A Possible Link Between Skeletal Muscle Mitochondrial Efficiency and Age-Induced Insulin Resistance

Susanna Iossa, Maria Pina Mollica, Lillà Lionetti, Raffaella Crescenzo, Rosaria Tasso, and Giovanna Liverini

The transition from young to adult age is associated with decreased insulin sensitivity. To investigate whether changes in skeletal muscle mitochondrial function could be involved in the development of insulin resistance, we measured the oxidative capacity and energetic efficiency of subsarcolemmal and intermyofibrillar mitochondria isolated from the skeletal muscle of 60- and 180-day-old rats. Mitochondrial efficiency was tested by measuring the degree of thermodynamic coupling and optimal thermodynamic efficiency, as well as mitochondrial proton leak, which was determined in both the absence (basal) and the presence (fatty acid induced) of palmitate. Serum glucose, insulin, and HOMA index were also measured. The results show that in adult rats, concomitant with increased HOMA index, skeletal muscle mitochondria display higher respiratory capacity and energy efficiency. In fact, thermodynamic coupling and optimal thermodynamic efficiency significantly increased and fatty acid–induced proton leak was significantly lower in the skeletal muscle mitochondria from adult than in younger rats. A deleterious consequence of increased mitochondrial efficiency would be a reduced utilization of energy substrates, especially fatty acids, leading to intracellular triglyceride accumulation and lipotoxicity, thus contributing to the onset of skeletal muscle insulin resistance. Diabetes 53: 2861–2866, 2004

Increasing age is associated with obesity and insulin resistance not only in humans but also in rodents (1–5). In fact, ad libitum–fed caged rats are a good animal model that simulates the situation (sedentary lifestyle and unrestricted diet) of people in the western world.

It has been previously shown that age-induced insulin resistance in rats is already detectable at 4 months of age, concomitant to a rapid rise in fat mass, and does not further increase later in life (3). This early decrease in insulin sensitivity could in turn lead to the metabolic disturbance typical of old age.

Our previous results also indicate that in the transition from postpubertal (60 days) to adult (180 days) age, rats display reduced sensitivity to the anorexic effect of leptin (6) and, at the cellular level, hepatic mitochondrial machinery shows some age-related biochemical impairment (7,8). From all the above findings, it can be deduced that adult rats can be considered a good experimental tool for studying the onset of the “aging” phenomenon.

It has been postulated that defects in mitochondrial performance could contribute to the development of insulin resistance (9), and mitochondrial oxidative capacity has been considered a good predictor of insulin sensitivity (10). These suggestions are not surprising since control of mitochondrial respiration and oxidative phosphorylation is fundamental for the maintenance of cellular homeostasis. The above control is exerted at two levels, i.e., regulation of the rate of oxygen consumption and ATP production and regulation of oxidative phosphorylation efficiency (11). The main determinant of oxidative phosphorylation efficiency is represented by the degree of coupling between oxygen consumption and ATP synthesis, which is always lower than 1 and can vary according to the metabolic needs of the cell (12). Among the factors that affect mitochondrial degree of coupling, the permeability of the mitochondrial inner membrane to H⁺ ions (leak) plays an important role. It is now well known that the mitochondrial inner membrane exhibits a basal proton leak pathway whose contribution to basal metabolic rate in rats has been estimated to be −20–25% (13). In addition, it is well known that fatty acids can act as natural uncouplers of oxidative phosphorylation by generating a fatty acid–dependent proton leak pathway (14–16), which is a function of the amount of unbound fatty acids in the cell.

Taking into account the above considerations, we considered it of interest to investigate mitochondrial performance and efficiency in 60- and 180-day-old rats. To this purpose, we measured basal and fatty acid–mediated proton leak and the parameters of thermodynamic coupling and efficiency in mitochondria isolated from skeletal muscle. In fact, skeletal muscle is the primary site of insulin action and is thus inherently linked to the development of whole-body insulin resistance (17). Analyses were carried out, taking into account that the skeletal muscle mitochondrial population is heterogeneous for localization, function, and regulation (18–21). In fact, mitochondria located beneath the sarcolemmal membrane (subsarcolemmal [SS]) exhibit lower respiratory rates than those located between the myofibrils (intermyofibrillar [IMF]), and aging has been shown to selectively affect...
IMF mitochondria in heart (22). A metabolic characterization of young and adult rats was obtained by measuring serum insulin, leptin, nonesterified fatty acid (NEFA), and glucose levels.

RESEARCH DESIGN AND METHODS
Two groups of eight male Wistar rats (Charles River, Calco, Como, Italy) aged 60 [young] and 180 [adult] days were used for this study. They were kept at 24°C under an artificial circadian 12-h light/12-h dark cycle, with ad libitum access to water and a standard stock diet (Mucedola 4RF21; Settimo Milanese, Milan, Italy). Treatment, housing, and killing met the guidelines of the Italian Health Ministry.

On the day they were killed and without any previous food deprivation, the rats were anesthetized by an injection of chloral hydrate (40 mg/100 g body wt i.p.) and blood was collected from the inferior cava vein. Immediately after blood collection, hind leg muscles were rapidly removed and used for the preparation of SS and IMF mitochondria (18), while the rat carcasses were used for determination of body composition (18).

Measurements of mitochondrial respiration parameters, thermodynamic coupling, and efficiency. Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, Yellow Springs, OH) at 30°C in a medium containing 30 mmol/l KCl, 6 mmol/l MgCl₂, 75 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l KH₂PO₄, pH 7.0, and 0.1% (wt/vol) fatty acid–free BSA. Substrates used were 10 mmol/l succinate plus 3.75 μmol/l rotenone, 10 mmol/l glutamate plus 2.5 mmol/l malate, or 40 μmol/l palmitoylCoA plus 2 mmol/l carnitine plus 2.5 mmol/l malate. Measurements were performed in the absence (state 4) and presence (state 3) of 0.6 mmol/l ADP. Respiratory control ratio (RCR) was calculated as the ratio between states 3 and 4.

The degree of thermodynamic coupling (q) was obtained by applying equation 11 by Cairns et al. (23).

\[
q = \frac{1 - (J_{o}^{\text{state 3}})/(J_{o}^{\text{state 4}})}{1 - (J_{s}^{\text{state 3}})/(J_{s}^{\text{state 4}})}
\]

where \(J_{o}\) represents oxygen consumption. \((J_{o})^{\text{state 3}}\) and \((J_{o})^{\text{state 4}}\) were measured as above using succinate plus rotenone in the presence of 2 μg/ml oligomycin or 1 μmol/l FCCP [carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone], respectively. The optimal thermodynamic efficiency of oxidative phosphorylation was calculated by using equation 13 by Cairns et al. (23).

\[
\eta = \frac{q^{2}}{1 + q^{2}}
\]

The adenine nucleotide translocase (ANT) content of SS and IMF mitochondria was determined by titrating state 3 respiration with increasing concentrations of carboxyatractyloside (24) in a medium containing 30 mmol/l KCl, 6 mmol/l MgCl₂, 75 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l KH₂PO₄, pH 7.0, and 0.1% (wt/vol) fatty acid–free BSA. Substrates and specific activity were measured polarographically with a Clark-type electrode (Yellow Springs Instruments) at 30°C and 0.5 mmol/l rotenone, 2 μmol/l oxaloacetate, 2 μmol/l malate, 2 μmol/l glycerophosphate, 2.5 μmol/l malate, 0.5 μmol/l dinitrophenol, 10 mmol/l Na-malate, and 75 mmol/l Hepes (pH 7.4) (25).

Measurements of basal proton leak kinetics. Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments) maintained at 30°C in a medium containing 30 mmol/l LiCl, 6 mmol/l MgCl₂, 75 mmol/l sucrose, 1 mmol/l EDTA, 20 mmol/l KH₂PO₄, pH 7.0, and 0.1% (wt/vol) fatty acid–free BSA. Titration of state 4 respiration was carried out by sequential additions of up to 5 mmol/l malonate in the presence of 10 mmol/l succinate, 3.75 μmol/l rotenone, 2 μg/ml oligomycin, 83.3 nmol/mg safranin O, and 80 ng/ml nigericin. Mitochondrial membrane potential recordings were performed in parallel with safranin O using a JASCO dual-wavelength spectrophotometer (511–533 nm) (26). The absorbance changes were transformed into mV membrane potential using the Nernst equation:

\[
\Delta \phi = 61 \text{ mV} \times \log \left( \frac{[\text{K}^{+}]_{\text{out}}}{[\text{K}^{+}]_{\text{in}}} \right).
\]

Calibration curves made for each rat provided the relationship between the absorption change caused by the addition of 3 μmol/l valinomycin and the calculated membrane potential.

Measurement of palmitate-induced proton leak kinetics. Mitochondrial membrane potential and oxygen consumption were measured as above in the presence of 10 mmol/l succinate, 3.75 μmol/l rotenone, 2 μg/ml oligomycin, 83.3 nmol/mg safranin O, and palmitate at a concentration of 45 μmol/l for SS mitochondria or 75 μmol/l for IMF mitochondria. Due to the presence of 0.1% BSA in the incubation medium, the above concentrations of palmitate correspond to 17 (for SS mitochondria) and 62 (for IMF mitochondria) nmol/l (9262 DIABETES, VOL. 53, NOVEMBER 2004)
with FAD-linked and lipid substrate tended to be higher. State 4 respiratory rates tended to be lower with all the substrates used in 180-day-old rats, although statistical significance was never reached. Consequently, a significant increase in RCR values was found in 180-day-old rats with all the substrates. In addition, ANT content significantly decreased, UCP3 protein content significantly increased, and no significant variation was found in COX specific activity in 180-day-old rats (Table 3).

The degree of thermodynamic coupling (q) and optimal thermodynamic efficiency (η) were calculated to allow evaluation of energetic efficiency of the mitochondrial populations from skeletal muscle. The results show that both SS and IMF mitochondria from 180-day-old rats exhibited higher q and η values compared with mitochondria from 60-day-old rats (Table 4).

Figures 1 and 2 illustrate titration of steady-state respiration rate as a function of mitochondrial membrane potential in SS and IMF skeletal muscle mitochondria. The above titration curves are an indirect measurement of proton leak, since steady-state oxygen consumption rate (i.e., proton efflux rate) in nonphosphorylating mitochondria is equivalent to the proton influx rate due to proton leak. The results show that no variation in basal proton leak was found in SS and IMF mitochondria from 180- compared with 60-day-old rats (Fig. 1). On the other hand, fatty acid–induced proton leak (Fig. 2) significantly decreased in SS and IMF mitochondria in 180- compared with 60-day-old rats.

**DISCUSSION**

This study reveals that, in adult rats, higher HOMA index is associated with an increase in oxidative capacity and energy efficiency in skeletal muscle mitochondria. Adult rats can be considered a good experimental tool for studying the onset of the aging phenomenon. In fact, we have found that at 180 days of age, rats display signs of age-induced metabolic disturbance, since they exhibit higher body weight and lipid mass, hyperinsulinemia, 

### TABLE 2
Respiratory capacities in SS skeletal muscle mitochondria from 60- and 180-day-old rats

<table>
<thead>
<tr>
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<th>60 days</th>
<th>180 days</th>
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<tbody>
<tr>
<td>FAD-linked substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>369.1 ± 9.5</td>
<td>539.3 ± 21.1*</td>
</tr>
<tr>
<td>State 4</td>
<td>85.3 ± 2.9</td>
<td>98.1 ± 2.9</td>
</tr>
<tr>
<td>RCR</td>
<td>4.3 ± 0.1</td>
<td>5.8 ± 0.2*</td>
</tr>
<tr>
<td>NAD-linked substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>257.0 ± 13.9</td>
<td>373.7 ± 20.0*</td>
</tr>
<tr>
<td>State 4</td>
<td>34.2 ± 3.2</td>
<td>35.1 ± 0.6</td>
</tr>
<tr>
<td>RCR</td>
<td>5.2 ± 0.1</td>
<td>10.7 ± 0.6*</td>
</tr>
<tr>
<td>Lipid substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>155.7 ± 10.9</td>
<td>248.1 ± 11.4*</td>
</tr>
<tr>
<td>State 4</td>
<td>30.4 ± 2.0</td>
<td>32.9 ± 1.4</td>
</tr>
<tr>
<td>RCR</td>
<td>5.2 ± 0.5</td>
<td>7.6 ± 0.3*</td>
</tr>
<tr>
<td>ANT content (nmol/mg)</td>
<td>5.33 ± 0.13</td>
<td>5.48 ± 0.15</td>
</tr>
<tr>
<td>COX-specific activity</td>
<td>7,029 ± 325</td>
<td>7,586 ± 450</td>
</tr>
<tr>
<td>UCP3 protein content</td>
<td>2,167.6 ± 258.6</td>
<td>4,882.6 ± 280.5*</td>
</tr>
</tbody>
</table>

Data are means ± SE of eight different rats. *P < 0.05 compared with 60-day-old rats. Substrate concentrations for states 3 and 4 respiration are FAD linked (10 mmol/l succinate + 3.75 μmol/l rotenone), NAD linked (10 mmol/l glutamate + 2.5 mmol/l malate), and lipid (40 μmol/l palmitoylCoA + 2 mmol/l carnitine + 2.5 mmol/l malate).

### TABLE 3
Respiratory capacities in IMF skeletal muscle mitochondria from 60- and 180-day-old rats

<table>
<thead>
<tr>
<th></th>
<th>60 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAD-linked substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>698.5 ± 24.5</td>
<td>748.6 ± 13.0</td>
</tr>
<tr>
<td>State 4</td>
<td>133.7 ± 6.0</td>
<td>121.4 ± 3.0</td>
</tr>
<tr>
<td>RCR</td>
<td>5.2 ± 0.1</td>
<td>6.2 ± 0.2*</td>
</tr>
<tr>
<td>NAD-linked substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>507.0 ± 17.5</td>
<td>638.8 ± 22.3*</td>
</tr>
<tr>
<td>State 4</td>
<td>40.4 ± 2.4</td>
<td>36.2 ± 0.8</td>
</tr>
<tr>
<td>RCR</td>
<td>13.0 ± 1.1</td>
<td>17.7 ± 0.7*</td>
</tr>
<tr>
<td>Lipid substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 3</td>
<td>283.1 ± 8.2</td>
<td>297.0 ± 11.8</td>
</tr>
<tr>
<td>State 4</td>
<td>34.6 ± 1.3</td>
<td>31.4 ± 1.2</td>
</tr>
<tr>
<td>RCR</td>
<td>8.2 ± 0.2</td>
<td>9.5 ± 0.3*</td>
</tr>
<tr>
<td>ANT content (nmol/mg)</td>
<td>3.85 ± 0.11</td>
<td>3.19 ± 0.12*</td>
</tr>
<tr>
<td>COX-specific activity</td>
<td>4,233 ± 256</td>
<td>4,130 ± 168</td>
</tr>
<tr>
<td>UCP3 protein content</td>
<td>1,738.1 ± 331.3</td>
<td>2,946.3 ± 940.8*</td>
</tr>
</tbody>
</table>

Data are means ± SE of eight different rats. *P < 0.05 compared with 60-day-old rats. Substrate concentrations for states 3 and 4 respiration are FAD linked (10 mmol/l succinate + 3.75 μmol/l rotenone), NAD linked (10 mmol/l glutamate + 2.5 mmol/l malate), and lipid (40 μmol/l palmitoylCoA + 2 mmol/l carnitine + 2.5 mmol/l malate).
hyperleptinemia, and a higher HOMA index. These meta-
bolic perturbations are in agreement with those previously
found by others. In fact, Barzilai and Rossetti (3) found
that an early fall in insulin responsiveness occurs in rats
between 2 and 4 months of age. Our present results also
show that, in adult rats, concomitantly with the above
metabolic perturbations, there is an increase in skeletal
muscle mitochondrial capacity and efficiency.

The generalized increase in state 3 oxidative capacity
found in SS mitochondria from 180-day-old rats suggests
an increase in one of the steps below complex II. The most
feasible target of the above increase should be at the level
of cytochromes and/or ATP synthetase, since COX activity

Previous results have shown that old rats display a
decrease in skeletal muscle mitochondrial activities (31–
33), and when SS and IMF mitochondria have been studied
separately in the heart, the above decrease was found to
be restricted to IMF organelles (22). In addition, we have
previously found that an aging-induced decline in hepatic
mitochondrial activity of complex II is already evident in
180-day-old rats (7), indicating that the liver is affected
early by the aging process. Our present results show that
skeletal muscle behaves very differently from liver and

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>SS</th>
<th>IMF</th>
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<tbody>
<tr>
<td></td>
<td>60 days</td>
<td>180 days</td>
</tr>
<tr>
<td>q</td>
<td>0.890 ± 0.003</td>
<td>0.916 ± 0.005*</td>
</tr>
<tr>
<td>η</td>
<td>0.374 ± 0.006</td>
<td>0.430 ± 0.013*</td>
</tr>
<tr>
<td>q</td>
<td>0.905 ± 0.002</td>
<td>0.925 ± 0.002*</td>
</tr>
<tr>
<td>η</td>
<td>0.403 ± 0.004</td>
<td>0.449 ± 0.006*</td>
</tr>
</tbody>
</table>

Data are means ± SE of eight rats. *P < 0.05 compared with
60-day-old rats.

FIG. 1. Kinetics of basal proton leak in SS (A) and IMF (B)
skeletal muscle mitochondria from 60- and 180-day-old rats. Results are
reported as means ± SE of eight different rats. Basal proton leak was not
significantly different in 180- compared with 60-day-old rats, as shown
by nonlinear regression curve fits.

FIG. 2. Kinetics of palmitate-induced proton leak in SS (A) and IMF (B)
skeletal muscle mitochondria from 60- and 180-day-old rats. Results are
reported as means ± SE of eight different rats. Palmitate-induced proton
leak was significantly (P < 0.05) lower in 180- compared with 60-day-old
rats, as shown by nonlinear regression curve fits.
that, at 180 days of age, oxidative capacity has not yet reached its maximum. In agreement, it has been found that complex II–IV activity of skeletal muscle mitochondria peaks in middle life (1 year) and then declines with increasing age (32).

As for mitochondrial efficiency, both mitochondrial populations display an increase in the degree of thermodynamic coupling and optimal thermodynamic efficiency as well as a decreased fatty acid–dependent proton leak. For IMF mitochondria, decreased fatty acid–dependent proton leak is in agreement with the decreased ANT content of this mitochondrial population, while in SS mitochondria, the reduced sensitivity to palmitate is probably due to the other carrier systems involved, since their ANT content was not affected. Our results also underline the dissociation between regulation of mitochondrial fatty acid–dependent proton leak and UCP3 levels. In fact, the increased UCP3 protein content of SS and IMF mitochondria from 180-day-old rats is in contrast with the lower fatty acid–dependent leak but is consistent with the increased serum NEFA levels, which have been shown to be closely linked to changes in UCP3 protein expression (34).

A number of recent human studies have focused the attention on the mitochondrial compartment as a cellular site involved in the pathogenesis of insulin resistance. In fact, mitochondrial dysfunction has been found in old men (9) and insulin-resistant young subjects (35). In addition, a significant direct relationship between skeletal muscle oxidative capacity and insulin sensitivity has been found (10). An alternative way to reduce mitochondrial oxidation of energy substrates is to increase the efficiency of oxidative phosphorylation. In fact, a higher efficiency implies that less substrate needs to be burned to obtain ATP. Since NEFA serum levels are significantly higher in adult rats, a deleterious consequence of reduced substrate burning could be intracellular triglyceride accumulation and lipotoxicity. The above consequence is of relevance because one of the main causes leading to insulin resistance in skeletal muscle is intramuscular triglyceride deposition (17,36). In addition, skeletal muscle is the primary site of insulin action and is thus inherently linked to the development of whole-body insulin resistance (17).

The increased mitochondrial efficiency and resulting lower thermogenesis in 180-day-old rats could be due to a reduced sensitivity of skeletal muscle cell to leptin. In fact, it has been shown that leptin directly stimulates thermogenesis in skeletal muscle (37) and that 180-day-old rats display age-induced leptin resistance (6). Therefore, leptin resistance could be regarded as one of the primary events leading to insulin resistance.

In conclusion, our present results show that although at 180 days of age no clear biochemical impairment of mitochondrial machinery can be detected, an increased efficiency in the production of ATP takes place. The impact of this modification on cellular metabolism could contribute to the onset of skeletal muscle insulin resistance.

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REFERENCES


