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Respiratory chain dysfunction in skeletal muscle does not cause insulin resistance

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Abstract

Insulin resistance in skeletal muscle is a characteristic feature of diabetes mellitus type 2 (DM2). Several lines of circumstantial evidence suggest that reduced mitochondrial oxidative phosphorylation capacity in skeletal muscle is a primary defect causing insulin resistance and subsequent development of DM2. We have now experimentally tested this hypothesis by characterizing glucose homeostasis in tissue-specific knockout mice with progressive respiratory chain dysfunction selectively in skeletal muscle. Surprisingly, these knockout mice are not diabetic and have an increased peripheral glucose disposal when subjected to a glucose tolerance test. Studies of isolated skeletal muscle from knockout animals show an increased basal glucose uptake and a normal increase of glucose uptake in response to insulin. In summary, our findings indicate that mitochondrial dysfunction in skeletal muscle is not a primary etiological event in DM2.

Keywords: Mitochondria; mtDNA; Insulin resistance; Diabetes; Respiratory chain; Oxidative phosphorylation

Several lines of evidence suggest that decreased mitochondrial oxidative phosphorylation capacity in skeletal muscle may be the underlying defect that causes peripheral insulin resistance in patients with diabetes mellitus type 2 (DM2) [1]: (i) global gene-expression studies of skeletal muscle of DM2 patients have shown a decreased expression of peroxisome-proliferation-activated receptor γ coactivator 1α (PGC1α) and its downstream nuclear target genes encoding mitochondrial proteins that are constituents of the respiratory chain [2,3]. Similar gene-expression changes are also present in non-diabetic individuals with impaired glucose tolerance and in healthy first-degree relatives to DM2 patients [2,3]. (ii) Decreased *in vivo* oxidative phosphorylation capacity has been reported by using nuclear magnetic resonance spectroscopy (MRS) of skeletal muscle of DM2 patients and their offspring [4–6]. (iii) Patients with mitochondrial diseases caused by certain mutations in their mitochondrial genome have a variety of neuromuscular phenotypes and an increased prevalence of diabetes [7]. (iv) Exercise is a potent inducer of mitochondrial biogenesis in skeletal muscle and thus increases oxidative phosphorylation capacity. Exercise is the most efficient non-pharmacological intervention that decreases insulin resistance, and it may even prevent diabetes development in subjects with impaired glucose tolerance [8].

There are thus many reports presenting correlative data that associate decreased oxidative phosphorylation capacity in skeletal muscle with peripheral insulin resistance. However, it is not known if the deficient mitochondrial function in skeletal muscle is directly responsible for the development of DM2. We have investigated this fundamental question by assessing glucose homeostasis in a strain of tissue-specific knockout mice with progressive respiratory chain dysfunction selectively in skeletal muscle [9].

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Materials and methods

Breeding of transgenic mice. We crossed mice expressing the *cre*-recombinase from the myosin light chain 1f promoter (*Mlc1f-cre* mice) with $Tfam^{loxP}/Tfam^{loxP}$ mice, to generate tissue-specific knockout mice (+/*Mlcf-cre*; $Tfam^{loxP}/Tfam^{loxP}$) and controls ($Tfam^{loxP}/Tfam^{loxP}$), as described [9].

Glucose and insulin tolerance tests. Insulin (Humulin Regular, Lilly) tolerance tests were performed at 4 months of age (control mice $n = 5$, tissue-specific knockout mice $n = 5$). The mice were subjected to overnight fasting for 14–16 h, followed by intraperitoneal insulin injection (0.5 U/kg body weight). Glucose tolerance tests were performed at the ages of 1, 2, and 4 months (control mice $n = 4$–7, tissue-specific knockout mice $n = 6$–7). The mice were subjected to overnight fasting for 14–16 h followed by intraperitoneal injection with glucose (1 g/kg body weight). Blood glucose concentrations were determined with an automatic glucometer (HemoCue AB) at the indicated time points.

Glucose uptake in isolated skeletal muscle. 2-Deoxy-D-[1,2-^{3}H]glucose (2-DG, Amersham) uptake was determined at 35 °C as described elsewhere [10]. Briefly, isolated EDL and soleus muscles were incubated in a shaking water bath in 1.5 ml Tyrode buffer devoid of glucose and supplemented with 2 mM pyruvate. The buffer was continuously gassed with 95% O_2/5% CO_2, which yields a pH of 7.4. After 30 min of preincubation, insulin (Actrapid, Novo Nordisk) was added (final concentration = 20 mU/ml) and after 60 min, 2-DG (final concentration = 1 mM; 1 mCi/mmol) and insulin (0.2 μCi/ml) were added. Twenty minutes later, the muscles were frozen and processed for scintillation counting.

AMPK activity and metabolites in skeletal muscle. Isolated muscles were incubated in 1.5 ml Tyrode buffer supplemented with 5.5 mM glucose in a shaking water bath at 25 °C for 60 min. Thereafter, the muscles were frozen in liquid N_2. Frozen muscles were freeze-dried, dissected free of non-muscle constituents, and powdered. Aliquots of powder were processed for analysis of glycogen and metabolites with enzymatic techniques adapted for fluorometry (changes in NAD[P]H) as described elsewhere [11]. For analysis of AMPK activity, muscle powder was homogenized in ice-cold buffer. The homogenate was centrifuged and the supernatant was diluted and analyzed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP (Amersham Biosciences) into SAMS peptide at 37 °C for 10 min as described elsewhere [12]. Protein concentrations were measured according to the Bio-Rad protein assay.

Quantitative real-time PCR. Total RNA was extracted using Trizol from muscle tissue samples of 4-month-old control and tissue-specific knockout mice, and further purified using an RNAeasy mini kit protocol (Qiagen). RNA quality and concentration was determined on a BioAnalyser 2100 (Agilent). Aliquots of cDNA (high capacity cDNA archive kit (Applied Biosystems)) were used for quantitative real-time PCR (qRT-PCR) amplifications in duplicate on a LightCycler 2.0 (Roche) using SyBr green technology and the FastStart DNA master SyBr Green 1 mix (Roche). Standard curves were generated by four 10-fold serial dilutions of a single control sample and values within the linear exponential phase were used to calculate relative concentrations after normalization. Primers were designed to span at least one intron as follows: GLUT1 Ex1F (5′-cagccggcacagctagag-3′) and GLUT1 Ex2-3R (5′-tcccacagccaacatgag-3′) (88 nt amplicon) (45 cycles at 95 °C (5 s), 60 °C (6 s), and 72 °C (2 s)); GLUT4 Ex6F (5′-ctgctgcccttctgtcct-3′) and GLUT4 Ex7R (5′-cggtcaggggctttagact-3′) (91 nt amplicon) (45 cycles at 95 °C (5 s), 60 °C (5 s), and 72 °C (5 s)); 18sF 5′-cggctaccacatccaaggaa-3′ and 18sR 5′-tgctggcaccagacttgccctc-3′ (169 nt amplicon) (30 cycles of 95 °C (5 s), 60 °C (5 s), and 72 °C (7 s)).

Western blot analyses. Total protein extracts were prepared as described [13]. Skeletal muscle protein lysates were separated by SDS–PAGE and transferred to nitrocellulose membranes by wet transfer. Membranes were then probed with primary Glut4 (1/2000, Chemicon), AMPK, pAMPK, ACC, pACC (1/1000, Cell signalling) or α-tubulin (1/1000, Calbiochem) overnight at 4 °C followed by incubation with the appropriate secondary horseradish peroxidase-conjugated antibodies. The Western blots were visualized using ECL detection kit (Amersham) and exposed to Hyperfilm (Amersham). Band densities were analyzed with ImageJ (NIH, USA; http//rsb.info.nih.gov/ij/).

Analyses of enzyme activities for glycolytic enzymes. Glycolytic enzyme activities were determined as previously described [14]. In brief, 10 mg frozen samples of skeletal muscle tissue were homogenized in ice-cold tris(hydroxymethyl)aminomethane 50 mmol/liter, EDTA 1 mmol/liter, and $MgSO_4$ 5 mmol/liter (pH 8.2) buffer. The homogenate was stored at −80 °C for subsequent analyses of the following enzyme activities: hexokinase, glucose-6-phosphate isomerase, and phosphoglycerate kinase. All enzyme activities were determined spectrophotometrically at 35 °C.

Statistical analysis. All data are expressed as means and SEM. Statistical analyses were performed using unpaired 2-tailed Student's *t* test.

Results

Normal insulin tolerance in mice with respiratory chain deficiency in skeletal muscle

We previously generated skeletal muscle-specific mitochondrial transcription factor A (Tfam) knockout mice, by exploiting the *cre*-loxP-recombination system [9]. These knockout mice have a progressive deterioration of respiratory chain function in fast-twitch skeletal muscle over a period of several months. The relative residual complex I (NADH dehydrogenase) activity is 59 ± 6% (means ± SEM; age 1 month), 45 ± 3% (2 months), and 7 ± 3% (4 months). The relative residual complex I–III (NADH cytochrome *c* reductase) activity is 51 ± 7% (age 1 month), 34 ± 4% (2 months), and 8 ± 3% (4 months). The relative residual complex IV (cytochrome *c* oxidase) activity is 84 ± 10% (age 1 month), 75 ± 6% (2 months), and 27 ± 8% (4 months) [9].

We injected control and tissue-specific knockout mice at 4 months of age with insulin and followed the decrease of blood glucose levels. There was no difference in the insulin tolerance between control and tissue-specific knockout mice (Fig. 1A). Thus, the 4-month-old knockout mice are not insulin resistant despite the severe respiratory chain deficiency in their skeletal muscle.

Increased glucose uptake in respiratory-chain-deficient skeletal muscle

We injected tissue-specific knockout and control mice at different ages with glucose and investigated the subsequent increase in blood glucose (Fig. 1B–D). Skeletal muscle knockouts at age 1 month had normal glucose tolerance (Fig. 1B), whereas the peripheral uptake of glucose was increased in knockouts at age 2 (Fig. 1C) and 4 months (Fig. 1D). The finding that impaired respiratory chain function in skeletal muscle leads to increased glucose uptake was unexpected and we therefore characterized glucose uptake further by assessing 2-DG uptake in isolated muscles *in vitro* from control and tissue-specific knockout mice at 4 months of age. The *Tfam* knockout is fiber type-specific, because the *Mlc1f* promoter expresses *cre*-recombinase exclusively in type II fibers [15]. Type II fibers

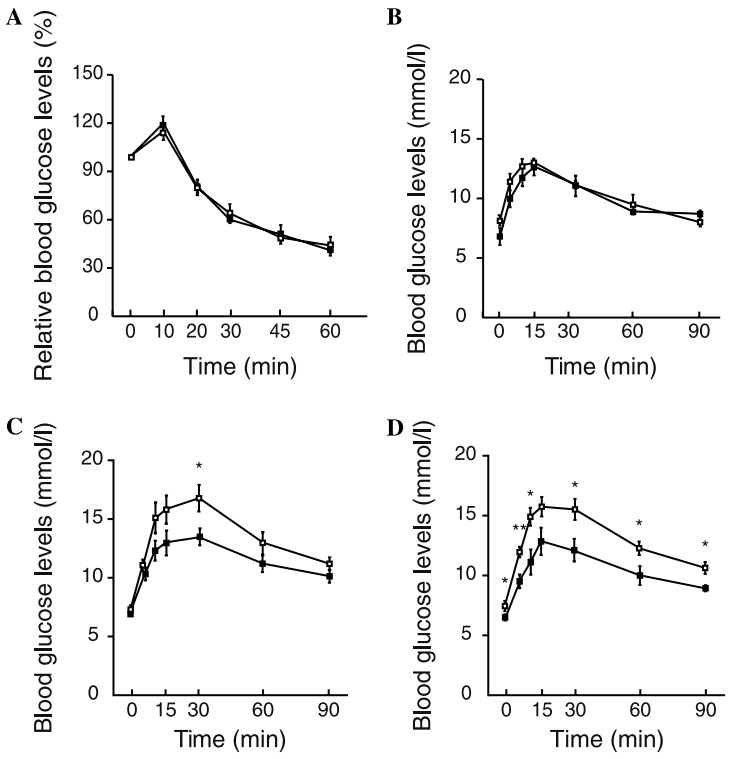

Fig. 1. Insulin and glucose tolerance tests. (A) Relative blood glucose levels after intraperitoneal injection of insulin in 4-month-old control ($n = 5$) and knockout mice ($n = 5$). (B–D) Intraperitoneal glucose tolerance tests in 1-month (B), 2-month (C), and 4-month-old (D) control ($n = 4$–7) and knockout mice ($n = 6$–7). Injections were performed at time 0 min. Control animals are represented as open and knockout animals with filled squares. Data are presented as means ± SEM; * and **, $P < 0.05$ and 0.01, respectively.

are the predominant fiber type in mouse skeletal muscles as exemplified by the fast-twitch extensor digitorum longus (EDL) muscle. In contrast some skeletal muscles in the mouse contain predominantly type I fibers as exemplified by the slow-twitch soleus muscle (data not shown). We found increased basal 2-DG uptake and normal increase of the insulin-stimulated glucose uptake in the EDL muscle (Fig. 2A), whereas the basal and insulin-stimulated 2-DG uptake was unaltered in the soleus muscle (Fig. 2B).

Minimal changes in expression of glucose transporters

Glucose uptake in skeletal muscle is mediated via glucose transporter 1 (GLUT1) and 4 (GLUT4), and we used quantitative real-time PCR analysis to measure the mRNA expression of these two transporters. The mRNA expression of GLUT1 was significantly increased and there was a tendency to increased expression of GLUT4 (Fig. 2C). However, the GLUT4 protein levels were unaltered in EDL and soleus of muscle-specific knockout mice (Fig. 2D).

AMPK activation may increase glucose uptake in knockout mice

There was no significant increase in basal AMPK activity in EDL and soleus muscles of tissue-specific knockout mice at 4 months of age (Fig. 3A). In accordance with these results, we found similar levels of total AMPK protein and phosphorylated AMPK (pAMPK) protein in EDL (Fig. 3B) and soleus muscle (not shown) of tissue-specific knockout and control mice at 4 months of age. The total levels of acetyl CoA carboxylase (ACC) protein were similar in knockout and control EDL muscle (Fig. 3C), but the levels of phosphorylated ACC (pACC) were increased in knockout EDL muscle. This finding is consistent with an activation of AMPK in EDL muscle of tissue-specific knockout mice, as AMPK has the capacity to inactivate ACC by phosphorylation at serine 79.

Several different types of metabolic stress, e.g. muscle contraction or hypoxia, lead to decreased levels of phosphocreatine and glycogen in muscle, which, in turn, activates AMPK and stimulates muscle glucose uptake [16–18]. We observed decreased levels of glycogen and phosphocreatine and increased inorganic phosphate in knockout EDL muscle (Table 1), consistent with metabolic stress.

We measured the activity of several glycolytic enzymes (hexokinase, phosphoglucose isomerase, and phosphoglycerate kinase), and found similar activities in skeletal muscle of control and knockout mice at age 4 months (Fig. 3D). However, these unaltered enzyme activities do not exclude activation of glycolysis. The increased basal glucose uptake (Fig. 2A) and the decreased glycogen content (Table 1) are suggestive of increased glycolysis in the respiratory-chain-deficient skeletal muscle.

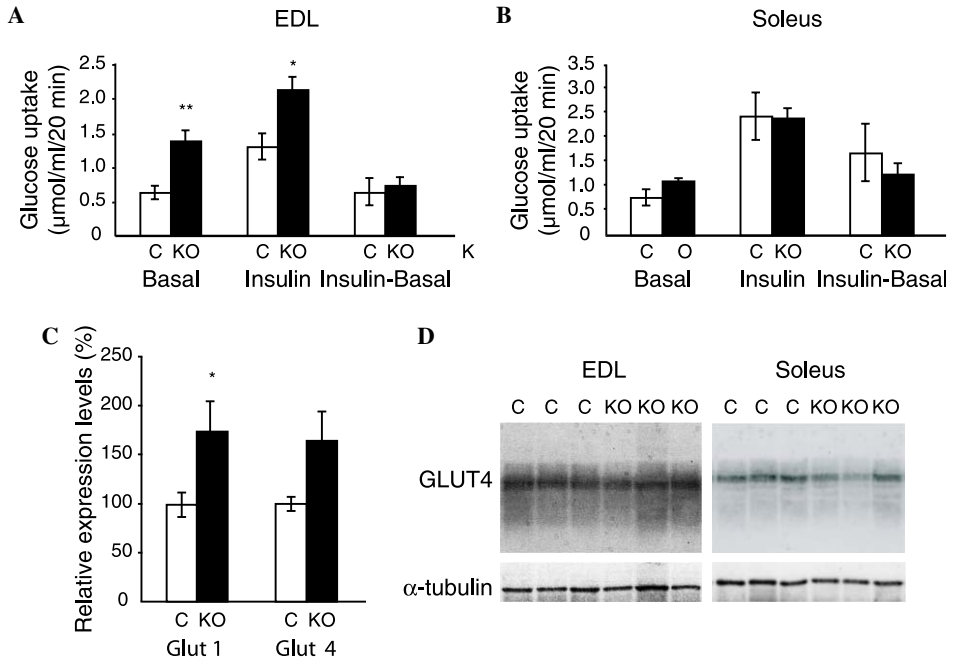

Fig. 2. *In vitro* glucose uptake and expression of glucose transporters in skeletal muscle. (A,B) Basal glucose uptake (basal), insulin-stimulated glucose uptake (insulin), and the difference between insulin-stimulated and basal glucose uptake (insulin-basal) in isolated EDL and soleus muscles from control ($n = 4$) and knockout ($n = 6$) mice at age 4 months. (C) Transcript levels of glucose transporter 1 (GLUT1) and (GLUT4) as determined by quantitative PCR of total RNA from skeletal muscle of control (C; $n = 8$) and knockout (KO; $n = 9$) mice at age 4 months. (D) Western blot analysis of GLUT4 protein levels in EDL (left panel) and soleus (right panel) muscles of 4-month-old animals. Data are presented as means ± SEM; * and **, $P < 0.05$ and 0.01, respectively.

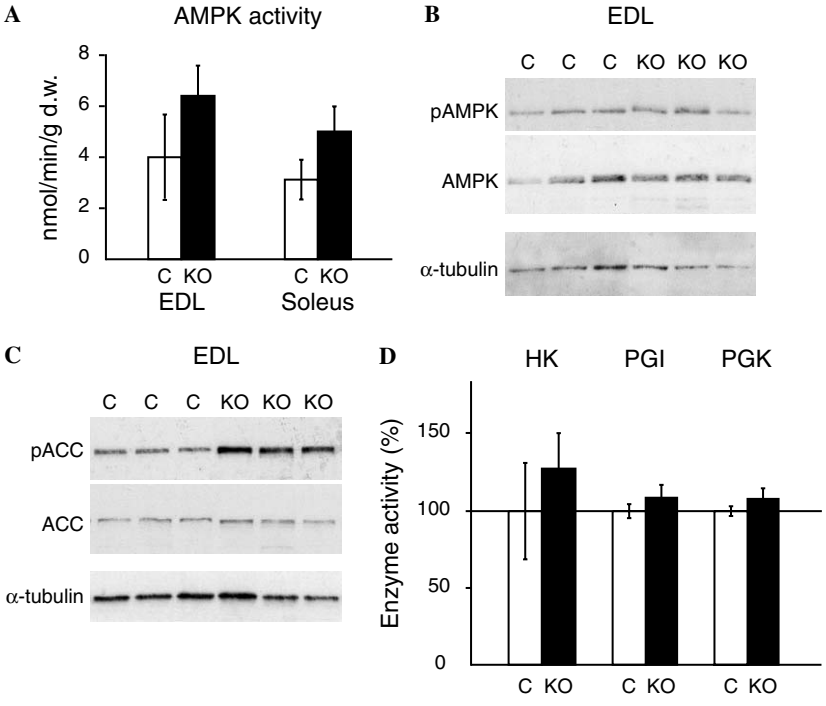

Fig. 3. Quantification of protein levels and enzyme activities. (A) AMPK activity in EDL and soleus muscles of control (C; $n = 5$) and knockout mice (KO; $n = 6$) at age 4 months. (B) Western blot analysis of phosphorylated (threonine 172), AMPK (pAMPK), and total AMPK protein levels in EDL muscle from control (C) and knockout mice (KO) at age 4 months. (C) Western blot analysis of phosphorylated (serine 79), ACC (pACC), and total ACC protein levels in EDL muscle from control (C) and knockout mice (KO) at age 4 months. (D) Activities of enzymes in the glycolytic pathway in control (C; $n = 4$) and knockout mice (KO; $n = 4$) at age 4 months. HK, hexokinase; PGI, phosphoglucose isomerase; PGK, phosphoglycerate kinase. Mean value in control set to 100%.

Table 1
Measurements of metabolites in EDL muscles

	Control	Knockout
Glycogen	66.5 ± 5.4	43.4 ± 4.2**
Glucose-6-P	0.35 ± 0.08	0.59 ± 0.07*
Lactate	1.3 ± 0.4	2.5 ± 0.8
PCr	67.6 ± 1.6	50.6 ± 1.7**
Cr	13.6 ± 1.6	32.8 ± 2.5**
Pi	10.1 ± 0.8	21.1 ± 2.0**
ATP	25.6 ± 2.2	24.2 ± 1.6

Measurements of metabolites (μmol/g dry weight) in EDL performed at rest. Values represent means ± SEM for control ($n = 4$–5) and knockout ($n = 5$–7). * and ** denote significant difference between controls and knockouts at $P < 0.05$ and <0.01, respectively.

Discussion

A wealth of correlative data have previously suggested that mitochondrial dysfunction in skeletal muscle may be causally related to the peripheral insulin resistance seen in patients with DM2 [5,6,19,20]. We have tested this hypothesis experimentally by studying glucose homeostasis in tissue-specific knockout mice with progressive respiratory chain dysfunction in skeletal muscle. Surprisingly, these knockout mice are neither diabetic nor insulin resistant, but instead display an increased peripheral glucose uptake *in vivo*. Studies of isolated respiratory-chain-deficient muscles *in vitro* show an increased basal insulin-independent glucose uptake and a normal response to insulin. These findings suggest that mitochondrial dysfunction in skeletal muscle is a secondary phenomenon not primarily involved in generating the insulin resistance of skeletal muscle seen in DM2.

Patients with mitochondrial diabetes are insulin deficient and have an ongoing β-cell death causing a pronounced age-dependent progressive deterioration of β-cell function [21–23]. Studies of tissue-specific knockout mice with disruption of *Tfam* in β-cells [24] and insulin-secreting cell lines lacking mtDNA [25,26] have demonstrated that mitochondrial function is essential for stimulus-secretion coupling. During this process glucose enters the β-cell and is metabolized to elicit signalling events that regulate insulin secretion. Prolonged respiratory chain deficiency in β-cells *in vivo* leads to reduction of β-cell mass and insulin deficiency [24]. Insulin resistance is not commonly found in mitochondrial diabetes despite the fact that patients with mitochondrial diseases frequently are respiratory chain deficient in skeletal muscle [22,27].

One striking result of the present study was the increased basal glucose uptake in respiratory-chain-deficient EDL muscles. An increased basal glucose uptake can be due to either an increased protein expression of GLUT1 or to a stress-related (e.g. exercise or hypoxia) translocation of GLUT4 to the plasma membrane. We found an increase in GLUT1 mRNA expression. Additionally, we investigated the stress-related pathway owing to the fact that the energy metabolic state of rested knockout muscles indicated that they were metabolically stressed. AMPK is considered to be a key metabolic sensor that accelerates glucose utilization [28,29]. AMPK is activated by AMPK kinase (AMPKK), which phosphorylates the α-subunit of AMPK at threonine 172. Activated AMPK, in turn, has been suggested to stimulate glucose uptake by increasing the translocation of GLUT4 to the plasma membrane [30]. We found no differences in total AMPK protein levels or in phosphorylation state at threonine 172, or in AMPK enzyme activity in respiratory-chain-deficient skeletal muscle of the knockout mice. However, AMPK can additionally be regulated by allosteric interactions, e.g. by increased AMP concentrations. In addition, AMP activates AMPK by preventing its dephosphorylation [29]. Glycogen may also be an allosteric regulator of AMPK [31,32]. The finding of normal AMPK activity in respiratory-chain-deficient skeletal muscle of the knockout mice could therefore be due to the fact that the used assays do not account for *in vivo* allosteric regulation. We found indirect evidence for activation of AMPK as one of its major targets, ACC, displayed increased phosphorylation at serine 79. Phosphorylation at this site inactivates ACC [33,34] and thereby increases β oxidation [35]. Furthermore, the metabolic profile of the knockout EDL muscle reflects conditions favoring AMPK activation.

In summary, our findings indicate that mitochondrial dysfunction in skeletal muscle is not necessarily a primary etiological event in insulin resistance or DM2, but rather represents a secondary event.

Acknowledgments

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