High-fat diets cause insulin resistance despite an increase in muscle mitochondria

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It has been hypothesized that insulin resistance is mediated by a deficiency of mitochondria in skeletal muscle. In keeping with this hypothesis, high-fat diets that cause insulin resistance have been reported to result in a decrease in muscle mitochondria. In contrast, we found that feeding rats high-fat diets that cause muscle insulin resistance results in a concomitant gradual increase in muscle mitochondria. This adaptation appears to be mediated by activation of peroxisome proliferator-activated receptor (PPAR)β by fatty acids, which results in a gradual, posttranslationally regulated increase in PPAR γ coactivator 1α (PGC-1α) protein expression. Similarly, overexpression of PPARδ results in a large increase in PGC-1α protein in the absence of any increase in PGC-1α mRNA. We interpret our findings as evidence that raising free fatty acids results in an increase in mitochondria by activating PPARδ, which mediates a posttranscriptional increase in PGC-1α. Our findings argue against the concept that insulin resistance is mediated by a deficiency of muscle mitochondria.

High-Fat Diets Induce an Increase in Skeletal Muscle Mitochondria. In our previous study of the effect of raising FFA on mitochondrial biogenesis in skeletal muscle, we fed rats a high-fat diet and gave them daily injections of heparin to raise serum FFA to high levels (16). The rationale for this approach was to try to mimic the effects of exercise on FFA levels. One purpose of the present study was to determine whether the more modest increase in fat oxidative capacity.

Overexpression of peroxisome proliferator-activated receptor (PPAR)δ in muscles of transgenic mice, or activation of PPARδ in skeletal muscle in vivo by using a specific PPARδ activator, has been shown to result in increases in skeletal muscle mitochondria (17, 18). In these studies, an increase in muscle mitochondria occurred in the absence of an increase in PGC-1α mRNA (17, 18). This finding led to the conclusion that the increase in mitochondria is mediated directly by PPARδ (17–19). We have shown that raising serum FFA, which are endogenous ligands of PPARδ (20), results in activation of PPARδ, as evidenced by a large increase in PPARδ binding to the PPAR response element in the carnitine palmitoyl transferase 1 (CPT-1) promoter (16). Muscle CPT-1 expression is regulated by PPARδ in skeletal muscle (21). This finding, viewed in the context of the previous studies of PPARδ overexpression and activation, makes it probable that activation of PPARδ is the first step in the pathway by which raising FFA leads to an increase in mitochondria. However, it did not seem possible that PPARδ could directly induce mitochondrial biogenesis, because it regulates expression of a limited number of mitochondrial proteins. It is well established that the biogenesis of normal, functional mitochondria requires the activation of a number of transcription factors in addition to PPARδ (22–24). The coordinated activation of these transcription factors and, thus, the regulation of mitochondrial biogenesis, is mediated by the transcription coactivator PGC-1 (22, 24).

In this context, another aim of this study was to test the hypothesis that raising FFA and overexpressing PPARδ both result in an increase in PGC-1α protein expression by a posttranscriptional mechanism.

Results

Body Weights, Intraabdominal Fat, and Serum Free Fatty Acids. Feeding rats the high-fat diets for 4–5 wk was not sufficiently long to result in a significantly greater increase in body weight than occurred in chow-fed controls. However, the omental, epididymal, and retroperitoneal fat depots were significantly greater in the high-fat diet-fed groups than in the chow-fed group (7.7 ± 0.5 g vs. 12.2 ± 1.1 g, P < 0.01). The high-fat diet-fed rats had higher serum FFA levels than the chow-fed animals (756 ± 68 μM vs. 249 ± 24 μM, P < 0.01).

High-Fat Diets Induce an Increase in Skeletal Muscle Mitochondria. In our previous study of the effect of raising FFA on mitochondrial biogenesis in skeletal muscle, we fed rats a high-fat diet and gave them daily injections of heparin to raise serum FFA to high levels (16). The rationale for this approach was to try to mimic the effects of exercise on FFA levels. One purpose of the present study was to determine whether the more modest increase in


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FFA that results from feeding a high-fat diet also induces an increase in muscle mitochondria. As shown in Fig. 1, there were significant increases in a range of mitochondrial proteins in epitrochlearis muscles of rats fed the flaxseed/olive oil diet for 5 wk. There was also a significant increase in the capacity of skeletal muscle to oxidize palmitate [10.2 ± 1.8 (chow) vs. 17.8 ± 2.5 (high fat) nmol/min per g, *P < 0.02], providing evidence for an increase in functional mitochondria. Further evidence for a high-fat diet-induced increase in muscle mitochondria is provided by the finding of an increase in mitochondrial DNA copy number, as evidenced by an increase in the ratio of cytochrome b to 18S DNA (Fig. 2).

Because previous studies in which high-fat diets were reported to result in decreases in PGC-1α and/or muscle mitochondria used either lard- or milk-based diets (8–10), we also examined the effect of a diet containing 32% lard and 18% corn oil. As shown in [supporting information (SI) Fig. S1], feeding rats the lard/corn oil diet for 4 wk resulted in increases in a range of mitochondrial proteins in muscle.

The Flax Seed/Olive Oil Diet Induces Insulin Resistance of Muscle Glucose Transport. The lard/corn oil diet and other high-fat diets induce insulin resistance of muscle glucose transport in rats within a few weeks and, depending on type of fat and genetic background, diabetes in rats (25–28). The flax seed/olive oil diet also results in decreases in insulin responsiveness of glucose transport in the slow-twitch red soleus muscle [2-deoxyglucose (2DG) transport: chow, 1.6 ± 0.2 (basal) and 5.9 ± 0.6 (insulin) μmol·ml⁻¹·30 min⁻¹ versus high fat, 1.4 ± 0.1 (basal) and 3.4 ± 0.3 (insulin) μmol·ml⁻¹·30 min⁻¹, *P < 0.05] and the fast-twitch white epitrochlearis muscle [2DG transport: chow, 0.52 ± 0.07 (basal) and 2.5 ± 0.2 (insulin) μmol·ml⁻¹·30 min⁻¹ versus high fat, 0.53 ± 0.05 (basal) and 1.8 ± 0.14 (insulin) μmol·ml⁻¹·30 min, *P < 0.05]. Because the high-fat diet-induced increases in mitochondria and in the capacity to oxidize fat occurred during the period in which insulin resistance developed, the muscle insulin resistance is clearly not due to mitochondrial deficiency.

Time Course of the Increase in Muscle Mitochondria. In contrast to induction of increases in mitochondrial biogenesis by other stimuli such as exercise (29–31), cold (32), or increases in cytosolic calcium (33), which occur rapidly (hours), the high-fat diet-induced increase in mitochondria occurs slowly (weeks). As shown in Fig. 3, no increases in expression of mitochondrial proteins were observed after 1 wk, and only small increases were present after 2 wk. Exceptions were long-chain acyl CoA dehydrogenase (LCAD) and uncoupling protein 3 (UCP3), which had increased 50–60% by 2 wk (data not shown). Transcription of LCAD and UCP3 is regulated by PPARδ (34, 35). A significant adaptive response of mitochondrial marker proteins had occurred by 4 wk (Fig. 3). The mRNA content of a range of mitochondrial markers was not different after 1 wk of fat feeding (data not shown), however, increases began to be evident by 2 wk and further increases were evident after 4 wk (Fig. S2 A and B).

PGC-1α Response to the High-Fat Diets. FFA are natural ligands of the PPARs (20). Both overexpression and activation of PPARδ result in increased mitochondrial biogenesis in the absence of an increase in PGC-1α mRNA in skeletal muscle in vivo (17–19). In these studies, PGC-1α protein was not measured, and it was concluded that overexpression or activation of PPARδ directly mediates mitochondrial biogenesis. It did not seem possible to us that PPARδ could directly mediate mitochondrial biogenesis, because it regulates expression of only a subset of mitochondrial proteins. Mitochondrial biogenesis requires the coordinated expression of genes encoded in both the nuclear and mitochondrial genomes, and this process requires activation and/or increased expression of a number of transcription factors in addition to PPARδ (22–24). These include the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) and PPARα.
NRF-2), mitochondrial transcription factor A (TFAM), the myocyte specific enhancer factors (MEFs), and the estrogen related receptors (ERRs) (22–24). The coordinated increase in transcription of genes encoding mitochondrial proteins is mediated by the transcription coactivator PGC-1α, which activates the NRFs, ERRs, PPARs, and MEFs and, thus, plays the key role in mediating adaptive mitochondrial biogenesis (22–24).

In this context, we hypothesized that the increase in muscle mitochondria induced by activation or overexpression of PPARδ is mediated by induction of an increase in PGC-1α protein expression by a posttranscriptional mechanism. We have previously reported that raising FFA by means of a high-fat diet plus daily heparin injections for 4 wk does not result in an increase in PGC-1α mRNA in muscle (16). The high-fat diets also did not cause an increase in PGC-1α mRNA (Fig. S3). In fact, we observed a significant decrease in PGC-1α mRNA after 1 wk of a high-fat diet, with a return to the control level after 2 wk (Fig. S3). Other investigators have also reported that high-fat diets (8–10) bring about a decrease in PGC-1α mRNA.

As shown in Fig. 4, there was a 2.5-fold increase in PGC-1α protein in muscle of rats fed the high-fat diets for 4 wk. In contrast to the induction of PGC-1α by other stimuli, such as exercise (29–31), cold (32), and calcium (33), which occurs rapidly (hours) and is mediated by increased transcription, the high-fat diet-induced increase in PGC-1α protein expression occurs slowly (weeks) and in the absence of an increase in PGC-1α mRNA. We used an anti-PGC-1α antibody obtained from Calbiochem in the Western blots shown in Fig. 4. To further confirm the finding of an increase in PGC-1α protein, we also used three other anti-PGC-1α antibodies directed at different amino acid sequences of the PGC-1α protein. The results obtained with all four antibodies show an increase in PGC-1α protein expression in muscles of the high-fat diet-fed animals (Fig. S4).

**Overexpression of PPARδ in Skeletal Muscle Results in an Increase in PGC-1α Protein.** The finding that overexpression of PPARδ in skeletal muscle of transgenic mice does not result in an increase in PGC-1α mRNA led to the conclusion that the increase in mitochondria is mediated directly by PPARδ (17–19). In light of our finding that raising fatty acids, which activate PPARδ, induces a posttranscriptionally mediated increase in PGC-1α protein, it seemed important to determine whether overexpression of PPARδ also results in an increase in PGC-1α protein. Three weeks after electric pulse-mediated transfer of a full-length PPARδ gene into tibialis anterior muscle, PPARδ protein was increased ~2-fold above control level (Fig. 5A). This increase in PPARδ protein resulted in a ~4-fold increase in PGC-1α protein expression (Fig. 5B). As in previous studies (17, 18), overexpression of PPARδ did not result in an increase in PGC-1α mRNA (Fig. S5).

**Discussion**

The major findings of the first phase of this study are that feeding rats high-fat diets results in an increase in mitochondria and in the capacity of muscle to oxidize fat concomitant with development of muscle insulin resistance. It has been hypothesized that skeletal muscle insulin resistance is mediated by a mitochondrial deficiency that limits fat oxidation and results in accumulation of intramyocellular lipids (2). This concept is based on the finding that muscles of insulin-resistant individuals generally contain ~30% less mitochondria than those of insulin-sensitive control subjects (3–7). This phenomenon has also been referred to as mitochondrial dysfunction (1, 2), although a detailed evaluation has provided evidence that the remaining mitochondria function normally (36). The present findings seem incompatible with the concept that muscle insulin resistance is mediated by mitochondrial deficiency.

Our findings that high-fat diets induce increases in PGC-1α protein, a range of mitochondrial proteins, and fat oxidative capacity in skeletal muscle of rats differ markedly from those of a recent study, in which feeding mice a high-fat diet for 3 wk was reported to decrease muscle mRNA levels of PGC-1α and mitochondrial respiratory chain constituents by ~90% and PGC-1α and cytochrome c protein levels by ~40% (8). However, a number of earlier studies provided evidence that high-fat diets result in an increase in mitochondrial enzymes in muscle (11–14), and we have shown that large increases in FFA induce an increase in mitochondrial biogenesis (16). Furthermore, Turner et al. (15) recently reported that, as in the present study, feeding rats a high-fat diet resulted in increases in mitochondrial enzyme activities, mitochondrial respiratory chain subunit protein levels, and fat oxidation capacity in skeletal muscle. Further evidence that fatty acids stimulate mitochondrial biogenesis comes from the finding that lowering serum FFA by giving acipimox to rats resulted in a ~30% decrease in mRNA content of mitochondrial proteins (37) and that overexpression of lipoprotein lipase in muscle results in a massive increase in mitochondria (38).

It is surprising that the mitochondrial deficiency causes insulin resistance concept has been so widely accepted, because it seems untenable in the context of what is known regarding the capacity of skeletal muscle for oxidative metabolism. The mechanism by which a 30% decrease in muscle mitochondria has been pro-
posed to cause muscle insulin resistance is an impairment in the ability to oxidize fat, resulting in accumulation of intramyocellular lipids (1, 2). Actually, the rate of substrate oxidation in resting muscle is not determined/limited by mitochondrial oxidative capacity but by the rate of ATP breakdown/ADP formation, which is regulated by the cells’ need for energy (39). The energy/substrate requirement of resting muscle cells is determined by “housekeeping” functions, such as maintenance of transmembrane potential by the Na+/K+ ATPase, protein synthesis, etc., and is very low relative to the maximal capacity of muscle for substrate oxidation. Increasing the supply of FFA or glucose to resting muscle can change the relative proportions of these substrates that are oxidized but does not result in an increase in substrate oxidation above that required to supply the energy needed for ATP repletion, regardless of its content of mitochondria.

Oxygen utilization by limb skeletal muscles when individuals are exercising at a work rate that elicits their maximal capacity to use oxygen (VO2max) during running or cycling is in the range of 200 mJ/kg muscle ± min^-1 for sedentary and 400 mJ/kg muscle ± min^-1 for well-trained individuals (40). Overweight, middle-aged patients with insulin resistance are generally in poor physical condition and have a low VO2max, in the range of 22–26 ml of O2/kg body wt ± min^-1 (7). It often assumes that such an individual has an extremely low muscle capacity to use oxygen of 100 mJ/kg ± min^-1 that is only 50% as great as that of normal sedentary individuals, this still represents a 33-fold increase above resting muscle oxygen uptake, which is ~3 ml/kg muscle ± min^-1. In light of the enormous difference in the capacity of muscle to oxidize substrate and the rate of substrate oxidation required to regenerate ATP in resting muscle, a 30% decrease in muscle mitochondria is irrelevant in terms of the ability of resting muscle to oxidize fat.

On the other hand, in pathological states in which mitochondrial number and/or function are so severely reduced as to limit the rate of substrate oxidation in resting muscle, both basal- and insulin-stimulated glucose transport are increased (41, 42) despite massive accumulation of intramyocellular lipid (41). Thus, mitochondrial deficiency and dysfunction sufficiently severe to limit fat oxidation increases, rather than decreases, insulin action. Similarly, severe hypoxia, which can be thought of as the ultimate degree of mitochondrial dysfunction, markedly increases basal- and insulin-stimulated glucose transport (43, 44). These effects of mitochondrial deficiency/dysfunction are mediated by activation of AMPK-dependent protein kinase (AMPK) (41, 42, 44), which stimulates glucose transport and induces an increase in expression of the GLUT4 glucose transporter (45, 46).

If increases in FFA do not mediate a decrease in mitochondria, what is responsible for the lower than usual amount of mitochondria in skeletal muscle of insulin-resistant patients? Nair and coworkers (6) have hypothesized that, rather than causing insulin resistance, a reduction in mitochondrial content is mediated by insulin resistance. Although our findings shed no light on this question, we think it is likely that exercise deficiency is the cause of the mitochondrial deficiency. The amount of mitochondria in muscle can vary over a considerable range, increasing with endurance exercise training and decreasing with physical inactivity (47). The insulin resistance syndrome and type 2 diabetes are, to a large extent, mediated by exercise deficiency in genetically predisposed individuals who do not balance reduced energy expenditure with reduced energy intake, resulting in obesity.

The central finding of the second phase of this study is that a high-fat diet induces an increase in muscle mitochondria by increasing the expression of PGC-1α protein via a posttranscriptional mechanism. We have shown that raising FFA levels activates PPARδ in skeletal muscle in vivo, as evidenced by increased binding to the PPAR response element in the carnitine palmitoyl transferase 1 promoter (16). This finding is not surprising because fatty acids are endogenous ligands responsible for activating the PPARs (20). In the studies on mouse skeletal muscle in vivo, in which PPARδ was overexpressed or activated by raising a PPARδ ligand, the following: (a) PGC-1α mRNA increased in the absence of an increase in PGC-1α mRNA (17, 18). Similarly, raising serum FFA (16) and feeding rats high-fat diets resulted in increased biogenesis of mitochondria in skeletal muscle despite no increase in PGC-1α mRNA. Because it did not seem possible that PPARδ could directly mediate mitochondrial biogenesis, we hypothesized that increases in PPARδ expression and/or activity bring about an increase in PGC-1α protein expression by a posttranscriptional mechanism. This hypothesis proved correct, because we found that both the high-fat diets and the increased expression of PPARδ induce increases in skeletal muscle PGC-1α protein expression.

It seems likely that the reason that the increase in PGC-1α protein was missed in previous studies is that it has become common practice to just measure mRNA levels and to refer to increases in gene transcription as increased gene expression. This approach ignores the fact that genes are expressed as proteins and that gene expression can be regulated at a number of steps during translation as well as posttranslationally. As an example of posttranslational regulation, it has been shown that overexpression of calpastatin, the endogenous inhibitor of the protease calpain, results in large increases in expression of GLUT4, CAMKII, and AMPK proteins despite a decrease or no change in their mRNA levels (48, 49).

The regulation of PGC-1α expression by PPARδ differs markedly between skeletal muscle in vivo and myotubes in culture. In contrast to the finding in mouse and rat skeletal muscle that PPARδ overexpression or activation does not result in an increase in PGC-1α mRNA, a number of studies have shown that PPARδ induces an increase in PGC-1α gene transcription, with a rapid increase in PGC-1α mRNA, in myotubes (19, 50, 51). This remarkable difference is currently unexplained, but it does show that skeletal muscle and myotubes have important differences that make it inappropriate to refer to myotubes as “skeletal muscle” and shows that findings on myotubes cannot always be directly extrapolated to skeletal muscle.

In conclusion, our results show that a high-fat diet that raises FFA results in a gradual increase in mitochondria in rat skeletal muscle, with an increase in the capacity for fat oxidation, concomitant with development of muscle insulin resistance. This finding argues against the concept that muscle insulin resistance is mediated by a deficiency of muscle mitochondria. We interpret our findings as evidence that the increase in muscle mitochondria induced by raising FFA levels is mediated by activation of PPARδ, which brings about a gradual increase in PGC-1α expression by a posttranscriptional mechanism.

Materials and Methods

Animal Care. This work was approved by the Animal Studies Committee of Washington University School of Medicine. Male Wistar rats weighing ~50 g were housed 2 or 3 per cage with a 12:12 light/dark cycle. Animals were given ad libitum access to one of three diets, a standard chow diet (PicoLab Rodent Diet 20, 5053) or one of two high-fat diets. The standard chow diet (chow) consisted of 23.5% calories from protein, 11.9% calories from fat, and 64.5% calories from carbohydrate. The high-fat flax seed/olive oil diet consisted of 20% calories from protein (243 g/kg casein), 50% calories from fat (187.5 g/kg flax seed oil and 93.7 g/kg olive oil), and 30% of calories from carbohydrate (210.8 g/kg corn starch, 105.4 g/kg sucrose, and 59.6 g/kg bran). The high-fat lard/corn oil diet consisted of the following: 23% calories from protein (254.3 g/kg casein), 50% calories from fat (180 g/kg lard and 100 g/kg corn oil), and 27% calories from carbohydrate (347.4 g/kg sucrose). Both of the high-fat diets included 22 g of vitamin mix (Teklad Premier #40077), 51 g of mineral mix
Insulin stimulated (2 milliunits/ml) glucose transport activity was measured by
were anesthetized with 50 mg/kg sodium pentobarbital and epitrochlearis
g of choline chloride per kg of diet.
Bioscience). The data were normalized to the housekeeping genes by the
pepstatin, 0.1 mM bpV(phen), and 2 mg/ml
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mediated metabolic remodeling of skeletal myocytes mimics exercise training and
drugs were transferred to a pAdCMV/ VS-DEST vector by using LR clonase (Invitrogen). Adenovirus was generated by
PGRC1
by ECL and quantified by densitometry.
PGC-1
by RT-PCR as described previously (57). The sense and antisense primers for
Muscle mitochondrial DNA content was determined as described previously (16). Briefly, DNA isolation was performed by using organic extraction (38). PCR was performed by using an 18S primer for a nuclear marker (catalog no. 1718; Ambion) and primers for cytochrome b
forward, ACAAAAATCCATTCTCATA; reverse, GTTGGGAATGGAGCTGAA,
PCR was performed with Promega master mix containing Taq polymerase,
dNTPs, MgCl2, and PCR buffers.
Plasmid DNA Constructs. To add myc-tag to the N terminus of PPARγ, PPARγ
cDNA (Origene) was PCR cloned with the forward primer, 5'-CACTATGCATTCAAGAGGATCTGAGCAACGAGAAGC-3', and reverse primer, 5'-TTGATCTAGTCTGGTATACTCC-3'. The PCR fragment was inserted into pcR2.1 Topo (Invitrogen). For the construction of the pCAG-
mvc-pppard, EcoRI fragment of pcR2.1 myc-pppard was inserted into EcoRI site of pcCAGEN (59). The plasmid DNAs were transformed and amplified by using
DH-5α bacteria and purified with a plasmid max kit (Qiagen). To generate
PGP genetic mapping was presented in the pEnder/D-Topo vector and cDNA inserts were transferred to a pAdCMV/ VS-DEST vector by using LR clonase (Invitrogen). Adenovirus was generated by

Plasmid DNA Electroporation. The overexpression of PPARγ in the tibial an- terior (TA) muscle was accomplished by using an electric pulse-mediated gene
transfection technique (60). Rats were anesthetized with isoflurane gas. The
tibial anterior (TA) muscle was injected with 100 µg of plasmid DNA con- taining the PPARγ-myc or empty vector in 0.1 ml of saline by using a 28-gauge needle connected to the 0.04 ml/min syringe. After the injection, an electric field was applied to
the TA muscle by using a 588 square-pulse stimulator (Grass) with a 533 model two-needle array (BTX). The electric field application consisted of eight pulses of 100-ms duration (at a frequency of 1 Hz and amplitude of 100 volts), which were applied perpendicularly to the long axis of the muscle. Muscles were harvested 3 wk after electroporation.

Immunoprecipitation of myc PPARγ. Three weeks after myc PPARγ gene trans- fer, tibial anterior muscles were homogenized in ice-cold buffer. Homoge- nates were rotated at 4°C for 1 h then centrifuged at 13,000 × g for 15 min. Supernatant fraction was collected and protein content was measured. Ho- mogenate samples containing 1 mg of protein were incubated overnight with anti-mycTag antibody (catalog no. 2276; Cell Signaling Technology). The next morning, 20 µl of protein A/G magnetic beads (Biolclone Inc.) were added, and samples were incubated for 3 h. After four 1-ml washes, samples were diluted in Laemmli buffer, boiled for 5 min, and subjected to SDS/PAGE.

Statistical Analysis. Results are presented as means ± standard error. Comparisons between control and treatment were made by using an unpaired Student t test.

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Fig. S1. A high-fat diet containing lard and corn oil induces an increase in skeletal muscle mitochondria. Western blot analysis of mitochondrial proteins from triceps muscle of rats fed the lard/corn oil diet for 4 wk. Values are means ± SE for six muscles. *, $P < 0.05$ for high fat vs. chow.
Fig. S2.  Effects of 2 (A) and 4 (B) wk of the lard/corn oil diet on mRNA levels of a range of mitochondrial enzymes. Values are means ± SE for four muscles per group.
Fig. S3. A high-fat diet does not induce an increase in PGC-1α mRNA in skeletal muscle. Effects of 1, 2, and 4 wk of a high-fat diet on PGC-1 mRNA level in triceps muscle. Values are means ± SE for four muscles per group.
Fig. S4. Effects of 4 wk of a high-fat diet on PGC-1α protein expression in muscle. PGC-1α was measured by Western blot analysis using four different antibodies in triceps muscle of rats fed the flax/olive oil diet for 4 wk. C, control; F, high fat. Values are means for four to eight muscles per group.
Fig. S5. PGC-1α mRNA is not increased in muscles overexpressing PPARδ. Values are means ± SE for four muscles per group.