

Mitochondrial dysfunction results from oxidative stress in skeletal muscle of diet-induced insulin resistant mice.

Charlotte Bonnard¹, Annie Durand¹, Simone Peyrol², Emilie Chanseume³, Marie-Agnes Chauvin¹, Béatrice Morio³, Hubert Vidal¹ and Jennifer Rieusset¹.

¹ INSERM, U870, IFR62, Lyon, F-69008 ; INRA, UMR1235, Lyon, F-69008 ; INSA-Lyon, RMND, Villeurbanne, F-69621 ; Université Lyon 1, Lyon, F-69003 ; Hospices Civils de Lyon, Lyon, F-69008, France.

² IFR62, Centre Commun d'Imagerie de Laennec, Université Lyon 1, Lyon, F-69008, France.

³ INRA, UMR 1019, Clermont-Ferrand, F-63000, France

Corresponding author : Rieusset Jennifer, INSERM U870, Faculté de médecine Lyon Sud, 165 chemin du grand Revoyet, 69600 Oullins. Tel: 33 4 26 23 59 20, Fax: 33 4 26 23 59 16, e-mail: jennifer.rieusset@univ-lyon1.fr

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Nonstandard abbreviations used: COX: cytochrome c oxidase, CS: citrate synthase, ERR α : estrogen related receptor α , HFHSD: high fat and high sucrose diet, HPRT : hypoxanthine guanine phosphoribosyl transferase, IRS1: insulin receptor substrate 1, Mfn2: mitofusin 2, mtDNA: mitochondrial DNA, mtTFA: mitochondrial transcription factor, NAC : N-acetylcysteine, NBT: nitroblue tetrazolium, NRF: nuclear respiratory factor, PGC1: PPAR gamma coactivator 1, POLG: polymerase gamma, ROS: reactive oxygen species, SD: standard diet, SSBP1: single strand binding protein, STZ: streptozotocin.

Abstract

Mitochondrial dysfunctions in skeletal muscle have been implicated in the development of type 2 diabetes. However, whether these changes are a cause or a consequence of insulin resistance is not clear. We have investigated the structure and functions of muscle mitochondria during the development of high fat and high sucrose diet-induced insulin resistance in mice. We found that mitochondrial dysfunctions were not present in glucose intolerant mice, whereas altered mitochondrial biogenesis, structure and functions were observed in muscle of diabetic mice. Furthermore, we demonstrated that muscle oxidative stress is one of the major determinants of these mitochondrial alterations since 1) there was an increase of ROS production specifically in skeletal muscle of hyperglycaemic and hyperlipidaemic diet-induced diabetic mice, 2) ROS production was also associated with mitochondrial alterations in muscle of hyperglycaemic streptozotocin-treated mice, 3) in this model, normalization of glycaemia or treatment with an antioxidant decreased muscle ROS production and restored mitochondrial integrity, and 4) glucose- or lipid-induced ROS production in muscle cells altered mitochondrial density and functions, and these effects were blocked by antioxidant treatment. Consequently, mitochondrial alterations do not precede the onset of insulin resistance and result from increased ROS production in muscle in diet-induced diabetic mice.

Introduction

The prevalence of type 2 diabetes increases dramatically in modern societies, in part due to ample food supplies coupled with sedentary lifestyle. Excess dietary fat and sugar plays a crucial role and is one of the determinants of the current epidemic. In peripheral tissues, increased flux of energy fuel substrates associated with such diets leads to ectopic lipid accumulation, generation of reactive oxygen species (ROS) and cellular dysfunctions, referred as gluco-lipo-toxicity (1).

Over the last few years, an increasing number of studies have linked lipid accumulation in skeletal muscle to reduced insulin sensitivity in various groups of subjects including type 2 diabetic patients (2-4). In addition, intracellular lipid metabolites, such as fatty acyl-CoA, diacylglycerol, or ceramide, have been shown to inhibit insulin action (5) via activation of serine/threonine kinases and serine phosphorylation of insulin receptor substrate-1 (IRS1) (6). Both increased fatty acid uptake and decreased fatty acid oxidation may induce lipid accumulation in skeletal muscle. Studies in humans (7) and rodents (8) have demonstrated that increased fatty acid uptake into muscle contributes to lipid accumulation in situations of insulin resistance. In addition, there is growing evidence that mitochondrial dysfunctions in skeletal muscle, and subsequent impaired ability to oxidize fatty acids, also play an important role in the development of insulin resistance (9). Indeed, in skeletal muscle, the oxidative capacity, which is mostly dependent on mitochondrial function, is directly correlated with insulin sensitivity (10), and reduced mitochondrial oxidative phosphorylation is associated with insulin resistance (11). A reduction in the number and changes in the morphology of mitochondria is observed in skeletal muscle of type 2 diabetic patients (12). In addition, a set of genes involved in oxidative phosphorylation exhibits reduced expression levels in the muscle of type 2 diabetic patients and of prediabetic subjects (13, 14). These changes may be mediated by decreased expression of PPAR gamma coactivator 1 (PGC1) α and nuclear respiratory factor (NRF) 1 genes, both controlling mitochondrial biogenesis. Interestingly, high fat diets down-regulated PGC1 α and PGC1 β as well as genes coding for proteins of the electron transport chain in human skeletal muscle (15), suggesting that excess dietary fat could alter mitochondrial functions. However, the effects of excess dietary lipids on mitochondrial biogenesis and functions have not been investigated in detail and the underlying mechanisms responsible for the reduced mitochondrial activity in the pathogenesis of insulin resistance are still unknown.

The purpose of this study was to determine the effects of a high fat and high sucrose diet (HFHSD) on mitochondrial density and functions and on insulin action in mice skeletal muscle, in order 1) to determine whether high fuel substrate availability contributes to mitochondrial

dysfunctions, 2) to monitor the relationship between mitochondrial alterations and insulin resistance and 3) to identify the molecular mechanisms linking excess dietary fuels and altered mitochondrial functions. Our data reveal that HFHSD-induced mitochondrial alterations in skeletal muscle are a consequence of hyperglycaemia and hyperlipidaemia-induced ROS production in mice, resulting from mitochondrial over-functioning and increased NAD(P)H oxidase enzyme in response to energy substrate overflow.

Results

Metabolic characteristics of mice under HFHSD feeding.

The characteristics of mice are summarized in Table 1. Four weeks feeding of C57Bl/6 mice with the HFHSD resulted in significant increase in body weight (20%, $p < 0.001$) and epididymal adipose tissue weight (336%, $p < 0.001$), as compared to the standard diet (SD)-fed mice. Plasma glucose, FFA and triglyceride levels were similar in both groups of mice, whereas plasma insulin (72%, $p < 0.05$) and leptin (79%, $p < 0.05$) levels were increased in HFHSD mice compared to SD mice. After 16 weeks of diet, body weight and epididymal fat weight gains in HFHSD animals were more marked. In addition, 16 week HFHSD mice were clearly hyperglycaemic ($p < 0.001$) and hyperinsulinaemic ($p < 0.001$), compared to 16 week SD mice. Plasma glucose levels of 16 week HFHSD mice were significantly higher ($p < 0.001$) than 4 week HFHSD mice. Plasma leptin ($p < 0.001$), FFA ($p < 0.05$) and triglyceride levels ($p < 0.001$) were all increased after 16 weeks of HFHSD feeding compared to SD mice.

Glucose and insulin tolerance tests showed that 4 week HFHSD mice were glucose intolerant, while their response to insulin injection remained unaltered compared to SD mice (Figures S1A and S1B). In contrast, after 16 weeks of HFHSD feeding, mice presented an altered response to both glucose and insulin injection compared to SD mice, indicating that 16 week HFHSD mice are insulin resistant (Figures S1A and S1B). Decreased insulin responsiveness in 16 week HFHSD mice was associated with intramyocellular lipid accumulation (Figure S2A), increased basal IRS-1 serine phosphorylation at Ser632 (45%, $p < 0.05$, Figure S2B) and with a decrease of ex vivo insulin-stimulated Akt phosphorylation at Ser473 (80%, $p < 0.01$, Figure S2C) in gastrocnemius muscle. In contrast, insulin-stimulated Ser473 phosphorylation of Akt was not modified in 4 week HFHSD mice compared to SD mice (Figure S2C).

Mitochondrial biogenesis is reduced in skeletal muscle of HFHSD mice.

Next, we investigated the impact of HFHSD on muscle mitochondrial density. As shown in Figure 1A, the mitochondrial DNA (mtDNA)/nuclear DNA ratio was significantly decreased in skeletal muscle of 16 week HFHSD mice compared to 16 week SD mice (3%, $p < 0.05$), whereas no change was observed after 4 weeks of diet. In agreement, the mRNA levels of the subunits 1 and 3 of cytochrome c oxidase (COX), two mitochondria-encoded genes, were significantly decreased in skeletal muscle of 16 week HFHSD mice (Figure 1B). Using transmission electron microscopy, we found that 16 week HFHSD resulted in a decrease of both subsarcolemmal (31%, $p < 0.05$) and

intermyofibrillar (41%, $p < 0.01$) mitochondria amounts in oxidative fibers when compared to SD mice (Figure 1C and 1D). These alterations were not observed after 4 weeks of HFHSD diet (Figure 1C). As shown in Figure 1E, citrate synthase (CS) activity was slightly decreased in isolated mitochondria from HFHSD mice, both after 4 weeks and 16 weeks of HFHSD feeding (13% and 16%, respectively, $p < 0.05$).

To clarify the mechanisms involved in the reduction of mitochondrial density in the muscle of 16 week HFHSD mice, we measured the mRNA levels of genes implicated in mitochondrial biogenesis, such as PGC1 α , PGC1 β , NRF1, NRF2, the mitochondrial transcription factor (mtTFA), the estrogen-related receptor α (ERR α) and mitofusin 2 (Mfn2). Only the mRNA levels of PGC1 α and Mfn2 were decreased in skeletal muscle of 16 week HFHSD mice compared to SD mice (~ 50% for both transcripts) (Figure 2A). This difference was not seen after 4 weeks of diet (Figure 2A). The protein levels of PGC1 α were also significantly decreased in skeletal muscle of 16 week HFHSD mice (data not shown). Concerning mtDNA replication and repair, we have investigated both gamma DNA polymerase (POLG1, the catalytic subunit and POLG2, the accessory subunit) and the single stranded DNA binding protein 1 (SSBP1), which play a key role in this process (16). As illustrated in Figure 2B, 16 weeks of HFHSD feeding induced a decrease of POLG2 and SSBP1 mRNA levels in skeletal muscle, whereas no effect was observed after 4 weeks of diet. POLG1 expression was not affected by HFHSD feeding.

Alteration of mitochondrial ultrastructure in skeletal muscle of HFHSD-fed mice.

In addition to reduced mitochondrial content, the transmission electron microscopy study demonstrated marked alterations of mitochondrial morphology in the gastrocnemius muscle of 16 week HFHSD mice. Area of both subsarcolemmal and intramyofibrillar mitochondria were decreased (45% and 35%, respectively, $p < 0.05$) in skeletal muscle of 16 week HFHSD mice compared to SD mice (Figure 3A and 3C). Higher magnification ($\times 100,000$) showed swelling of both types of mitochondria associated with increased number of disarrayed cristae and reduced electron density of the matrix (Figure 3B). No alteration in mitochondrial morphology was observed after 4 weeks of HFHSD (data not shown).

Altered mitochondrial functions in skeletal muscle of HFHSD mice.

To investigate whether alterations in mitochondrial density and ultrastructure was associated with mitochondrial dysfunction in skeletal muscle of HFHSD mice, we measured substrate-driven oxygen consumption in saponin-skinned skeletal muscle fibers. When mice were fed with a HFHSD

for 4 weeks, the respiration rates were not different from SD mice, whatever were the tested substrates (Table 2). In muscle fibers from 16 week HFHSD mice, respiration with complex 1-linked substrates (Glutamate/Malate), but not with complex 2-linked substrates (Succinate/Rotenone), was significantly reduced, both during state 3 and state 4, when compared to SD mice (Table 2). In addition, we observed a significant decrease of oxidation capacities when using octanoyl or palmitoyl-carnitine as substrates in fibers of 16 week HFHSD mice. Taken together, these data demonstrate that complex 1-linked respiration and β -oxidation were decreased specifically in diet-induced diabetic mice. Reduced oxidation of fatty acids was probably not related to altered availability of the substrates since genes involved in muscle fatty acid uptake (FAT/CD36) and entry in the mitochondria (CPT1) were significantly up-regulated in skeletal muscle of 16 week HFHSD mice (Figure S3A). Further supporting a reduction of the mitochondrial functions, a decreased activity of succinate dehydrogenase was evidenced by succinate dehydrogenase staining in gastrocnemius histological sections in 16 week-, but not in 4 week HFHSD mice (Figure S3B).

Increased oxidative stress in skeletal muscle of HFHSD mice.

Since mitochondrial alterations were observed in HFHSD mice only when they were hyperglycaemic and hyperlipidaemic, and since both glucose and lipids are known to induce oxidative stress, we tested whether ROS levels were increased during HFHSD feeding. Plasma H_2O_2 concentrations (Table 1) and muscular protein carbonylation levels (Figure 4A), a marker of protein oxidation, were elevated in 16 week HFHSD mice compared to 16 week SD mice. No difference was observed after 4 weeks of diet (Figure 4A).

In addition, mRNA levels of uncoupling proteins 2 and 3 (markers reflecting increased mitochondrial ROS production in condition of lipid oversupply (17)) and of four subunits of NAD(P)H oxidase (gp91, p67, p40, p47) were induced in skeletal muscle of 16 weeks HFHSD mice, suggesting an increase of both mitochondrial and cytoplasmic ROS production (Figure 4B). Only the p67 subunit of NAD(P)H oxidase was significantly induced after 4 weeks of HFHSD feeding. Concerning the antioxidant system, the mRNA levels of glutathione reductase and catalase were increased in skeletal muscle of 16 week HFHSD mice, whereas expression of other antioxidant enzymes, including glutathione peroxidase, superoxide dismutase 2, peroxiredoxin 3 and peroxiredoxin 5 were not modified (Figure 4B). None of these genes showed a modification of expression after 4 weeks of HFHSD.

Exposure to ROS leads to apoptosis and cell damages in a variety of experimental systems. As shown in Figure 4C, we observed an increase in cytochrome C levels in the cytosol with a concomitant decrease in the mitochondria fraction in skeletal muscle of 4 week HFHSD mice,

indicating a cytochrome C release from mitochondria. Due to the strong alterations in the number and functions of mitochondria in the muscle of 16 weeks HFHSD-fed mice, this phenomenon was difficult to evidence after 16 weeks of feeding (Figure 4C). Nevertheless, the activity of caspase 3, another index of apoptosis, was markedly increased in skeletal muscle of 16 week HFHSD mice (90%, $p < 0.05$), whereas no difference was observed after 4 weeks of diet (Figure 4D).

We have also investigated ROS production and mitochondrial dysfunction in KKA^y mice, a genetic model of obesity and diabetes. KKA^y mice were obese, hyperglycaemic, hyperinsulinaemic, and hypertriglyceridaemic, compared to age-matched control mice (Table S1), but they showed normal plasma FFA levels (Table S1). Plasma H_2O_2 levels were increased in KKA^y mice compared to C57Bl/6 mice (Table S1), but not skeletal muscle protein carbonylation (Figure S4A). Interestingly, in contrast to the HFHSD model, mitochondrial density and structure were not altered in KKA^y mice compared to age-matched control mice (Figures S4B and S4C). Taken together, these data suggest that oxidative stress in skeletal muscle is a determinant parameter for mitochondrial alterations in diabetic mice.

Alterations of mitochondria biogenesis and structure in skeletal muscle of streptozotocin-treated mice.

To test whether ROS production is a key feature in HFHSD-induced mitochondrial dysfunctions, we investigated mitochondrial structure and functions in streptozotocin (STZ)-treated mice, a model of hyperglycaemia-associated oxidative stress without insulin-resistance and obesity. Eleven days after STZ administration, mice were hyperglycaemic ($p < 0.001$), and hypoinsulinaemic ($p < 0.001$) and showed unaltered plasma FFA levels and a reduced body weight ($p < 0.005$) (Table 1). Insulin injection (STZ+INS) rapidly decreased plasma glucose levels and 24 hours after insulin injections, plasma glucose were lower ($p < 0.001$), body weight were higher ($p < 0.05$) and FFA were undetected in STZ+INS mice compared to STZ mice (Table 1). Phlorizin injection (STZ+PHL) reproduced the effect of insulin on glycaemia (decrease of 25% compared to STZ mice, $p < 0.05$).

In agreement with hyperglycaemia-induced oxidative stress, protein carbonylation levels were elevated in skeletal muscle of STZ mice and insulin treatment restored the extent of protein carbonylation close to the levels observed in control mice (Figure 5A). Furthermore, STZ treatment induced a release of cytochrome C from mitochondria and insulin treatment reversed this proapoptotic process (Figure 5B). Regarding mitochondrial density, the mtDNA/nuclear DNA ratio (Figure 5C) and mitochondria amount per area (Figure 5D) were reduced in the muscle of STZ mice compared to control mice. The morphology of both types of mitochondria was also affected in skeletal muscle of STZ mice, with altered cristae and reduced electron density of the matrix (Figure

5D). Importantly, density and structural abnormalities of mitochondria in the muscle of STZ mice were restored by insulin and phlorizin treatments (Figures 5C and 5D).

To verify whether mitochondrial alterations were related to ROS production, we treated STZ mice with N-acetylcysteine (NAC), a general antioxidant. NAC treatment, did not modify systemic oxidative stress (Figure 6A), but decreased muscle protein carbonylation to the levels of control mice (Figure 6B), and restored mitochondria density (Figure 6C) and structure (Figure 6D) in gastrocnemius muscle of STZ mice.

Taken together, these results demonstrate that oxidative stress in hyperglycaemic mice is associated with altered mitochondrial structure and function in skeletal muscle and that both amelioration of glycaemia and antioxidant treatment restore mitochondrial structure.

Reactive oxygen species induce mitochondrial alterations and dysfunction in cultured myotubes.

We examined the effects of high glucose and lipid levels on ROS production and mitochondria density and functions in C₂C₁₂ muscle cells. ROS production was markedly increased by glucose (25mM) and by palmitate (200 μ M) treatments for 96 hours, and NAC (10mM) addition blocked these effects (Figure 7A). H₂O₂ (100 μ M) addition for 96 hours decreased mtDNA levels (Figure 7B) and reduced CS activity (Figure 7C) in C₂C₁₂ cells. Incubation with glucose or with palmitate also decreased CS activity (Figure 7C), but the effects on mtDNA were not significant (Figure 7B). Furthermore, POLG2, SSBP1 and PGC1 α mRNA levels were decreased in myotubes treated with H₂O₂, glucose or palmitate for 96 hours. NAC addition counteracted all these effects, indicating that ROS contributed to the observed mitochondrial alterations in cultured muscle cells (Figures 7B-7D). Finally, we also performed experiments in primary cultures of human myotubes and found similar results (Figure S5), suggesting that these effects could also take place in human muscle cells. Transmission electronic microscopy studies nicely illustrated that both H₂O₂ and glucose addition for 96 hours altered mitochondria structure in myotubes, compared to their respective control cells (Figure S5D).

Discussion

Cumulative evidences strongly suggest that alterations of mitochondrial density and function in skeletal muscle are associated with both insulin resistance and type 2 diabetes (9). However, whether these changes are a cause, a consequence or a parallel process of insulin resistance is not clear. Here, we have investigated the amount, structure and functions of skeletal muscle mitochondria during the development of HFHSD-induced insulin resistance in mice. Our data indicate that mitochondrial defects do not appear before insulin resistance since no alteration was observed in a pre-diabetic state (after 4 weeks of diet), whereas mitochondrial dysfunctions were present in skeletal muscle of diabetic mice (after 16 weeks of diet). Furthermore, we found that oxidative stress in skeletal muscle is probably one of the major determinants of the mitochondrial alterations. This is supported by data showing that 1) increase in muscle ROS production occurred specifically after 16 weeks of HFHSD when mice were hyperglycaemic and hyperlipidemic; 2) ROS production was also associated with mitochondrial alterations in muscle of hyperglycaemic STZ mice; 3) in this model, normalization of glycaemia by insulin or phlorizin and treatment with an antioxidant (NAC) decreased muscle ROS production and restored mitochondrial integrity; 4) incubation of cultured muscle cells with high glucose or lipid concentrations induced ROS production and altered mitochondrial density and functions; 5) these effects were blocked by antioxidant treatment.

We have investigated mitochondrial structure and functions in skeletal muscle of mice at two different stages of HFHSD-induced metabolic disturbances. After 4 weeks, mice were overweight, normoglycaemic and normolipidaemic, with no systemic or muscle oxidative stress. However, they showed hyperinsulinaemia and altered glucose clearance during a glucose tolerance test, but normal *in vivo* and *ex-vivo* insulin-responsiveness. Consequently, 4 week HFHSD-fed mice could be considered in a prediabetic state, with glucose intolerance but no diabetes. At this time, we did not observe modification of mitochondrial density and structure in skeletal muscle, as assessed by electronic microscopy. Mitochondrial DNA copy number, as well as expression of mitochondria-encoded genes and key regulators of mitochondrial biogenesis, was not altered in skeletal muscle of these prediabetic mice. Furthermore, mitochondrial functions in muscle of 4 week HFHSD mice were normal, as assessed by substrate-driven respiration and lipid oxidative capacities measurements. Taken together, these data clearly indicate that mitochondrial alterations do not precede the onset of insulin resistance and diabetes. The only early perturbations observed in the skeletal muscle of 4 week HFHSD mice were 1) a slight increase of lipid stores (data not shown), indicating a preferential orientation of muscle metabolism towards lipid esterification in pre-diabetic

state, as reported in humans (18), 2) a release of cytochrome C from mitochondria, suggesting that early pro-apoptotic events in skeletal muscle could precede mitochondrial alterations during HFHSD, 3) a decrease of CS activity that could be attributed to HFHSD-induced down-regulation of CS gene expression, as supported by reduced CS mRNA levels in muscle of 4 week HFHSD mice compared to SD mice (CS/HPRT mRNA: 0.29 ± 0.03 vs. 0.45 ± 0.03 , respectively, $p < 0.01$). Although these results suggest that 4 week HFHSD was associated with some metabolic perturbations, which may reflect initiation of deleterious processes, there was no major functional and structural alteration of the mitochondria in the skeletal muscle of the prediabetic mice. Recent studies in humans, however, indicated impaired mitochondrial activity and density in skeletal muscle of offspring of type 2 diabetic patients (11, 19), leading to the concept that early mitochondrial alterations could predispose to intramyocellular lipid accumulation and insulin resistance. Nevertheless, these studies have been conducted in subjects who were already insulin-resistant (11, 19) and thus then they did not allow determining whether mitochondrial alterations precede the development of insulin resistance.

HFHSD feeding for 16 weeks was associated with hyperglycaemia, hyperinsulinaemia, increased plasma and muscle lipid levels, and altered in vivo and ex vivo insulin responsiveness, indicating that 16 week HFHSD mice are diabetic. These metabolic alterations were associated with systemic and muscle oxidative stress, probably due to an increase of both mitochondrial and cytoplasmic ROS production, rather than to reduced antioxidant defences. Furthermore, these disturbances were associated with striking mitochondrial changes in gastrocnemius muscle. There was a significant decrease in mitochondria number associated with a reduction in mtDNA content and reduced expression levels of mitochondria-encoded genes (COX1 and COX3), suggesting that the control of mitochondrial biogenesis and/or mtDNA replication is altered in diabetic mice. PGC1 α is one of the master regulators of mitochondrial biogenesis and oxidative phosphorylation gene expression (20). Two DNA microarray studies have found a coordinated reduction in the expression of genes regulated by PGC1 α in the skeletal muscle of type 2 diabetic patients (13, 14) and expression of PGC1 α itself is decreased in the muscle of the patients (14, 21). In agreement, we found that PGC1 α was down-regulated in skeletal muscle of 16 week HFHSD mice, but we did not observe significant change in downstream targets of PGC1 α , such as NRF1, NRF2, mtTFA and ERR α . We observed, however, a decreased expression of Mfn2, a protein participating to the mitochondrial network. This observation is in agreement with previous reports in other models of obesity and diabetes and also in humans (22). In addition, we reported, for the first time, a decrease in POLG2 and SSBP1 expression in skeletal muscle of diabetic mice, which was reproduced in vitro

by H₂O₂, glucose and lipids treatments and restored by NAC treatment. These results suggest that POLG2 and SSBP1 altered expression was probably a consequence of increased ROS production. Together with decreased mtDNA content, these data indicate alterations of mtDNA replication in skeletal muscle of diabetic mice. HFHSD feeding was also associated with decreased mitochondrial functions since substrate-driven oxygen consumption was altered, specifically in muscle fibers of 16 week HFHSD mice. State 3 and state 4 respiration rates were reduced when glutamate/malate were used as substrates, indicating decreased oxidation of NADH₂ at complex 1. Furthermore, decreased oxygen consumption, with octanoyl- and palmitoyl-carnitine as substrates, suggested an impaired β -oxidation rate in muscle of 16 week HFHSD mice. These defects in the respiratory functions could be secondary to decreased mitochondria content. However, the fact that respiration with succinate/rotenone was not altered, indicating normal oxidation rates of FADH₂ at complex 2, strongly suggests that specific alterations of mitochondria functions occurred in muscle of 16 week HFHSD mice.

A striking phenotype of skeletal muscle in diabetic mice resided in the structural anomalies of the mitochondria as revealed by electron microscopy. Both subsarcolemmal and intramyofibrillar mitochondria were affected, indicating common alterations independent of the subcellular localization. A number of mitochondria appeared swollen, with less cristae, and the inner and/or outer membranes were sometimes disrupted in muscle of 16 week HFHSD mice. The same mitochondrial alterations, including decreased mitochondrial density, mitochondrial swelling and disruption and reduced mtDNA copy number, were observed in skeletal muscle of STZ-treated mice, a well-known model associated with hyperglycaemia-induced oxidative stress. STZ mice were hyperglycaemic and hypoinsulinemic, but they were not insulin resistant. In agreement, administration of exogenous insulin improved circulating concentrations of glucose, restored glycogen and lipid stores in muscle, and decreased oxidative injury, as assessed by reduction of protein carbonylation. In parallel, mitochondrial density, structural alterations and mtDNA copy number were improved in skeletal muscle of insulin-treated STZ mice. Confirming that mitochondrial restoration was secondary to improvement of glycaemia, phlorizin treatment decreased glycaemia and was associated with increased mtDNA content and improvement of mitochondrial density and structure in skeletal muscle. To confirm the implication of oxidative stress in skeletal muscle mitochondrial alterations, we demonstrated that antioxidant treatment of STZ mice restored mitochondrial density and structure. The strong analogies between HFHSD and STZ regarding the changes in mitochondria structure and integrity in skeletal muscle strongly suggested common underlying mechanisms. Oxidative stress in skeletal muscle, induced by hyperglycaemia in STZ mice and the combination of hyperglycaemia and hyperlipidaemia in HFHSD mice, could be

the culprit. Supporting this assumption, *in vitro* data in cultured skeletal muscle cells demonstrated that treatment with high glucose or high fatty acid concentrations induced ROS production and mitochondrial damages in myotubes. This is also consistent with several reports indicating that high glucose levels (23) as well as elevated fatty acids (24) increase oxidative stress in various models. Interestingly, the absence of ROS production in muscle of KKA^y mice further suggests that weak hyperglycaemia, in the absence of elevated FFA levels, is not sufficient to increase ROS production and mitochondrial dysfunction. At this stage, we cannot determine whether ROS production is the only factor contributing to mitochondrial dysfunctions. However, the fact that H₂O₂ addition induced a decrease of mtDNA amount and CS activity in cultured myotubes, with a concomitant reduction of POLG2 and SSBP1 expression, and that these effects were reversed by antioxidant treatment, supports a critical role of ROS in mediating mitochondria alterations in skeletal muscle. In agreement with this conclusion, it has been demonstrated that glucose-induced ROS production and oxidative stress in dorsal root ganglion neurons paralleled changes in mitochondrial size and function (25). In addition, mitochondrial DNA polymerase has been shown to be one of the targets of oxidative damage (26).

What is the mechanism of ROS-induced mitochondrial dysfunctions? It seems that increased ROS production into skeletal muscle is crucial for the induction of mitochondrial alterations since mitochondrial density and structure were not altered in genetically obese and diabetic KKA^y mice, which were hyperglycaemic with mild elevation of plasma H₂O₂, but without intramuscular oxidative stress, as evidenced by low levels of muscle protein carbonylation. In addition, restoration of mitochondria damages in NAC treated STZ mice was associated with decrease in the index of skeletal muscle oxidative stress, but not in plasma H₂O₂ levels, also suggesting that local oxidative stress might be determinant for mitochondria alterations in skeletal muscle. Moreover, investigations of the changes in the expression of several enzymatic systems involved in the regulation of oxidative stress in muscle revealed increases in the mRNA levels of uncoupling proteins and of almost all the subunits of NAD(P)H oxidase, strongly suggesting that locally increased ROS production was probably due to *de novo* mitochondrial and cytoplasmic generation, rather than to reduced antioxidant defences during HFHSD. We propose the following working hypothesis to explain how skeletal muscle oxidative stress could induce mitochondria dysfunctions during HFHSD in mice: chronic elevation of plasma glucose and fatty acid levels leads to energy substrates overflow in muscle, promoting intramyocellular lipids accumulation and inducing ROS production through increased mitochondrial uncoupling (17) and increased NAD(P)H oxidase enzyme (23). This intramuscular oxidative stress then causes mitochondria alterations and decreases mitochondria functions through damages of proteins, lipids and DNA (27). Particularly, increased ROS level could

lead to decreased expression of PGC1 α , POLG2 and SSBP1, altering mitochondrial biogenesis and mtDNA replication, which in turn contributes to the mitochondrial dysfunctions. Consequently, fatty acid oxidation is dampened, amplifying the deposition of lipids in muscle. This initiates a vicious cycle in which increased intramuscular lipids, prone to ROS-induced formation of lipid peroxides (28), could foster mitochondrial damage. Another potential mechanism, which needs further investigations, could also implicate a ROS-mediated regulation of sirtuin activity. Indeed, it has been recently demonstrated that muscle mitochondrial function is controlled by the activation of both the deacetylase sirtuin 1 and PGC1- α (29, 30). Moreover, resveratrol treatment, which likely increases sirtuins activity, decreases PGC1- α acetylation, improves mitochondrial function and protects mice against diet-induced obesity and diabetes (30). Since resveratrol has antioxidant capacities, it is tempting to speculate that ROS-induced mitochondrial dysfunctions could involve decreased sirtuins activity and increased acetylation of proteins, including PGC1- α . In agreement, acetylation of PGC1- α is increased in high-fat diet fed mice (30).

This working hypothesis assumes that increased intake of a high energy diet for a prolonged period of time might be an initiating factor for the generation of ROS locally in skeletal muscle. In agreement with this assumption, skeletal muscle mitochondria are generally not altered in genetic models of obesity. Indeed, we did not observe mitochondrial dysfunction in muscle of KKA^y mice. Similarly, a recent report indicates that mitochondria are not altered in skeletal muscle of genetically obese (ob/ob) and diabetic (db/db) mice (31). However, it cannot be excluded that leptin receptor mutation and decreased leptin signalling could play a protective role in these models, since leptin has been shown to increase the production of ROS (32).

In summary, the present study demonstrates that mitochondrial dysfunction is not an early event in the development of insulin resistance in diabetic mice, but rather a complication of hyperglycaemia/hyperlipidaemia-induced ROS production in skeletal muscle. If similar mechanism occurs in human, our data suggest that mitochondrial dysfunction, as observed in skeletal muscle of type 2 diabetic and prediabetic patients (11, 12, 18), is probably not the initial event which triggers decreased oxidative capacities, lipid accumulation and inhibition of insulin action. Under such conditions, increased oxidative stress in the skeletal muscle might be a unifying mechanism promoting in parallel mitochondria alterations, lipid accumulation and insulin resistance. Because increased ROS levels also play an important role in altered insulin secretion by pancreas (33), oxidative stress might contribute to the two prominent features of type 2 diabetes, insulin resistance and pancreatic β -cell dysfunction. Therefore, therapeutic strategies to limit mitochondrial radical

production and to counteract their damaging effects may provide a useful complement to conventional therapies designed to normalize blood glucose and lipids.

Methods

Animals

Male C57Bl/6 mice of 4 weeks (diet protocol) and 10 weeks (STZ protocol) were purchased from Harlan. Male C57Bl/6J and KKA^y mice of 9 weeks were purchased from the Jackson Laboratory. Animals were housed in the common animal center from Laennec faculty medicine (IFR62, Lyon) at 22°C and with a 12h light/dark cycle. Animal procedures were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. After one week of acclimatization, mice in the diet protocol were divided into two groups: one with free access to a standard chow diet (SD, 57% carbohydrate, 5% fat, 18% protein, Harlan) and one with free access to pelleted high-fat and high-sucrose diet (HFHSD, 36% fat, 35% carbohydrate of which 50% sucrose, 19.8% protein, TD99249, Harlan). Animals were studied after 4 and 16 weeks of feeding. At the end of the protocols, blood was withdrawn at the fed state from the orbital sinus of anesthetized animals, by using heparinized microcapillary tubes. Then, animals were sacrificed by cervical dislocation, and gastrocnemius muscles were rapidly excised and frozen in liquid nitrogen.

For the STZ study, 10 week-old C57Bl/6 mice were given daily an intraperitoneal dose of streptozotocin dissolved in sodium citrate buffer (100 mg/kg body weight, Sigma), for 3 consecutive days. Glucose levels were monitored daily and when mice achieved fed glucose levels of >500 mg/dl for 3 consecutive days (day 11). One group was treated with insulin (*Insulatard*[®], 3mU) and another one with phlorizin (0.2g/kg), both twice daily at 8 hours intervals. Control groups for each treatment were infused with the respective vehicle. Twenty-four hours after the first injection of insulin or phlorizin, animals were sacrificed and gastrocnemius muscles were removed and frozen. Another group of STZ mice was treated by a general antioxydant, N-acetylcysteine (NAC, 10 mM in drinking water), starting from the 7th day after the first injection of STZ. Mice were sacrificed after 5 days of NAC treatment, and blood and samples were obtained as described above.

Measurement of metabolites and hormones

Blood glucose levels were measured using a glucometer (Roche Diagnostics). Serum levels of insulin (Linco Research) and leptin (BioVendor) were determined using the murine ELISA kits. Total serum triglycerides (Biomérieux) and FFA (Roche Diagnostics) were assayed by using enzymatic methods. Plasma H₂O₂ levels were measured using an Amplex Red hydrogen peroxide assay kit (Invitrogen).

Transmission electronic microscopy

Gastrocnemius muscle was cut into small pieces and fixed in 2% glutaraldehyde for 2h at 4°C, postfixed in 1% Osmium tetroxide for 1h at 4°C, dehydrated and embedded in Epon at either a longitudinal or transverse orientation. The tissue was then cut using a RMC/MTX ultramicrotome (Elexience) and ultrathin sections (60-80nm) were mounted on copper grids, contrasted with 8% uranyl acetate and lead citrate, and observed with a Jeol 1200 EX transmission electron microscope (Jeol LTD) equipped with MegaView II high resolution TEM camera. Analysis was performed with Soft Imaging System (Eloïse SARL). Selection of oxidative fibers was based on the size of fibers and the amount of mitochondria.

Mitochondrial DNA analysis

Total DNA was extracted from muscle using phenol/chloroform/isoamyl alcohol (25:24:1) followed by ethanol precipitation. The content of mitochondrial DNA (mtDNA) was calculated using real time quantitative PCR by measuring the threshold cycle ratio (ΔC_t) of a mitochondrial-encoded gene (COX1, fwd 5'-ACTATACTACTACTAACAGACCG-3', rev. 5'-GGTTCTTTTTTCCGGAGTA-3') versus a nuclear-encoded gene (cyclophilin A, fwd 5'-ACACGCCATAATGGCACTGG-3', rev. 5'-CAGTCTTGGCAGTGCAGAT-3').

Real time quantitative RT-PCR analysis

Total RNA was extracted with the Trizol Reagent (Invitrogen). The level of target mRNAs was measured by RT followed by real-time PCR using a LightCycler (Roche). A standard curve was systematically generated with six different amounts of purified target cDNA and each assay was performed in duplicate (34). We measured hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA as a reference gene so that the results are expressed as a ratio referred to the expression of HPRT. Primer sequence and RT-qPCR conditions are available upon requested.

Measurement of mitochondrial respiration on skinned fiber preparation

Mitochondrial respiration was studied in saponin-skinned fibers (35). Fiber bundles were mechanically separated with tongs and permeabilized with saponin (60 mg/l, 20 minutes). Bundles were then washed three times for 10 minutes to remove ADP, creatine phosphate, soluble enzymes and metabolites. Fiber respiration rates were measured at 25°C using an oxygraph system (Hansatech Instruments). Different substrates were used as follows: glutamate 5mM + malate 2mM as complex 1 substrates; succinate 5mM + rotenone 2.2 μ M as a complex 2 substrates with inhibition of complex 1 by rotenone; octanoyl-carnitine (110 μ M) or palmitoyl-carnitine (55 μ M) in presence of 1mM

malate, as β -oxydation substrates. State 3 was measured in the presence of respiratory substrates after the addition of 1mM ADP and state 4 was measured after the addition of 60 μ M of atractyloside, a potent inhibitor of the ATP/ADP carrier. State 4 was considered as the control state of respiration. Finally, fibers were dried for 24 hours at 100°C and weighted. Respiration rates were expressed as nanoatoms (nat) O/(min.mg dried fiber). Respiratory control ratio was calculated by dividing state 3 by state 4 respiration rates.

Mitochondria isolation

Muscle was thawed in isolation buffer (Mannitol 210mM, Saccharose 70mM, Tris 50mM, EDTA 10mM and BSA 0.5%, pH=7.4) and cut in small pieces. Then, it was digested 15 minutes with trypsin, under agitation, and washed 2 times with the isolation buffer. After each wash, the tissue was centrifuged two minutes, at 70g. The tissue was homogenised with a conical glass grinder (VWR International) in 1ml of isolation buffer. The homogenate was centrifuged 10 minutes at 820g. Then the supernatant was centrifuged 20 minutes at 6800g. The pellet was resuspended in 1ml of suspension buffer (Mannitol 225mM, Saccharose 75mM, Tris 10mM, and EDTA 0.1mM, pH=7.4) and centrifuged 10 minutes at 820g. The mitochondria were then pelleted by centrifuging the supernatant 20min at 6800g, and resuspended in 50 μ l of the same buffer. Western-blotting analysis was used to detect cytochrome C (Santa Cruz, 1/100) content in cytosolic and mitochondrial fractions.

Measurement of enzyme activities

CS activity was measured spectrophotometrically (36) in purified mitochondria from gastrocnemius muscle. For measurement of caspase 3 activity, gastrocnemius muscle was lysed in ice-cold lysis buffer (1% NP40, 20mM Tris-HCl, 138mM NaCl, 2.7mM KCl, 1mM MgCl₂, 5% glycerol, pH=8.0, supplemented with 5mM EDTA, 1mM Na₃VO₄, 20mM NaF, 1mM DTT and protease inhibitors). After centrifugation for 10 minutes at 4°C, aliquots of supernatant (100 μ g) were incubated with 10 μ l DEVD-pNA for 1 hour at 37°C, according to the instructions of the manufacturer (Clinisciences). The pNA light emission was quantified using a spectrophotometer at 400 nm.

Protein carbonylation

The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon. The carbonyl groups in the protein side chains are derivatized to DNP-hydrazone by reaction with DNPH, following the manufacturer's instructions. After the derivatization of the protein sample, one

dimensional electrophoresis was carried out on a 10% SDS-PAGE gel. Proteins were transferred to PVDF-membranes. After incubation with anti-DNP antibody, the blot was developed using a chemiluminescence detection system.

Muscle cells culture

C₂C₁₂ myoblasts were grown to confluence in DMEM supplemented with 10% FCS. They were induced to differentiate into myotubes, by switching to DMEM containing 2% horse serum. Primary cultures of human skeletal muscle cells were initiated from satellite cells of quadriceps samples obtained from organ donors (3 men, 27 ± 7 years, 23 ± 1.7 kg/m²). Differentiated myotubes were prepared according to the procedure previously described in details (37). Myotubes were then cultured for 96 hours, with or without H₂O₂ (100µM), with mannitol (25mM) or glucose (25mM), and with BSA (1%) or palmitic acid (200µM), in the presence or not of N-acetylcysteine (NAC, 10mM).

ROS production in C₂C₁₂ cells

ROS production was detected by the nitroblue tetrazolium (NBT, Sigma) assay. NBT is reduced by ROS to a dark-blue, insoluble form of NBT called formazan. After treatment, myotubes were incubated for 90 minutes in DMEM containing 0.2% NBT. Formazan was dissolved in 50% acetic acid, and the absorbance was determined at 560 nm. Optical density values were normalized by protein levels.

Statistical analysis

The student's t-test was used to analyze the difference between control and experimental groups. P < 0.05 was considered to be significant.

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Figure Legends

Figure 1: Decreased mitochondrial density in skeletal muscle of 16 week HFHSD mice. A: mtDNA quantity calculated as the ratio of COX1 to cyclophilin A DNA levels, determined by real time PCR, in skeletal muscle of SD and HFHSD mice, after 4 and 16 weeks of diet (n=6). Note that the y-axis scale is between 0.5 and 0.8. B: mRNA expression of mitochondria-encoded COX1 and COX3 genes, determined by real-time RT-PCR in skeletal muscle of 16 week HFHSD mice (n=6). Results were normalized by the mean value of the 16 week SD condition set to one unit. C: mitochondrial density assessed by electron microscopy, in skeletal muscle from SD and HFHSD mice, after 4 and 16 weeks of diet. D: Quantification of subsarcolemmal and intermyofibrillar mitochondria number per image area in gastrocnemius muscle of 16 week HFHSD mice (analysis of 5 images in 3 mice per group). Results were normalized by the mean value of the 16 week SD condition set to one unit. E- Citrate synthase (CS) activity in isolated mitochondria from gastrocnemius of SD and HFHSD mice, after 4 and 16 weeks of diet (n=6). * p<0.05, ** p<0.01 relative to SD mice, \$ p<0.05 relative to 4 week SD mice.

Figure 2: Expression of genes implicated in mitochondrial biogenesis and in mtDNA replication. mRNA levels of mitochondrial biogenesis (A) and mtDNA replication (B) genes, determined by real-time RT-PCR, in gastrocnemius muscle of SD and HFHSD mice, after 4 and 16 weeks of diet (n=6). Results were expressed as fold change versus the SD conditions set to one unit (dotted line). * p< 0.05. PGC1: PPAR gamma coactivator 1, NRF: nuclear respiratory factor, mtTFA: mitochondrial transcription factor, ERR α : estrogen-related receptor α , Mfn2: mitofusin 2, POLG: polymerase gamma, SSBP1: single strand binding protein.

Figure 3: Alterations in mitochondrial structure in skeletal muscle of 16 week HFHSD-fed mice. A-B: Transmission electronic microscopy images (magnification x 25,000 (A) and x 100,000 (B)) of subsarcolemmal and intermyofibrillar mitochondria in gastrocnemius muscle of 16 weeks SD and HFHSD-fed mice. C: Quantification of subsarcolemmal and intermyofibrillar mitochondria area in gastrocnemius muscle of 16 week HFHSD mice (analysis of 5 images in 3 mice per group). Results were normalized by the mean value of the 16 week SD condition set to one unit.* p<0.05, **p<0.01.

Figure 4: Chronic HFHSD feeding induces an oxidative stress in skeletal muscle. A: Immunoblots showing total protein carbonylation in gastrocnemius muscle of SD and HFHSD mice, after 4 and 16 weeks of diet. B: mRNA levels of oxidant stress-related genes, determined by real-time RT-PCR, in gastrocnemius muscle of SD and HFHSD mice, after 4 and 16 weeks of diet (n=6). Results were expressed as fold change versus the SD conditions set to one unit (dotted line). * p< 0.05. C: Immunoblot showing cytochrome C protein in mitochondrial (MF) and cytosolic (CF) fractions of gastrocnemius muscle of SD and HFHSD mice, after 4 and 16 weeks of diet. D: Caspase 3 activity measured in gastrocnemius muscle of 4 and 16 week HFHSD mice (n=6). Results were normalized by the mean values of the 4 and the 16 week SD conditions, respectively. * p<0.05. UCP: uncoupling protein, GSR: glutathione reductase, GPx: glutathione peroxidase, CAT: catalase, SOD: superoxide dismutase, Prdx: peroxiredoxin.

Figure 5: Streptozotocin-induced oxidative stress alters mitochondria density and structure in skeletal muscle. A: Immunoblots showing total protein carbonylation in gastrocnemius muscle of control (Co), streptozotocin-treated (STZ) and insulin-treated STZ mice (STZ+INS). B: Immunoblot showing cytochrome C protein in mitochondrial (MF) and cytosolic (CF) fractions of gastrocnemius muscle of Co, STZ and STZ+INS mice. C: mtDNA copy number was calculated as the ratio of COX1 to cyclophilin A DNA levels, determined by real time PCR, in skeletal muscle of Co, STZ, STZ+INS, and phlorizin-treated STZ mice (STZ+PHL) (n=6). Note that the y-axis scale is between 0.5 and 1. Results were normalized by the mean value of the control condition set to one unit. * p< 0.01 STZ vs. Co, # p<0.05 compared to STZ mice. D: Transmission electronic microscopy images (x 25,000) of gastrocnemius muscle from Co, STZ, STZ+INS, and STZ+PHL mice, pointing both subsarcolemmal and intermyofibrillar mitochondria.

Figure 6: Antioxidant treatment restores mitochondrial alterations in STZ mice. A: Plasma H₂O₂ levels in control (Co) and streptozotocin mice, treated (STZ+NAC) or not (STZ) with N-acetylcysteine (10mM in drinking water). B: Immunoblots showing total protein carbonylation in gastrocnemius muscle of Co, STZ and STZ+NAC mice. C: mtDNA copy number was calculated as the ratio of COX1 to cyclophilin A DNA levels, determined by real time PCR, in skeletal muscle of Co, STZ and STZ+NAC mice. Note that the y-axis scale is between 0.5 and 1. Results were normalized by the mean value of the control condition set to one unit. * p< 0.01 STZ vs. Co, # p<0.05 compared to STZ mice. D: Transmission electronic microscopy images (x 25,000) of gastrocnemius muscle of STZ and STZ+NAC mice, pointing both subsarcolemmal and intermyofibrillar mitochondria.

Figure 7: ROS-induced mitochondrial alterations in C₂C₁₂ muscle cells. A- ROS production, measured by NBT reduction, measured in differentiated C₂C₁₂ myotubes incubated with glucose (25mM) or palmitate (200μM), in presence or not of NAC (10mM), for 96 hours (n=4). Data are expressed relative to respective control (dotted line). B- Effect of H₂O₂ (0.1mM) , glucose (25mM) and palmitate (200μM) on mtDNA levels in differentiated C₂C₁₂ myotubes. Myotubes were treated for 96 hours in presence or not of 10mM NAC (n=4). Data are expressed relative to control condition (dotted line). C: Citrate synthase (CS) activity measured in total lysates of myotubes treated for 96 hours with H₂O₂ (0.1mM), glucose (25mM) or palmitate (200μM), in presence or not of NAC (10mM) (n=4). D: mRNA levels of POLG2, SSBP1 and PGC-1α genes, determined by real-time RT-PCR, in H₂O₂, glucose or palmitate-treated myotubes, in presence or not of NAC, for 96 hours (n=4). All results are expressed as fold change relative to the values of untreated cells set to one unit (dotted line). *p<0.05, ** p<0.01 vs. respective control. # p<0.05 with and without NAC for each treatment.

Table 1**Characteristics of the mice**

	SD/HFHS protocol				STZ/INS protocol		
	SD 4w	HFHSD 4w	SD16w	HFHSD16w	Co	STZ	STZ+INS
Body weight (g)	24.08 ± 0.38	28.9 ± 0.6**	27.7 ± 0.36	44 ± 1.02**	23.9 ± 0.1	19.6 ± 0.1 [§]	22 ± 0.4 [§]
Fat weight (g)	0.25 ± 0.02	1.1 ± 0.07**	0.61 ± 0.05	2.5 ± 0.08**	nd	nd	nd
Glucose (mg/dl)	167 ± 4.7	170 ± 4.7	149 ± 4.1	210.7 ± 7**	160 ± 4	598.5 ± 21.6 ^{§§}	484.3 ± 22 ^{§§}
Insulin (ng/ml)	0.87 ± 0.2	1.5 ± 0.4*	0.8 ± 0.22	7.4 ± 1.6**	0.8 ± 0.2	0.3 ± 0.02 ^{§§}	15.4 ± 3.3 ^{§§}
Leptin (pg/ml)	3.1 ± 0.3	5.5 ± 1.7*	8.4 ± 0.9	55 ± 3.7**	nd	nd	nd
TG (g/l)	0.3 ± 0.07	0.2 ± 0.03	0.16 ± 0.03	0.4 ± 0.06**	nd	nd	nd
FFA (mM)	0.5 ± 0.04	0.6 ± 0.13	0.45 ± 0.06	0.9 ± 0.1*	0.11 ± 0.01	0.12 ± 0.05	ud
H ₂ O ₂ (μM)	29.9 ± 8	33.02 ± 5.7	28.8 ± 3.4	44 ± 4.5*	nd	nd	nd

Data represent the means ± sem of 10-20 mice per group.

* p<0.05, ** p<0.001 vs. the respective SD mice.

§ p<0.05, §§ p< 0.001 between Co and STZ mice.

\$ p<0.05, \$\$ p<0.001 between STZ and STZ+INS mice.

nd: not determined, ud: undetected, SD: standard diet, HFHSD: high fat and high sucrose diet, Co: control, STZ: streptozotocin-treated mice, STZ+INS: insulin-treated STZ mice, TG: triglyceride, FFA: free fatty acids.

Table 2

Respiration rates and respiration control ratio (RCR) in permeabilized muscle fibers from SD and HFHSD mice, after 4 and 16 weeks of feeding.

	Respiration rates (nat O/(min.mg dry weight))				
		SD 4w	HFHSD 4w	SD 16w	HFHSD 16w
Glutamate /Malate	State 4	2.45 ± 0.3	2.55 ± 0.2	2.23 ± 0.1	1.62 ± 0.2 *
	State 3	6.43 ± 0.9	6.15 ± 0.8	6.34 ± 0.4	4.5 ± 0.4 *
	RCR	2.79 ± 0.5	2.47 ± 0.3	2.86 ± 0.2	2.87 ± 0.3
Succinate/Rotenone	State 4	5.02 ± 0.6	6.61 ± 0.9	5.25 ± 0.5	5.55 ± 0.7
	State 3	10.7 ± 1.4	14.4 ± 1.2	11.85 ± 1.4	11.1 ± 0.9
	RCR	2.79 ± 0.5	2.47 ± 0.4	2.86 ± 0.2	2.87 ± 0.3
Octanoyl-carnitine/Malate	State 4	1.86 ± 0.1	1.36 ± 0.1	1.4 ± 0.1	0.99 ± 0.1
	State 3	3.93 ± 0.4	3.52 ± 0.5	3.38 ± 0.5	1.73 ± 0.2 *
	RCR	2.11 ± 0.18	2.66 ± 0.5	2.29 ± 0.4	1.85 ± 0.2
Palmitoyl-carnitine/Malate	State 4	1.18 ± 0.2	1.49 ± 0.1	1.47 ± 0.2	1.01 ± 0.1
	State 3	2.19 ± 0.3	2.99 ± 0.3	3.68 ± 0.6	1.72 ± 0.26 *
	RCR	1.95 ± 0.2	2.07 ± 0.3	2.65 ± 0.4	2.08 ± 0.3

Respiration rates were measured on saponin-skinned fibers as described under Materials and methods. State 4 was the control state of respiration in presence of atractyloside and state 3 was the ADP-stimulated respiration. RCR was the respiratory control ratio (state3/state 4). Values are means ± sem for 6 animals per group. * p<0.01 different from the respective SD mice.