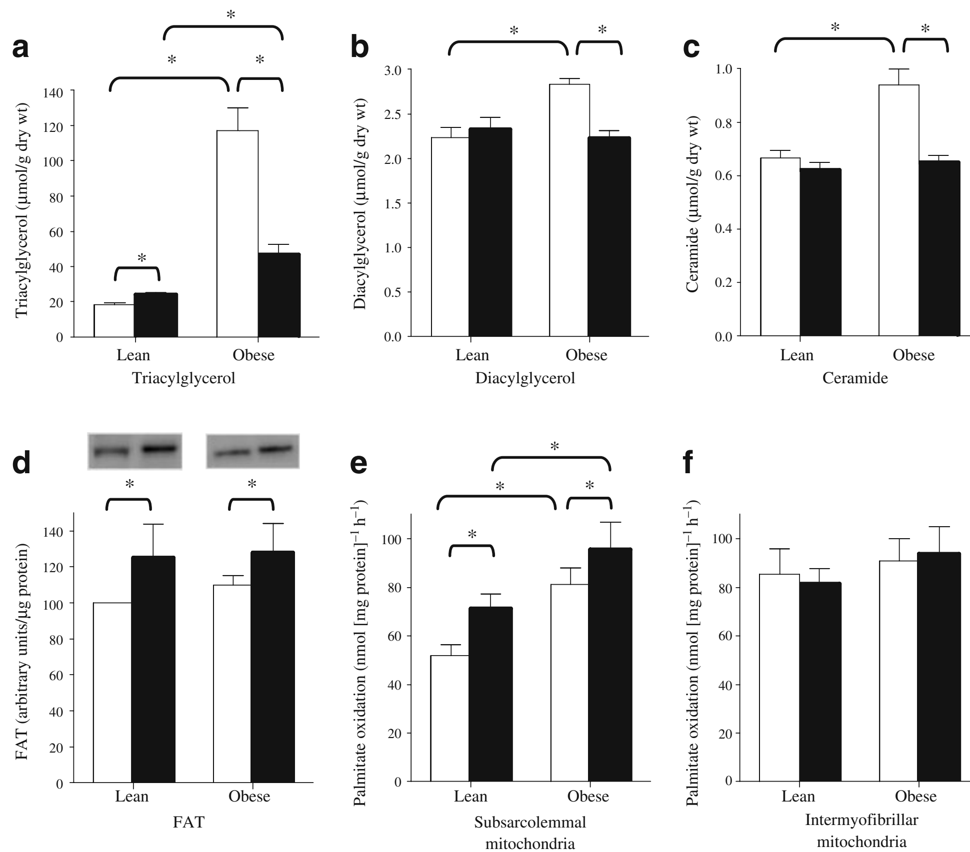

Fig. 4 Effects of a modest increase in PGC-1α protein on the fatty acid transporter FAT, intramuscular lipids accumulation and mitochondrial fatty acid oxidation in lean and obese Zucker rat muscles. **a** Triacylglycerol, (**b**) diacylglycerol, (**c**) ceramide, (**d**) FAT protein and (**e**) palmitate oxidation in isolated subsarcolemmal and (**f**) intermyofibrillar mitochondria. **a–c** Five muscles and (**e, f**) three muscles were pooled for each of 6–7 independent determinations. **d** Equal protein concentrations were loaded for each muscle. White bars, control (muscles transfected with empty vector); black bars, *PGC-1α*-transfected muscles. Data are shown as mean ± SEM and were compared using paired analyses within each group and unpaired analyses between groups. *$p<0.05$ for comparisons indicated with the brackets

a 20% to 25% increase in insulin-stimulated glucose transport after 7 to 14 days, either when PGC-1α [3] or carnitine palmitoyltransferase 1 (CPTI) [4] were overproduced in healthy animals [3] or in diet-induced, insulin-resistant animals [4]. Thus, a modest PGC-1α protein increase improves insulin sensitivity rapidly within ranges observed in other experimental models. We recognise that PGC-1α did not fully normalise insulin sensitivity. This was also observed in insulin-resistant, CPTI-transfected muscles [4]. However, other than studies in cell lines [7], our work is the first to show that modest PGC-1α overexpression improves insulin sensitivity in vivo in healthy (as shown by the present and another study [3]) and in insulin-resistant muscle (present study).

FAT, intramuscular lipids and mitochondrial palmitate oxidation

FAT is a key fatty acid transporter. The plasmalemmal content of this protein is correlated with intramuscular lipid accumulation in obesity and type 2 diabetes [46]. A large muscle-specific increase in *Pgc-1α* (mRNA increased by 600%) provoked diet-induced insulin resistance, which was attributed to a large increase in *Fat* (also known as *Cd36*) (mRNA increased by 300%) and the consequent increases in intramuscular diacylglycerol and long-chain fatty acyl CoAs [11]. In contrast, when we modestly overexpressed *Pgc-1α* (~25%), insulin sensitivity was improved, as seen in the present and another study [3]. A key reason is that this limited the extent of PGC-1α-mediated FAT upregulation. While a large increase in FAT contributes to intramuscular lipid accumulation [11], a small increase in muscle FAT is not deleterious, as this increases fatty acid oxidation only [34]. Thus to improve insulin sensitivity in muscle via PGC-1α, it is essential, as we have previously suggested [3, 13], to limit the increase in PGC-1α protein in order to limit the upregulation of FAT protein.

It is not clear why intramuscular diacylglycerol and ceramide were only reduced in the obese Zucker rats.

Nevertheless, these reductions may have contributed to the improved insulin-stimulated phosphorylation of insulin signalling proteins (Akt, AS160) in the obese animals. However, a similar rationale cannot be used for lean animals or in CPTI transfected muscles in chow-fed animals [4], as in these animals intramuscular lipid metabolites were not reduced. Recent data indicate that changes in insulin-stimulated phosphorylation of signalling proteins can occur independently of changes in intracellular lipids [28], including restoration of muscle insulin sensitivity [40]. Thus, while PGC-1α-mediated reductions in intramuscular lipids may have contributed to improved insulin-stimulated phosphorylation of Akt and AS160 in obese animals, this is less certain for the lean animals.

It is known that subsarcolemmal mitochondria are more malleable than intermyofibrillar mitochondria. With changes in aerobic fitness induced via exercise training or inactivity [47, 48], as well as in obesity and type 2 diabetes [9, 49], changes in mitochondrial function and density are largely confined to subsarcolemmal mitochondria [47–49]. Although the bases for selective changes in subsarcolemmal mitochondria are not known, our work, i.e. the present and previous studies [3, 24], indicates that PGC-1α targets subsarcolemmal mitochondria. The PGC-1α-mediated upregulation of subsarcolemmal fatty acid oxidation was not attributable to concurrent changes in CPTI (data not shown; [3]). PGC-1α targeting of subsarcolemmal mitochondria may be an attempt to efficiently remove, via β-oxidation, fatty acids as soon as they cross the sarcolemma, thus minimising their intramuscular accumulation. However, the relationship between skeletal muscle fatty acid oxidation and intramuscular lipid accumulation is not necessarily a simple reciprocal one [14, 50], as has been widely thought.

Insulin sensitivity depends on the level of PGC-1α overproduction

The effects of *Pgc-1α* overexpression on insulin sensitivity in vivo can be strikingly different. When *Pgc-1α* overexpression is maintained within a low range (<100%), improvements in insulin sensitivity are observed (Fig. 5), but when muscle-specific *Pgc-1α* overexpression is induced far beyond normal physiological limits, insulin-stimulated glucose disposal is reduced (Fig. 5). These different PGC-1α-mediated responses in insulin sensitivity are likely to be related to differences in the PGC-1α-induced increases in FAT levels (see above).

Exercise and PGC-1α

Our work in the present and a previous study [3] showing that modest PGC-1α overproduction is sufficient to improve lipid metabolism and insulin action evolved from observations that exercise training-induced upregulation of insulin sensitivity is accompanied by a modest PGC-1α increase. While it may be desirable to seek a pharmacological means to upregulate PGC-1α, it is difficult to ignore the fact that the simplest way to upregulate PGC-1α is via exercise.

Summary

By increasing PGC-1α protein only modestly, an approach based on physiological and metabolic considerations, we found that insulin-stimulated glucose transport was improved in healthy and insulin-resistant skeletal muscle. The underlying mechanisms appear to be: (1) PGC-1α-induced increases in GLUT4 protein levels; (2) improvements in lipid

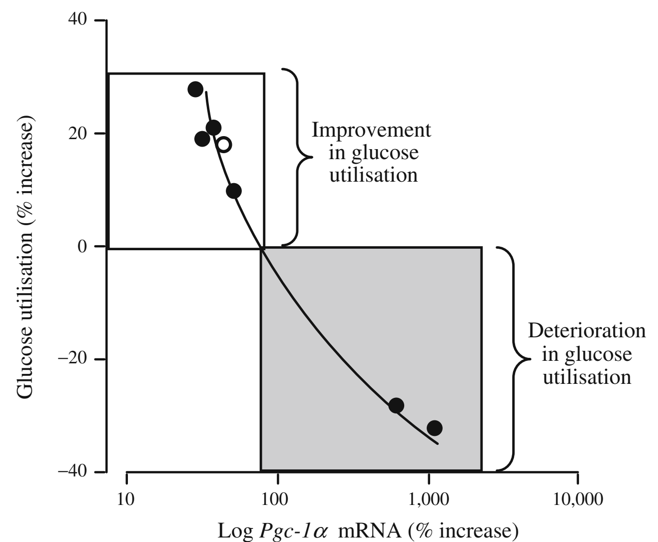

Fig. 5 Comparison between relative changes in *Pgc-1α* overexpression (%) and changes in insulin-stimulated glucose utilisation (%) in healthy animals. Data are from the present study and recently published studies [3, 10, 11, 41] in which *Pgc-1α* was overexpressed to varying levels in transgenic animals [10, 11, 41] or in electrotransfected muscles (as here and in a previous study [3]). *Pgc-1α* mRNA increase (%) was calculated relative to controls in the studies mentioned. Insulin-stimulated glucose utilisation in the various studies was based on various methods [3, 10, 11, 41] and therefore the increase (%) was calculated relative to controls. White circles, relative increase (%) in obese Zucker rats in the present study (these data were not include in the derived regression line). These data suggest that when *Pgc-1α* mRNA level is maintained continuously at a high level (i.e. >100% increase), insulin-stimulated glucose utilisation deteriorates sufficiently to result in insulin resistance (grey box). In contrast, maintaining *Pgc-1α* mRNA expression more modestly (<100%) increases insulin sensitivity (clear box). These contrasting responses may reflect, in part, the differential effects of PGC-1α protein (depending on its increase) on FAT-mediated intramuscular lipid accumulation, which can interfere with insulin signalling (see the Discussion)

metabolism and insulin-stimulated phosphorylation of insulin signalling proteins; and (3) limitation of PGC-1α-induced increase in FAT. In obese muscles, the improved insulin-stimulated glucose transport and insulin signalling were less robust, while improvements in lipid metabolism were greater. Nevertheless, our work demonstrates for the first time the therapeutic potential of PGC-1α for improving insulin action in insulin-resistant skeletal muscle.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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