Early mitochondrial dysfunction in glycolytic muscle, but not oxidative muscle, of the fructose-fed insulin-resistant rat

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Warren BE, Lou PH, Lucchinetti E, Zhang L, Clanachan AS, Affolter A, Hersberger M, Zaugg M, Lemieux H. Early mitochondrial dysfunction in glycolytic muscle, but not oxidative muscle, of the fructose-fed insulin-resistant rat. Am J Physiol Endocrinol Metab 306: E658–E667, 2014. First published January 14, 2014; doi:10.1152/ajpendo.00511.2013.—Although evidence that type 2 diabetes mellitus (T2DM) is accompanied by mitochondrial dysfunction in skeletal muscle has been accumulating, a causal link between mitochondrial dysfunction and the pathogenesis of the disease remains unclear. Our study focuses on an early stage of the disease to determine whether mitochondrial dysfunction contributes to the development of T2DM. The fructose-fed (FF) rat was used as an animal model of early T2DM. Mitochondrial respiration and acylcarnitine species were measured in oxidative (soleus) and glycolytic [extensor digitorum longus (EDL)] muscle. Although FF rats displayed characteristic signs of T2DM, including hyperglycemia, hyperinsulinemia, and hypertriglyceridemia, mitochondrial content was preserved in both muscles from FF rats. The EDL muscle had reduced complex I and complex II respiration in the presence of pyruvate but not glutamate. The decrease in pyruvate-supported respiration was due to a decrease in pyruvate dehydrogenase activity. Accumulation of C14:1 and C14:2 acylcarnitine species and a decrease in respiration supported by long-chain acylcarnitines but not acetyl carnitine indicated dysfunctional β-oxidation in the EDL muscle. In contrast, the soleus muscle showed preserved mitochondrial respiration, pyruvate dehydrogenase activity, and increased fatty acid oxidation, as evidenced by overall reduced acylcarnitine levels. Aconitase activity, a sensitive index of reactive oxygen species production in mitochondria, was reduced exclusively in EDL muscle, which showed lower levels of the antioxidant enzymes thioredoxin reductase and glutathione peroxidase. Here, we show that the glycolytic EDL muscle is more prone to an imbalance between energy supply and oxidation caused by insulin resistance than the oxidative soleus muscle.

insulin resistance; type 2 diabetes; mitochondrial dysfunction; skeletal muscle; fatty acid oxidation; aconitase; sirtuin-3

Worldwide, the prevalence of type 2 diabetes mellitus (T2DM) has reached epidemic proportions, which is due largely to increasing age and increasing incidence of obesity (21). Insulin resistance is a major characteristic of T2DM and refers to the inability of cells to respond adequately to insulin, which consequently leads to impaired glucose uptake, reduced glucose clearance, and hyperinsulinemia (15, 51). Because about 80% of glucose uptake in the postprandial state occurs in skeletal muscle, skeletal muscle is the largest “glucose sink” in the body, and therefore, it is considered a key player in the pathogenesis of insulin resistance. Although many studies demonstrate that mitochondrial dysfunction may accompany insulin resistance in skeletal muscle, the role of mitochondrial dysfunction in the pathogenesis of T2DM remains unclear. The difficulty lies in determining exactly which aspects of mitochondrial dysfunction are involved as well as the chronology of mitochondrial/cellular events that lead to T2DM (39, 44).

The primary function of mitochondria is ATP production via oxidative phosphorylation (OXPHOS), and it relies on an adequate substrate supply (fuel) as well as on the efficiency of its electron transport system (ETS) to convert substrate into ATP. In resting skeletal muscle, about 40% of oxygen uptake is normally used for carbohydrate oxidation, whereas the remaining 60% is used for fatty acid oxidation (10). A mismatch between fatty acid availability and rates of oxidation has been proposed as an important factor leading to cellular overload of fatty acyl-CoAs and other fatty acid metabolites, which interfere with and ultimately disrupts insulin signaling and glucose homeostasis (51). Based on currently available studies in humans (e.g., Refs. 8, 47, and 50), a general consensus on how mitochondrial dysfunction is linked to T2DM has not been reached mainly because of the scarce metabolic data on skeletal muscle during the early stages of T2DM. An additional complication is related to the variable skeletal muscle fiber composition in human subjects (22) that may rapidly change in response to physical activity and lifestyle changes associated with the metabolic syndrome and diabetes (42, 53). Evaluation of mitochondrial function in distinct skeletal muscle types (glycolytic vs. oxidative) during the early stages of diabetes is required to provide essential insights as to whether dysfunction in mitochondrial fatty acid oxidation is indeed the actual cause of the accumulation of intracellular lipids.

The interpretation of studies from animal models of T2DM is equally challenging due to differences in animal diets, experimental protocols, and examined tissues such as skeletal muscle (oxidative, glycolytic, or mixed), liver, adipose tissue, or heart. In this study, we have chosen the fructose-induced rat model of insulin resistance for our experiments (14, 54), since increasing consumption of sugar and high-fructose corn syrup is a major contributing factor to the metabolic syndrome and T2DM and thus is a useful dietary model of early changes associated with T2DM. Moreover, fructose-induced insulin resistance resembles an early stage of T2DM, which is largely reversible upon fructose withdrawal (14) and is devoid of the severe maladaptive consequences typically observed in genetic or inbred models. After 6 wk of fructose feeding (10% fructose in the drinking water), rats develop symptoms of the metabolic
syndrome similar to those observed in humans, including fasting hyperglycemia, hyperinsulinemia, insulin resistance, hypertriglyceridemia, and arterial hypertension (14). Although fructose-fed rats do not gain significantly more weight than age-matched littermates, they do develop considerable hepatic and abdominal fat deposits (central obesity), rendering the fructose-fed rat an animal model that exhibits nearly all criteria of the human metabolic syndrome (1, 12). Unfortunately, characterization of mitochondrial function in this model is scarce and limited mostly to the liver (13).

In the rat, the soleus muscle comprises 90% of slow-twitch muscle fibers as well as a more robust expression of enzymes involved in lipid metabolism than the extensor digitorum longus (EDL), which displays 80% fast glycolytic and 20% mixed glycolytic-oxidative fibers (3). Thus we hypothesized that during the early stages of diabetes due to fructose feeding, OXPHOS as well as fatty acid β-oxidation would be differentially affected in soleus and EDL.

Our results show that mitochondrial dysfunction in an early model of T2DM occurs in a muscle-specific pattern. The glycolytic EDL muscle showed 1) a decrease in pyruvate-supported respiration due to a reduction in pyruvate dehydrogenase activity, 2) a higher fat overload leading to a dysfunctional fatty acid oxidation, and 3) increased mitochondrial oxidative stress, as evidenced by reduced aconitase activity. In contrast, in the oxidative soleus muscle, pyruvate dehydrogenase activity was preserved and fatty acid oxidation increased, as evidenced by lower acylcarnitine levels.

METHODS

Animals. Male Sprague-Dawley rats 6 wk of age were housed under a 12:12-h light-dark cycle and divided in two groups. The control group received a standard rodent diet (Picolab Laboratory Rodent diet 5LOD, which provides 28% kcal protein, 12% kcal fat, and 60% kcal carbohydrates mainly in form of starch; the metabolizable energy is 3 kcal/g) and water ad libitum, whereas the fructose-fed (FF) animals received a fructose-enriched diet (standard rodent diet and 10% fructose in the drinking water, equivalent to a diet where ~50% of the calories are derived from fructose) for 6 wk. At the end of the experimental period, the animals were euthanized with pentobarbital sodium (150 mg/kg ip). Soleus and EDL muscles were collected and either used immediately or rapidly frozen and stored at −80°C. This study conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication, 8th ed., 2011) and was approved by the University of Alberta Animal Policy and Welfare Committee.

Chemicals. All chemicals were from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated.

High-resolution respirometry. Respirometric measurements were made in freshly collected muscle samples using the Oroboros system (Oxygraph-2k, Innsbruck, Austria) with 2–5 mg of permeabilized muscle fibers, as described previously (37). Nine mitochondrial titration protocols were applied to evaluate mitochondrial function and capacity at three different respiratory states (37): 1) LEAK is the respiration in the presence of substrate but without ADP and represents the respiration compensating for proton leak and slip (9); 2) OXPHOS is the coupled oxidative phosphorylation at saturating ADP (2.5 mM); and 3) ETS capacity is the noncoupled state of maximum oxygen flux at optimum uncoupler concentration. The first three sets of substrates used for complex I were first measured in LEAK and OXPHOS states, and then succinate was added to measure respiration with complex I and II substrates under OXPHOS and ETS states. Cytochrome c was added after ADP in complex I protocols to test for the integrity of the outer mitochondrial membrane (35); an increase of respiration of only 0–5% was observed, confirming the integrity of the mitochondrial outer membrane. Maximal ETS capacity was measured in the presence of complex I and II substrates after titration of dinitrophenol (10 μM steps) up to an optimum concentration of 10–30 μM. In complex IV (CIV) assays, the chemical background that was measured after the addition of azide (15 mM) was subtracted from the OXPHOS rate. The fatty acid protocols are according to Puchowicz et al. (48). Octanoylcarnitine was purchased from Cedarlane (no. 0605; Burlington, ON, Canada). Respiratory fluxes are expressed in picomoles of O2 per second per milligram wet weight.

Enzyme activity assays. Citrate synthase (CS) activity was determined at 37°C in tissue extracts [in 110 mM sucrose, 60 mM K-lactobionate, 0.5 mM EGTA, 1 g/l bovine serum albumin (fatty acid free), 3 mM MgCl2, 20 mM taurine, 10 mM KH2PO4, and 20 mM K-N-2-hydroxyethylpiperazine-N2-ethanesulphonate, pH 7.1], as described previously (52). Pyruvate dehydrogenase (PDH) enzyme activity assay was performed on freshly prepared tissue homogenates using the PDH Enzyme Activity Microplate Assay Kit, following the manufacturer’s recommendation (Abcam, ab109902). PDH activity results were normalized to CS activity. Aconitase activity was determined from mitochondria isolated from frozen tissue in the presence of 5 mM sodium citrate and 0.6 mM MnCl2, which were included to protect aconitase from further oxidation during isolation. Mitochondrial preparations were diluted to a protein concentration of 0.1 mg/ml in 50 mM Tris-HCl (pH 7.4) containing 0.05% Triton X-100. Aconitase activity was measured at 37°C according to Gardner (20). One unit of aconitase converted 1.0 nmol/min of citrate to isocitrate. Activity was also measured in the presence of oxalacatate (2 mM), an inhibitor of aconitase, to ensure specificity of this assay.

Immunoblot analyses. Samples of total tissue lysate were resolved by SDS-PAGE. The following antibodies were used to detect their respective proteins: PDH kinase-2 (PDK2; rabbit monoclonal, Abcam, ab68164), PDH kinase-4 (PDK4; rabbit polyclonal, Abcam, ab89295), peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α; rabbit polyclonal, Abcam, ab54481), thioredoxin reductase-2 (rabbit polyclonal, Thermo Scientific, PA1–20940), glutathione peroxidase-1 (goat polyclonal, Acris Antibodies, AP32028PU-N), NAD-dependent deacetylase sirtuin-3 (Sir3; H-40, rabbit polyclonal, sc-99143; Santa Cruz Biotechnology), cytochrome c oxidase IV (COX-IV; mouse monoclonal, ab14744, Abcam), or tubulin (mouse monoclonal, Sigma-Aldrich, T6074). Denitrogenated and dialysed tissue homogenates was carried out using Image J software (National Institutes of Health). Protein concentrations of tissue lysates were measured with the Bradford assay.

Acylcarnitine profile by mass spectrometry. The levels of acylcarnitine species from frozen tissue samples were measured using electrospray ionization tandem mass spectrometry (56). Acylcarnitines were extracted from muscle tissues with methanol and quantified using eight isotopically labeled internal standards (Cambridge Isotopes Laboratories, Andover, MA).

Data analysis. SigmaPlot 12.0 (Systat Software, San Jose, CA) was used for statistical analyses. Results are presented as means (SE) for independent observations. Differences between control and FF rats were evaluated by Student’s t-test or Mann-Whitney rank sum tests, depending on the underlying data distribution. Differences were considered significant when $P < 0.05$.

RESULTS

Characteristics of control and FF rats. After 6 wk of fructose-enriched diet, FF rats showed significantly higher 1) fasting plasma glucose and insulin, 2) systolic and diastolic blood pressures, and 3) plasma triglycerides (Table 2). The quantitative insulin sensitivity check index was significantly reduced in FF rats (Table 2) (11). These changes are consistent
with previously reported results with the FF model (14). The final body mass of FF rats was slightly but significantly lower compared with control rats (Table 2) and consistent with previous data showing no weight gain during 4–8 wk of fructose feeding (see review in Ref. 54).

**CS activity is preserved in the EDL of FF animals.** CS activity was not significantly different between control and FF rats in either the soleus muscle (5.1 ± 0.7 vs. 5.3 ± 0.4 U/g) or the EDL muscle (3.9 ± 0.7 vs. 4.5 ± 1.2 U/g).

**Muscle-specific differences in mitochondrial respiration between control and FF rats.** In the EDL muscle, significant differences in complex I between control and FF rats were evident with pyruvate/malate but not with glutamate or glutamate/malate as respiratory substrates (Fig. 1A). Respiratory activities for other complexes were similar between control and FF rats (Fig. 1A). In soleus muscle, complex II OXPHOS capacity was significantly lower in the FF rats compared with control rats (Fig. 1B). However, in the EDL muscle, octanoylcarnitine oxidation was significantly reduced in FF rats compared with control rats, whereas rates of oxidation of the other fatty acid substrates showed no significant differences between control and FF groups (Fig. 2A).

**LEAK in the presence of complex I substrates (glutamate or glutamate/malate; Table 2) or fatty acid substrates (results not shown) was not significantly different between control and FF animals for both muscle types.** In the presence of pyruvate/malate, the soleus muscle showed a lower LEAK in the FF animals (Table 3). The LEAK in the presence of fatty acid substrates was similar or lower compared with the LEAK measured in the presence of carbohydrate substrates, indicating no fatty acid-induced uncoupling. LEAK flux normalized for ETS capacity (Table 3) provides an expression of uncoupling.

**Table 1. Assays for mitochondrial respiration in permeabilized skeletal muscle fibers**

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Substrates</th>
<th>Complexes, Transporters, Systems, and Enzymes Measured</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>PM: pyruvate (5 mM) + malate (5 mM)</td>
<td>Complex I, PDC, pyruvate transporter</td>
</tr>
<tr>
<td>2</td>
<td>G: glutamate (10 mM)</td>
<td>Complex I, PDC, pyruvate transporter</td>
</tr>
<tr>
<td>3</td>
<td>GM: glutamate (10 mM) + malate</td>
<td>Complex I, glutamate dehydrogenase</td>
</tr>
<tr>
<td>4</td>
<td>S(Rot): succinate (10 mM) + rotenone (0.5 μM)</td>
<td>Complex II, succinate dehydrogenase</td>
</tr>
<tr>
<td>5</td>
<td>AsTm: ascorbate (2 mM) + TMPD (0.5 mM) + rotenone</td>
<td>Complex IV</td>
</tr>
<tr>
<td>6</td>
<td>PalCar: palmitoylcarnitine (0.04 mM) + malate</td>
<td>Carnitine translocase, CPT-II, long-chain FAO</td>
</tr>
<tr>
<td>7</td>
<td>PalCoA: palmitoyl-CoA (0.02 mM) + carnitine (1.25 mM) + malate</td>
<td>Carnitine translocase, CPT-II, long-chain FAO</td>
</tr>
<tr>
<td>8</td>
<td>OctCar: octanoylcarnitine (0.2 mM) + malate</td>
<td>Carnitine translocase, CPT-II, medium-chain FAO</td>
</tr>
<tr>
<td>9</td>
<td>ActCar: acetylcarnitine (5 mM) + malate</td>
<td>Carnitine translocase, carnitine acetyltransferase</td>
</tr>
</tbody>
</table>

ETS, electron transport system; PDC, pyruvate dehydrogenase complex; FAO, fatty acid oxidation system; CPT-I, carnitine palmitoyltransferase-I; CPT-II, carnitine palmitoyltransferase-II; TMPD, N,N,N,N-tetramethyl-p-phenylenediamine. Protocols 1–3: complex I (LEAK) → complex I (OXPHOS) → complex I + II (OXPHOS) → complex I + II (ETS).
and may indicate increased shunting of (excessive) long-chain fatty acids toward triacylglycerol synthesis, as shown previously (28). In addition, significant declines in short odd-chain acylcarnitine species were found in the EDL of FF rats relative to healthy age-matched littermates (Table 4), consistent with a decreased branched-chain amino acid metabolism. The C5-acylcarnitine, representing acylcarnitine esters of both isovaleryl-CoA and α-isomethylbutyryl-CoA from the decarboxylation of leucine and isoleucine ketoacids, respectively, was reduced by 63%; tiglylcarnitine, an isoleucine-specific catabolite, was reduced by 47%; and propionylcarnitine, a catabolite of valine and isoleucine, was reduced by 21% in the EDL of FF rats. Other short-chain acylcarnitines (methylmalonylcarnitine/succinylcarnitine, glutarylcarnitine), which are produced distal to α-ketoglutarate in the tricarboxylic acid (TCA) cycle, were also significantly reduced in EDL tissue from FF rats.

The profile of tissue acylcarnitine species in the soleus showed a general reduction in acylcarnitine species in the soleus of FF rats compared with control rats (Fig. 4B and Table 4). This indicates enhanced β-oxidation in the oxidative muscle without concomitant storage. The desaturation index showed only a trend toward an increase in the soleus muscle in early T2DM.

**Key regulators of metabolic flexibility in EDL and soleus muscle: PGC1α and Sirt3.** To better understand the metabolic regulation in the two different muscle types, we determined the levels of two key regulators of metabolic flexibility, the transcriptional coactivator PGC-1α (33, 38, 57) and the mitochondrial protein NAD⁺-dependent deacetylase Sirt3 (26, 30, 31). We found similar levels of PGC-1α protein in EDL and soleus of healthy rats (Fig. 5A). In early T2DM, PGC-1α protein levels decrease in both EDL and soleus, with a more robust decline in the latter (Fig. 5B). In contrast, Sirt3 protein was expressed at very low levels in the EDL but was abundant in the soleus (Fig. 6A). Sirt3 protein levels were increased significantly in FF rats (when normalized to COX-IV, one of the nuclear-encoded polypeptide chains of cytochrome c oxidase; Fig. 6B).

**Antioxidant enzymes.** We found evidence for increased oxidative stress in the EDL but not in soleus of FF rats. The activity of the redox-sensitive enzyme aconitase was significantly reduced (Fig. 7A), a finding that indicated an imbalance between production and scavenging of reactive oxygen species (20). Thus we determined the protein levels of the cellular antioxidant enzyme glutathione peroxidase 1 and of the mitochondrial antioxidant enzyme thioredoxin reductase-2 (Fig. 6B).

**Table 2. Basic characteristics of control and FF rats**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>FF</th>
<th>*P&lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight, g</td>
<td>249 (3.2)</td>
<td>247 (3.3)</td>
<td></td>
</tr>
<tr>
<td>Final body weight, g</td>
<td>565 (10.9)</td>
<td>496 (9.2)*</td>
<td></td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>117 (3)</td>
<td>149 (2)*</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>68 (4)</td>
<td>104 (4)*</td>
<td></td>
</tr>
<tr>
<td>Plasma fasting glucose, mg/dl</td>
<td>114 (4)</td>
<td>156 (5)*</td>
<td></td>
</tr>
<tr>
<td>Plasma fasting insulin, ng/ml</td>
<td>0.41 (0.08)</td>
<td>1.00 (0.12)*</td>
<td></td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.33 (0.009)</td>
<td>0.28 (0.006)*</td>
<td></td>
</tr>
<tr>
<td>Plasma triglycerides, mg/ml</td>
<td>85 (9)</td>
<td>177 (14)*</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means (SE); n = 6 rats/group. FF, fructose-fed; QUICKI, quantitative insulin sensitivity check index. QUICKI was calculated from fasting glucose (in mg/dl) and fasting insulin concentrations (in μIU/ml) as follows: QUICKI = 1/log [fasting insulin] + log [fasting glucose]. *P < 0.05.

**Mechanism of selective reduction in pyruvate-supported respiration in EDL muscle.** The selective decrease in pyruvate-supported respiration in EDL muscle from FF rats (Fig. 1A) could be explained by a significant reduction in PDH activity without any concomitant changes in protein levels of the related regulating kinases PDK-2 and PDK-4 (Fig. 3) (23).

**Mitochondrial fatty acid overload and incomplete fatty acid oxidation in the EDL muscle of FF rats.** In the EDL muscle, we observed an acylcarnitine pattern, indicating mitochondrial overload and/or decelerated β-oxidation flux with accumulation of long-chain intermediates of β-oxidation. The levels of C14:1 and C14:2 were increased in the EDL from FF rats relative to control animals, and the C14:1/C16 ratio was also significantly higher in EDL from FF-fed animals compared with their healthy counterparts (Fig. 4A and Table 4). The “desaturation index” (i.e., the C16:1/C16:0 and C18:1/C18:0 ratios) was significantly increased in FF-fed animals (Fig. 4A)

**OXPHOS**

**Fig. 2.** Mitochondrial OXPHOS capacities in the presence of fatty acid substrates in permeabilized fibers from skeletal muscle of control and FF rats. Substrates for fatty acid oxidation measurements were palmitoylcarnitine + malate (PalCar), palmitoyl-CoA + carnitine + malate (PalCoA), octanoylcarnitine + malate (OctCar), and acetyl carnitine + malate (ActCar). All data are for the OXPHOS state. Data are for EDL (A) and soleus muscles (B). Black bars, C animals; open bars, FF animals. Data are means ± SE for 8 rats/group. *Significance of P < 0.05.
opposed to glucose, feeds directly into the pool of C2 bodies, distance, and T2DM. In the liver, the lipogenic sugar fructose, as fructose corn syrup (18 kg/capita) is indeed one of the major First, increased consumption of fructose in the form of high-
feeding offers a number of advantages for our study (14, 54).
metabolic flexibility of the latter.
oxidative muscle (soleus), which is likely due to the greater
occur specifically in glycolytic muscle (EDL) rather than in
are as follows:
changes in mitochondrial function in skeletal muscle (the main
site of glucose disposal) of insulin-resistant, fructose-fed rats
The salient findings of this study pertaining to the early
changes resulted in a significant difference (P = 0.03) in
thioredoxin reductase-2 content in the EDL of FF rats. These
did not lead to any changes in thioredoxin reductase-2 protein
levels in the soleus. In contrast, there was a trend toward lower
thioredoxin reductase-2 content in the EDL of FF rats. These
changes resulted in a significant difference (P = 0.03) in
thioredoxin reductase-2 protein levels between soleus and EDL
in FF rats.

**DISCUSSION**

The salient findings of this study pertaining to the early changes in mitochondrial function in skeletal muscle (the main site of glucose disposal) of insulin-resistant, fructose-fed rats are as follows: 1) mitochondrial content and ETS function are not affected; and 2) early defects associated with early T2DM occur specifically in glycolytic muscle (EDL) rather than in oxidative muscle (soleus), which is likely due to the greater metabolic flexibility of the latter.

The fructose-induced diabetes rat model with 6 wk of feeding offers a number of advantages for our study (14, 54). First, increased consumption of fructose in the form of high-fructose corn syrup (18 kg/capita) is indeed one of the major factors causing the current pandemic of obesity, insulin resistance, and T2DM. In the liver, the lipogenic sugar fructose, as opposed to glucose, feeds directly into the pool of C2 bodies, and hyperlipidemia occurs as a result of increased hepatic triglyceride formation (13). This dietary model of insulin resistance reflects an early stage of T2DM due to its reversibility for up to 12 wk of fructose feeding (14) and the absence of severe maladaptive changes as observed in genetic or inbred animal models. The animals exposed to fructose feeding for 6 wk consistently exhibited characteristics of the metabolic syndrome and early T2DM such as abdominal fat accumulation (central obesity, despite the absence of weight gain), arterial hypertension, increased fasting glucose, hyperinsulinemia, hyperperoxidase, and insulin resistance.

**Mitochondrial content is not affected after 6 wk of fructose feeding.** Our results show that CS activity and CIV respiration with ascorbate/TMPD as substrates, two validated markers of mitochondrial content in permeabilized fibers (36), are unaffected and indicate that mitochondrial content in both EDL and soleus muscle is not affected during early T2DM. Most studies on T2DM subjects report that mitochondrial content in skeletal muscle is either decreased or unchanged, depending on the severity of diabetes. During the early stage of T2DM, mitochondrial content in skeletal muscle is typically unchanged or increased in both rodent models of insulin resistance (high-fat diet, high-fat/high-sucrose diet) (6, 19, 24, 55) and human subjects (16). A significant reduction in mitochondrial content is observed only at later stages of diabetes (8, 27, 41, 50). Taken together, our results support the view that late-stage reduction in mitochondrial content in T2DM occurs as a consequence of prolonged exposure to insulin resistance and progression of the disease rather than being a cause of the disease.

**The ETS is unaffected in early T2DM, irrespective of fiber type.** After 6 wk of fructose feeding and the development of insulin resistance, the ETS is largely unaffected in both the glycolytic (EDL) and oxidative muscles (soleus). Moreover, using complex IV as an index of overall mitochondrial oxidative capacity, as suggested by Larsen et al. (36), the absence of changes in both muscles of FF rats when compared with

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**Table 3. Respiratory coupling in the permeabilized fibers from the soleus and EDL muscle of the control and FF rats**

<table>
<thead>
<tr>
<th>Muscles</th>
<th>State/Ratios</th>
<th>Control</th>
<th>FF</th>
<th>Control</th>
<th>FF</th>
<th>Control</th>
<th>FF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyruvate + Malate</td>
<td></td>
<td></td>
<td>Glutamate</td>
<td></td>
<td>Glutamate + Malate</td>
<td></td>
</tr>
<tr>
<td>Soleus</td>
<td>LEAK</td>
<td>17.3 (1.5)</td>
<td>9.2 (1.0)*</td>
<td>14.4 (1.5)</td>
<td>12.2 (0.7)</td>
<td>20.2 (2.7)</td>
<td>14.6 (1.7)</td>
</tr>
<tr>
<td>RCR</td>
<td>6.0 (0.3)</td>
<td>9.7 (0.8)*</td>
<td>4.5 (0.5)</td>
<td>3.5 (0.2)</td>
<td>2.9 (0.5)</td>
<td>2.9 (0.2)</td>
<td></td>
</tr>
<tr>
<td>LEAK/ETS</td>
<td>0.11 (0.01)</td>
<td>0.07 (0.01)*</td>
<td>0.10 (0.01)</td>
<td>0.11 (0.01)</td>
<td>0.21 (0.03)</td>
<td>0.17 (0.02)</td>
<td></td>
</tr>
<tr>
<td>EDL</td>
<td>LEAK</td>
<td>17.5 (1.9)</td>
<td>14.7 (1.8)</td>
<td>12.8 (1.7)</td>
<td>10.0 (1.5)</td>
<td>18.6 (2.2)</td>
<td>17.1 (2.3)</td>
</tr>
<tr>
<td>RCR</td>
<td>6.22 (0.8)</td>
<td>4.9 (0.5)</td>
<td>4.9 (0.3)</td>
<td>6.7 (1.2)</td>
<td>3.5 (0.5)</td>
<td>3.6 (0.5)</td>
<td></td>
</tr>
<tr>
<td>LEAK/ETS</td>
<td>0.12 (0.01)</td>
<td>0.18 (0.03)</td>
<td>0.11 (0.01)</td>
<td>0.09 (0.02)</td>
<td>0.24 (0.03)</td>
<td>0.23 (0.03)</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as means (SE). RCR, respiratory control ratio; ETS, electron transport system. LEAK respiration is measured in the presence of substrates but without adenylates. RCR is the ratio of OXPHOS (in the presence of saturating ADP, without cytochrome c) over LEAK capacity. The LEAK/ETS ratios is the LEAK normalized to the maximal ETS capacity in the presence of complex I + II substrates (succinate and uncoupler added to the substrates). *P < 0.05.

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7B). Glutathione peroxidase 1 was expressed at very low levels in the EDL compared with the soleus. Thioredoxin reductase-2 enzyme content in the soleus of healthy rats was slightly higher compared with the EDL (P = 0.07). The fructose-enriched diet did not lead to any changes in thioredoxin reductase-2 protein levels in the soleus. In contrast, there was a trend toward lower thioredoxin reductase-2 content in the EDL of FF rats. These changes resulted in a significant difference (P = 0.03) in thioredoxin reductase-2 protein levels between soleus and EDL in FF rats.

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Fig. 3. **A**: pyruvate dehydrogenase (PDH) activity in soleus and EDL muscles of C and FF rats. B and C: immunoblots of pyruvate dehydrogenase kinase isoforms 2 (PDK2; A) and 4 (PDK4; B) from soleus and EDL muscles of C and FF rats. Tubulin was used as loading control. Black bars, C animals; open bars, FF animals. Data are means ± SE for 6 rats/group. *Significance of P < 0.05.
control animals also indicates that changes in ETS do not occur in early T2DM. However, accumulation of metabolic intermediates from incomplete fatty acid oxidation occurred in the glycolytic EDL muscle only.

Substrate-selective reduction in complex I respiration.
Changes in mitochondrial function, other than the ETS, did occur in the glycolytic EDL muscle and included a reduced complex I respiration, but only in the presence of pyruvate/malate and not in the presence of the other complex I-linked substrate, glutamate/malate. Even under simultaneous electron input via complexes I and II, respiration rate was still reduced whenever pyruvate was present in the EDL muscle of FF rats. Complex II respiratory activity (with succinate) was unaffected. Therefore, the observed reduction in complex I respiratory activity is substrate dependent and not a consequence of diminished complex I activity. This observation suggests a reduced mitochondrial pyruvate dehydrogenase activity, which is consistent with the notion that increased acyl-CoA levels from fatty acid oxidation (see below) impose a negative feedback on PDH (17, 32). The glutamate/malate substrate combination and succinate are not dependent on PDH to enter the TCA cycle and thus preserve the production of reducing equivalents. Although we also observed reduced aconitase activity in EDL muscle from fructose-fed rats, the oxidative capacity of the TCA cycle was not yet significantly affected. We anticipate that a more pronounced loss of aconitase activity at a later stage of the disease may further aggravate insulin resistance and metabolic derangements. Mechanistically, it has been proposed that nutrient excess from hyperglycemia and hyperlipidemia produce a high a NADH/NAD⁺ ratio, leading to feedback inhibition of oxidative phosphorylation, increased production of reactive oxygen species (46), and inactivation of the iron/sulfur-containing TCA enzyme aconitase (20). The reduced aconitase activity may lead to increased levels of citrate. Excess citrate is exported to the cytoplasm, where it supports lipid synthesis and inhibits glycolysis and glucose uptake (2). It has been suggested that accumulation of citrate may play a key role in the development of insulin resistance.

Fig. 4. A: markers of accumulation of long-chain fatty acid intermediates (laureoylcarnitine, myristdienoylcarnitine, and the laureoylcarnitine/palmitoylcarnitine ratio) and of fatty acid desaturation (oleoylcarnitine/stearoylcarnitine and palmitoleoyl-carnitine/palmitoylcarnitine ratios) in the EDL. B: increased fatty acid oxidation as measured by levels of free carnitine, the total amount of acylcarnitines, and the ratio thereof in the soleus. Markers of fatty acid desaturation are also presented for comparison to the EDL. Black bars, C animals; open bars, FF animals. *Significance of P < 0.05; **significance of P < 0.01; ***significance of P < 0.001.
Production of reactive oxygen species is particularly detrimental in the absence of sufficient antioxidant enzyme capacity in the EDL, as reported previously in the literature (43) and confirmed in our experiments.

Despite decreased respiration with the complex II-linked substrate (succinate) in the soleus, we did not observe a significant impact on muscle energy production under physiological conditions of convergent electron input (OXPHOS I and II; pyruvate/malate/succinate and glutamate/malate/succinate).

Fatty acid oxidation is dysfunctional in EDL muscle but increased in soleus muscle. Acylcarnitine profiling revealed accumulation of C14 fatty acid intermediates and reduced respiration in the presence of octanoylcarnitine, albeit not significant, and in the presence of palmitoylcarnitine in the EDL muscle from fructose-fed rats, consistent with decreased activity of the long-chain hydroxyl acyl-CoA dehydrogenase (LCAD) enzyme. Recent observations link dysfunction of LCAD to insulin resistance and progression to T2DM (60). Alternatively, the accumulation of C14 fatty acid intermediates may indicate an imbalance between an overloaded fatty acid oxidation pathway and a TCA cycle depleted of C4 carbon bodies. Optimal levels of TCA intermediates are essential for the efficient functioning and regulation of the TCA cycle.

Interestingly, our data show significantly reduced levels of acylcarnitine species derived from amino acid metabolism in Table 4.

### Table 4. Concentrations of carnitine and acylcarnitine in the soleus and EDL muscles of control and FF rats

<table>
<thead>
<tr>
<th>Acylcarnitine Species</th>
<th>Soleus</th>
<th>EDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free carnitine (C0)</td>
<td>Control FF</td>
<td>Control FF</td>
</tr>
<tr>
<td>Total acylcarnitines</td>
<td>925 (55)</td>
<td>776 (23)*</td>
</tr>
<tr>
<td>Acetylcarnitine (C2)</td>
<td>346 (37)</td>
<td>191 (10)*</td>
</tr>
<tr>
<td>Propionoylcarnitine (C3)</td>
<td>299 (33)</td>
<td>158 (9)*</td>
</tr>
<tr>
<td>Butyrylcarnitine (C4)</td>
<td>12.5 (2.3)</td>
<td>8.4 (0.4)</td>
</tr>
<tr>
<td>3-OH-butyrylcarnitine/malonanoylcarnitine</td>
<td>6.48 (1.51)</td>
<td>3.88 (0.18)</td>
</tr>
<tr>
<td>Methylmalonylcarnitine (C4-DC)</td>
<td>2.23 (0.33)</td>
<td>1.57 (0.13)</td>
</tr>
<tr>
<td>Isovalerylcaritnine (C5)</td>
<td>0.59 (0.08)</td>
<td>0.40 (0.04)</td>
</tr>
<tr>
<td>Valerylcaritnine (C5-DC)</td>
<td>0.30 (0.002)</td>
<td>0.23 (0.03)</td>
</tr>
<tr>
<td>Hexanoylcaritnine (C6)</td>
<td>0.97 (0.17)</td>
<td>0.66 (0.04)</td>
</tr>
<tr>
<td>Methylglutarylacarnitine (C6-DC)</td>
<td>0.09 (0.01)</td>
<td>0.05 (0.008)*</td>
</tr>
<tr>
<td>Octanoylcaritnine (C8)</td>
<td>0.30 (0.04)</td>
<td>0.24 (0.03)</td>
</tr>
<tr>
<td>Octenoylcaritnine (C8-1)</td>
<td>0.08 (0.02)</td>
<td>0.05 (0.009)</td>
</tr>
<tr>
<td>Suberylcaritnine (C8-DC)</td>
<td>0.04 (0.02)</td>
<td>0.04 (0.003)</td>
</tr>
<tr>
<td>Decanoylcaritnine (C10)</td>
<td>0.26 (0.06)</td>
<td>0.19 (0.02)</td>
</tr>
<tr>
<td>Decenoylcaritnine (C10:1)</td>
<td>0.19 (0.01)</td>
<td>0.12 (0.02)*</td>
</tr>
<tr>
<td>Laurinylcaritnine (C12)</td>
<td>0.25 (0.06)</td>
<td>0.17 (0.03)</td>
</tr>
<tr>
<td>Lauroylcaritnine (C12:1)</td>
<td>0.18 (0.02)</td>
<td>0.16 (0.01)</td>
</tr>
<tr>
<td>Myristinoylcaritnine (C14:2)</td>
<td>0.24 (0.04)</td>
<td>0.13 (0.01)*</td>
</tr>
<tr>
<td>Myristoylcaritnine (C14)</td>
<td>0.64 (0.10)</td>
<td>0.54 (0.10)</td>
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<tr>
<td>Myristoylcaritnine (C14)</td>
<td>0.90 (0.18)</td>
<td>0.65 (0.12)</td>
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<td>3-OH-myristoylcaritnine (C14-OH)</td>
<td>0.94 (0.10)</td>
<td>0.85 (0.02)</td>
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<td>3-OH-myristoylcaritnine (C14:1-OH)</td>
<td>0.93 (0.14)</td>
<td>0.72 (0.05)</td>
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<tr>
<td>Palmitoylcaritnine (C16:1)</td>
<td>1.03 (0.18)</td>
<td>1.02 (0.14)</td>
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<td>Palmitoylcaritnine (C16)</td>
<td>2.08 (0.44)</td>
<td>1.69 (0.37)</td>
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<td>3-OH-palmitoylcaritnine (C16:1-OH)</td>
<td>1.45 (0.19)</td>
<td>1.23 (0.08)</td>
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<tr>
<td>3-OH-palmitoylcaritnine (C16:1-OH)</td>
<td>0.99 (0.12)</td>
<td>1.11 (0.05)</td>
</tr>
<tr>
<td>Linoleoylcaritnine (C18:2)</td>
<td>3.22 (0.90)</td>
<td>1.41 (0.15)</td>
</tr>
<tr>
<td>Oleoylcaritnine (C18:1)</td>
<td>3.60 (0.83)</td>
<td>2.79 (0.47)</td>
</tr>
<tr>
<td>Stearoylcaritnine (C18)</td>
<td>0.90 (0.14)</td>
<td>0.60 (0.07)</td>
</tr>
<tr>
<td>3-OH-stearoylcaritnine (C18:1-OH)</td>
<td>0.46 (0.04)</td>
<td>0.33 (0.02)*</td>
</tr>
</tbody>
</table>
| Data are presented as means (SE); n = 6/group. *P < 0.05.

Fig. 5. A: representative immunoblot of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) in soleus and EDL muscles of control rats. Tubulin served as loading control. B: representative immunoblots of PGC-1α in EDL and soleus from C and FF rats. Black bars, C animals; open bars, FF animals. ***Significance of P < 0.001.
the EDL from fructose-fed rats. Propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA are anaplerotic substrates derived mainly from the metabolism of branched-chain amino acids (i.e., valine, isoleucine) and feed into the TCA cycle (propionyl-CoA and methylmalonyl-CoA after conversion to succinyl-CoA). Therefore, we postulate that insulin resistance in the EDL muscle leads to a reduced uptake of glucose and amino acids or a decreased mobilization of amino acids from intracellular protein stores (5). Hence, anaplerosis of the TCA cycle with C4 units may indeed be reduced. Inefficient oxidation rate of C2 carbon units may subsequently become more apparent at the rate-limiting step of fatty acid oxidation, i.e., LCAD. The observed significant increase in the desaturation index confirms data by Crescenzo et al. (13), who found a significant increase in the activity of hepatic fatty acid synthase and stearoyl-CoA desaturase-1, a fatty acid desaturase that catalyzes the conversion of 16:0 and 18:0 into the monounsaturated fatty acids palmitoleic (16:1n7) and oleic acid (18:1n9), respectively, in FF rats compared with controls. In contrast, acylcarnitines showed reduced levels in diabetic soleus muscle, consistent with increased flux through fatty acid oxidation, and no depletion of propionyl-carnitine/CoA, implying a balanced interplay between fatty acid oxidation and tricarboxylic acid cycle.

**Metabolic adaptation vs. metabolic stress.** Postural oxidative muscles such as soleus display a higher insulin sensitivity, higher insulin-stimulated glucose uptake and glycogen synthesis, and higher levels of acyl-CoA-binding protein than glycolytic muscles such as the EDL (18, 25, 29). It is thus conceivable that nutrient excess may lead to less metabolic derange-

ments in the soleus due to its higher oxidative capacity and higher storage capacity, at least in the early stages of T2DM. Metabolic flexibility and PGC-1α expression go hand in hand and have been shown to be normal in the metabolic syndrome but decreased in overt diabetes (40). Overexpression of PGC-1α in skeletal muscle results in fiber type switching toward oxidative type I fibers, which rely on mitochondrial OXPHOS for the generation of ATP rather than glycolysis (38). Furthermore, it protects mice against age-induced muscle wasting and metabolic disease (58). Consistent with these data, in our study both soleus and EDL showed reduced PGC-1α protein levels in response to fructose feeding, with a more pronounced and significant decrease in the soleus. These data, together with the observed changes in the desaturation index, an indicator of stearoyl-CoA desaturase 1 activity, are also consistent with the findings that PGC-1α protein expression is downregulated when triacylglycerol synthesis rates are increased (4). Because PGC-1α is also considered the master regulator of mitochondrial biogenesis (59), reduced levels of this transcriptional coactivator would over time lead to a progressive loss in mitochondrial mass. During early T2DM, however, FF animals do not yet show significant changes in the measured markers of mitochondrial content.

Our study further shows a strong differential expression of Sirt3 protein, which is expressed abundantly in the soleus but...
is nearly absent in the EDL, thus confirming a previous report (45). The observed increased expression of Sirt3 in the soleus of fructose-fed rats may support glucose oxidation by deacetylating PDH and increasing PDH activity, as shown recently (31). Moreover, Sirt3 has been shown to deacetylate MnSOD, an important modulator of mitochondrial oxidative stress, enhancing its activity (49). This, along with our data on reduced expression of antioxidant enzymes in the EDL, may explain why early signs of mitochondrial oxidative stress (reduced aconitate activity) and fat overload (accumulation of long-chain acylcarnitines, increased desaturation index) can first be detected in the glycolytic EDL muscle rather than the oxidative soleus of fructose-fed rats.

Although we did not quantify fat pads in the FF rats in our study, central fat accumulation with fructose feeding is well established in the literature (1, 12). Consistent with fat accumulation, data from our study show hypertriglycerideremia and an increased desaturation index in the EDL muscle where metabolic changes were observed, indicating increased shunting of long-chain fatty acids toward triacylglycerol synthesis. The definition of metabolic syndrome in humans encompasses insulin resistance, but not all individuals with metabolic syndrome suffer from overt T2DM. Also, in many animal models of T2DM, the transition from insulin resistance to overt T2DM is poorly defined compared with the human model, where clear definitions distinguish insulin resistance from overt T2DM.

Conclusions. Our data suggest that the soleus, a muscle that depends largely on mitochondrial oxidative phosphorylation for energy production, but not the EDL can activate compensatory mechanisms in early diabetes. However, this counter-regulation becomes insufficient after a more extended exposure of the muscle to nutrient excess (34).

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DISCLOSURES

No conflict of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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