Reduced Mitochondrial Oxidative Capacity and Increased Mitochondrial Uncoupling Impair Myocardial Energetics in Obesity

Sihem Boudina, PhD; Sandra Sena, PhD; Brian T. O’Neill, BS; Prakash Tathireddy, BS; Martin E. Young, DPhil; E. Dale Abel, MB, BS, DPhil

Background—Obesity is a risk factor for cardiovascular disease and is strongly associated with insulin resistance and type 2 diabetes. Recent studies in obese humans and animals demonstrated increased myocardial oxygen consumption (MV\textsubscript{O2}) and reduced cardiac efficiency (CE); however, the underlying mechanisms remain unclear. The present study was performed to determine whether mitochondrial dysfunction and uncoupling are responsible for reduced cardiac performance and efficiency in ob/ob mice.

Methods and Results—Cardiac function, MV\textsubscript{O2}, mitochondrial respiration, and ATP synthesis were measured in 9-week-old ob/ob and control mouse hearts. Contractile function and MV\textsubscript{O2} in glucose-perfused ob/ob hearts were similar to controls under basal conditions but were reduced under high workload. Perfusion of ob/ob hearts with glucose and palmitate increased MV\textsubscript{O2} and reduced CE by 23% under basal conditions, and CE remained impaired at high workload. In glucose-perfused ob/ob hearts, mitochondrial state 3 respirations were reduced but ATP/O ratios were unchanged. In contrast, state 3 respiration rates were similar in ob/ob and control mitochondria from hearts perfused with palmitate and glucose, but ATP synthesis rates and ATP/O ratios were significantly reduced in ob/ob, which suggests increased mitochondrial uncoupling. Pyruvate dehydrogenase activity and protein levels of complexes I, III, and V were reduced in obese mice.

Conclusions—These data indicate that reduced mitochondrial oxidative capacity may contribute to cardiac dysfunction in ob/ob mice. Moreover, fatty acid but not glucose-induced mitochondrial uncoupling reduces CE in obese mice by limiting ATP production and increasing MV\textsubscript{O2}. (Circulation. 2005;112:2686-2695.)

Key Words: obesity ■ metabolism ■ energetics ■ fatty acids ■ mitochondria

The prevalence of obesity is increasing in the United States, with approximately one third of the adult population being reported as obese. Obesity is an independent risk factor for the development of heart failure. It has been proposed that a mismatch between fatty acid (FA) uptake and FA oxidation may result in intramyocardial accumulation of lipids. This may induce lipotoxic injury and cardiac dysfunction via a number of mechanisms, including ceramide-activated apoptosis. Moreover, an association between increased lipid accumulation and diastolic dysfunction (with preserved systolic function) has been reported in hearts isolated from ob/ob mice, which are a model of severe obesity, and myocardial lipid accumulation has been associated with left ventricular hypertrophy and impaired septal contractility in humans with obesity.
ment of db/db mice with peroxisome proliferator-activated receptor (PPAR)-α and -γ agonists failed to restore cardiac function.9,10 Thus, additional defects are likely to persist in the hearts of genetically obese and diabetic mice that cannot be reversed by short-term normalization of systemic metabolic abnormalities.

A potential mechanism for increased oxygen consumption and increased FA utilization in the hearts of obese animals is mitochondrial uncoupling. Although streptozotocin diabetes has been shown to be associated with increased expression of uncoupling protein-3 (UCP3) mRNA in the heart,11 no studies have directly evaluated whether mitochondrial uncoupling directly contributes to myocardial dysfunction in either diabetes or obesity. Second, increased FA concentrations have been associated with increased expression of UCP3 in skeletal muscle and cardiac muscle,12,13 but whether or not increased FA delivery uncouples mitochondria in the heart in obesity remains to be established definitively.

The present studies were undertaken to test the hypothesis that abnormal substrate metabolism and contractile dysfunction in the hearts of obese mice are associated with or result from impaired mitochondrial energetics. We demonstrate that multiple mitochondrial defects impair ATP generation in the hearts of obese animals, and mitochondrial energetics is further impaired by FA-induced uncoupling.

**Methods**

**Animals**

Nine week-old male ob/ob mice on the C57BL/6J background and their lean C57BL/6J controls were obtained from the Jackson Laboratory (Bar Harbor, Maine). Mice were housed in conventional cages and maintained with a 12-hour light/12-hour dark photoperiod in humidity- and temperature-controlled rooms with free access to water and food. Experimental procedures in animals were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Utah.

**Heart Perfusion**

Mice were anesthetized by intraperitoneal injection of 15 mg of chloral hydrate, and the heart was rapidly excised and arrested in ice-cold buffer. The aorta was then cannulated and retrogradely perfused at constant pressure of 60 mm Hg with 37°C Krebs buffer containing (in mmol/L): K 2EGTA 7.23, K CaEGTA 2.77, MgCl 6.56, imidazole 20, dithiothreitol 0.5, K-methanS 53.3, taurine 20, Na,A TP 5.3, PCr 15, and KH2PO4 3, pH 7.1 adjusted at 25°C. The fibers were then washed twice for 10 minutes in buffer B containing (in mmol/L) K EGTA 7.23, K CaEGTA 2.77, MgCl 1.38, imidazole 20, dithiothreitol 0.5, K-methanS 100, taurine 20, KH2PO4 3, and BSA 2 mg/mL, pH 7.1 at 25°C.

**Respiration and ATP Measurements**

The respiratory rates of saponin-permeabilized fibers were determined with the same oxygen sensor probe used for the MV2 measurements in 2 mL of buffer B at 25°C with continuous stirring. Studies were performed with 3 independent substrates (in mmol/L): (1) glutamate 5 and malate 2, (2) pyruvate 10 and malate 5, or (3) palmitoyl-carnitine 0.02 and malate 2. The solubility of oxygen in buffer B is 215 nmol of O2 per mL at 25°C. Oxygen consumption rates were expressed as nmol of O2 - min-1 · mg dry fiber weight-1. Respiratory parameters were defined as follows. Basal respiration rates before the addition of ADP (V0) were defined at state 2. Maximal ADP (1 mmol/L)-stimulated respiration rates (VADP) were defined as state 3, and respiration rates in the absence of ADP phosphorylation and measured in the presence of 1 µg/mL oligomycin (VADP). Were termed state 4. This nomenclature has been used in prior studies15–17 but differs from other studies in which state 4 respirations were measured either in the absence of added ADP or after a period of time during which ADP was believed to be depleted. ATP concentration was determined by a bioluminescence assay based on the luciferin/luciferase reaction with the ATP assay kit (ThermoLabsystems).

**Determination of Mitochondrial Enzyme Activities**

Total carnitine palmitoyl-transferase (CPT; CPT 1+CPT 2) activity was measured in mitochondria that were isolated as described previously.10 Mitochondria (~200 µg) were assayed in 1 mL of reaction buffer containing (in mmol/L) HEPES 20, EGTA 1, sucrose 220, KCl 40, 5,5’-dithio-bis (2-nitrobenzoic acid) (DTNB) 0.1, BSA 1.3 mg/mL, and palmitoyl-CoA 40 µmol/L, pH 7.4 at 25°C. The reaction was started by adding 1 mmol/L carnitine and was monitored at 412 nm for 4 minutes with an Ultrospec 3000 spectrophotometer. CPT 1 activity was measured with the same reaction as total CPT but after addition of malonyl-CoA (10 µmol/L), which completely inhibits CPT 1 activity. CPT 1 activity was calculated by subtracting CPT 2 activity from total CPT activity. Citrate synthase (CS) activity was assessed in frozen cardiac tissue (~10 mg). Hearts were homogenized on ice in 20% (wt/vol) homogenization buffer containing (in mmol/L) HEPES 20, EDTA 10, pH 7.4. The homogenates were then frozen for 1 hour to liberate CS from mitochondrial matrix and were then diluted 1:10. The reaction was performed in 1 mL of reaction buffer containing (in mmol/L) HEPES 20, EGTA 1, sucrose 220, KCl 40, DTNB 0.1, and acetyl-CoA 0.1 µmol/L, pH 7.4 at 25°C, and was started by the addition of 0.05 mmol/L oxaloacetate and finally monitored at 412 nm for 3 minutes with an Ultrospec 3000 spectrophotometer. β-Hydroxyacyl-CoA dehydrogenase activity was measured in the same homogenate as used for CS assay but diluted 1:4. The reaction was performed in 1 mL of reaction buffer containing (in mmol/L) HEPES 20, EGTA 1, KClN 1, and NADH 0.15, pH 7.4 at 25°C. The reaction was started by the addition of 0.1 mmol/L acetoacetyl-CoA and monitored at 340 nm for 4 minutes with an Ultrospec 3000 spectrophotometer. Pyruvate dehydrogenase
(PDH) activity was assayed as described before19 with a radioactiv-

enzyme method. Frozen heart tissue was homogenized in a buffer

containing (in mmol/L) HEPES 25 (pH 7.4), EDTA 3, sodium

dichloroacetate 5, N-α-tosyl-L-lysylchloromethane 1, ADP 1, dithio-

threitol 5, CaCl2 2, MgCl2 5, and potassium fluoride (KF) 25,

supplemented with 1.5% Triton X-100 and 5 μmol/L leupeptin. For
determination of the total fraction of the PDH (PDHt), the HEPES
formed, with the percentage of active PDH calculated with the

termination of the reaction by addition of 2 mol/L acetic acid in 2%

GNoms and the KF was omitted from the homogenization buffer to allow for PDH dephosphorylation

domulation of the total fraction of the PDH (PDHt), the HEPES

centrifuged for 1 hour at 4°C at 47 000 rpm. For
centrifuged for 15 minutes at 9000 rpm at 4°C. The supernatant was

samples were then

sodium orthovanadate 10 supplemented with 1% Triton X-100 and

Polytron in sample buffer containing (in mmol/L) HEPES 30, pH7.4,

cationic ATPase activity was determined by adding 10 mmol/L

(Ca2+), CaCl2 2, MgCl2 5, and KF 25, supplemented with 1.5%

Triton X-100 and 5 μmol/L leupeptin. For
determination of the total fraction of the PDH (PDHt), the HEPES

centrifuged for 10 minutes at 8000
g at 4°C. The corresponding

mitochondrial pellets were then suspended in 50
mL of sample

buffer. Protein concentration was measured with Micro BCA reagent

ersham Biosciences).

formed with the appropriate horseradish peroxidase–conjugated

secondary antibody and ECL or ECL Plus detection systems (Am-

ersham Biosciences).

Statistical Analysis

Data are presented as mean±SE. Significance (P<0.05) was deter-
mimed by ANOVA followed by Fisher’s least protected squares test.
Statistical calculations were performed with the Statview 5.0.1
software package (SAS Institute).

Results

As previously described, body weights were increased in

ob/ob mice (50.62±0.92 versus 23.09±0.37 g; P<0.00001).

We also have reported previously that these mice are hyper-

insulinemic and exhibit impaired glucose tolerance but are

not diabetic.6 Wet and dry heart weights were 13% and 20%

(P<0.001) higher in ob/ob mice than in wild-type controls,

which is consistent with previous reports of cardiac hyper-

trophy in these mice. We hypothesized that FA specifically

contributes to changes in the bioenergetics and oxygen

consumption in ob/ob hearts and mitochondria. We therefore

initially determined cardiac performance and MV O2, as well

as mitochondrial function, in the absence of FA by examining

these parameters in hearts perfused with glucose as sole

substrate. Subsequent analysis after perfusion of hearts with

glucose plus palmitate then allowed us to ascertain the

specific additive effects of FA exposure.

Cardiac Function in Glucose-Perfused Hearts

Basal cardiac function was similar in ob/ob and controls

perfused with glucose only (Table 1). The relationship

between MVO2 and rate pressure product (RPP) was then

investigated at different workload conditions. Increasing
calcium in the perfusate caused a 2.2-, 2.1-, and 1.9-fold

increase in left ventricular developed pressure (LVDP), RPP,

and MVO2, respectively, in controls. In contrast, the inotropic

TABLE 1. Contractility, MVO2, and CE in Glucose-Perfused Hearts at Baseline and After Calcium-Induced

Workload Increase

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (n=10)</th>
<th>ob/ob (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Workload</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>362.1±2.1</td>
<td>347.6±9.9</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>67.8±4.1</td>
<td>133.67±11.8</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>11.7±1.2</td>
<td>8.6±0.9</td>
</tr>
<tr>
<td>Developed pressure, mm Hg</td>
<td>56.1±4.2</td>
<td>125.11±3.3</td>
</tr>
<tr>
<td>RPP, mm Hg X bpm</td>
<td>20 307.4±1475.3</td>
<td>42 658.2±2725.8</td>
</tr>
<tr>
<td>dP/dtmax, mm Hg · s−1</td>
<td>−2249.3±97.2</td>
<td>−3175.3±229.3</td>
</tr>
<tr>
<td>dP/dtmin, mm Hg · s−1</td>
<td>2734.7±136.8</td>
<td>4465.9±341.4</td>
</tr>
<tr>
<td>MVO2, μmol · min−1 · g−1</td>
<td>22.1±1.8</td>
<td>41.8±2.6</td>
</tr>
<tr>
<td>CE, %</td>
<td>28.2±2</td>
<td>30.6±1.6</td>
</tr>
</tbody>
</table>

Values are mean±SE. Baseline corresponds to 2 mmol/L calcium concentration in the perfusate; workload corresponds to 4
mmol/L calcium concentration in the perfusion. Hearts were paced at 6 Hz.

*P<0.05; †P<0.005 vs wild type under equivalent workload conditions; ‡P<0.05; §P<0.005 vs baseline.
Relative to glucose-perfused control hearts, MVO$_2$ increased by 18% in palmitate-perfused controls. This contrasts with ob/ob hearts in which MVO$_2$ increased by 62% relative to glucose-perfused ob/ob hearts. Inotropic responses in wild-type hearts perfused with glucose and palmitate were similar to changes in glucose-perfused control hearts. Thus, calcium stimulation resulted in a 2.3-fold increase in LVDP and RPP, which was associated with doubling of MVO$_2$ (Table 2; Figure 1B). In ob/ob hearts perfused with glucose and palmitate, the inotropic response to increased calcium was greater than that observed in glucose-perfused ob/ob hearts (1.96- versus 1.5-fold increase in RPP), but peak RPP remained lower in palmitate-perfused ob/ob hearts than in wild-type hearts (Table 2). In contrast to glucose-perfused hearts, MVO$_2$ in palmitate-perfused ob/ob hearts increased by 55%, and MVO$_2$ was equivalent to wild-type hearts at high workload (50.1 ± 4.1 versus 52.1 ± 1.4 μmol · min$^{-1}$ · g$^{-1}$). The relationship between RPP and MVO$_2$ was similar in wild-type hearts perfused with or without palmitate, but the relationship was shifted to the left in ob/ob hearts perfused with glucose and palmitate so that any given RPP was associated with greater oxygen utilization (Figure 1B). Thus, CE was reduced significantly in ob/ob hearts perfused with glucose and palmitate both at baseline and after inotropic stimulation (Table 2).

**Mitochondrial Function in Glucose-Perfused Hearts**

To test the hypothesis that mitochondrial dysfunction contributed to reduced oxygen consumption in glucose-perfused ob/ob hearts after calcium stress, mitochondrial respiration and ATP synthesis rates were measured in saponin-permeabilized cardiac fibers isolated from glucose-perfused hearts at [2 mmol/L] Ca$^{2+}$. Experiments were performed with 3 different substrates to isolate potential defects in the tricarboxylic acid (TCA) cycle and electron transport flux with glutamate, in carbohydrate-dependent flux with pyruvate, and in the carnitine shuttle and/or β-oxidation with palmitoyl-carnitine. Permeabilized fibers that were obtained from ob/ob hearts perfused with glucose as sole substrate exhibited reduced mitochondrial respiration. In glutamate-treated fibers, state 3 and state 4 (Vogomycin) mitochondrial respirations were reduced by 39% (Figure 2). ATP synthesis and ATP/O ratios were unchanged. State 3 mitochondrial respirations were also reduced in ob/ob fibers incubated with palmitoyl-carnitine (20% reduction, P < 0.05 versus controls), but the ATP synthesis rates and ATP/O ratios were again similar to controls (Figure 3). The most pronounced changes in mitochondrial function were obtained with pyruvate. State 2 respirations (V$_b$) were reduced by 23% in ob/ob mice compared with controls (P < 0.05). State 3 (V$_{ATP}$) and state 4 (V$_{digermycin}$) respiration rates were also reduced by 29% and 33% in ob/ob mice versus controls (Figure 4). In contrast to glutamate- and palmitoyl-carnitine–exposed fibers, ATP production was depressed by 35% in obese animals (40.1 ± 5.33 versus 61.65 ± 5.02 nmol ATP · min$^{-1}$ · mg dry weight$^{-1}$). Because ATP and oxygen consumption rates were reduced proportionally, the ATP/O ratios were not different from controls.
Mitochondrial Function in Glucose- and Palmitate-Perfused Hearts

Mitochondrial analyses were repeated in fibers obtained from hearts that were perfused with glucose and palmitate at [2 mmol/L] Ca\(^{2+}\)/H\(_{100}\) to test the hypothesis that FA-induced mitochondrial uncoupling would further impair mitochondrial function in the hearts of ob/ob mice. Compared with glucose-perfused wild-type hearts, state 3 mitochondrial respiration rates and ATP production declined in permeabilized fibers obtained from palmitate-and-glucose–perfused wild-type hearts exposed to glutamate and pyruvate as substrates (Figures 2 and 4, respectively). These changes were most pronounced with pyruvate, for which state 3 was 19.77 ± 1.78 in glucose-perfused wild-type hearts versus 15.08 ± 0.89 nmol O\(_2\) · min\(^{-1}\)· mg dry weight\(^{-1}\) (P < 0.05) in glucose-and-palmitate–perfused wild-type hearts (Figure 4). ATP production and state 3 respiration rates were proportionally reduced, and thus, the ATP/O ratios were not different in glucose-

Table 2. Hemodynamics, MV\(_{\text{O}2}\), and CE in Glucose-and-Palmitate–Perfused Hearts at Baseline and After Calcium-Induced Workload Increase

<table>
<thead>
<tr>
<th></th>
<th>Wild Type Baseline (n=4)</th>
<th>Workload (n=4)</th>
<th>ob/ob Baseline (n=4)</th>
<th>Workload (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>363.1 ± 1.9</td>
<td>364.2 ± 1.1</td>
<td>362 ± 0.5</td>
<td>365.9 ± 1</td>
</tr>
<tr>
<td>Systolic blood</td>
<td>61.8 ± 1.4</td>
<td>132.7 ± 5.7</td>
<td>58.9 ± 1</td>
<td>107 ± 6.8‡§</td>
</tr>
<tr>
<td>pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diastolic blood</td>
<td>7.4 ± 1</td>
<td>5.4 ± 0.3</td>
<td>6.8 ± 0.8</td>
<td>5.6 ± 0.9</td>
</tr>
<tr>
<td>pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Developed blood</td>
<td>54.4 ± 0.8</td>
<td>127.4 ± 5.9§</td>
<td>52.1 ± 0.9</td>
<td>101.4 ± 7.5§</td>
</tr>
<tr>
<td>pressure, mm Hg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPP, mm Hg×bpm</td>
<td>19,754.4 ± 327.3</td>
<td>46,368.1 ± 2025.2§</td>
<td>18,873.2 ± 325</td>
<td>37,100.9 ± 2756.5§</td>
</tr>
<tr>
<td>dP/dt(_{\text{max}}), mm Hg · s(^{-1})</td>
<td>-2499.5 ± 104.9</td>
<td>-4129 ± 158.8§</td>
<td>-2362.5 ± 86.6</td>
<td>-3426.8 ± 134.3§</td>
</tr>
<tr>
<td>dP/dt(_{\text{max}}), mm Hg · s(^{-1})</td>
<td>2894 ± 173.7</td>
<td>5018.8 ± 552.2§</td>
<td>2602.8 ± 101.5</td>
<td>3718 ± 134.1‡</td>
</tr>
<tr>
<td>MV(_{\text{O}2}),</td>
<td>26.2 ± 0.4</td>
<td>52.1 ± 1.4§</td>
<td>32.3 ± 1.2</td>
<td>50.1 ± 4.1§</td>
</tr>
<tr>
<td>μmol · min(^{-1}) · g(^{-1})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CE, %</td>
<td>22.4 ± 0.5</td>
<td>26.4 ± 1‡</td>
<td>17.4 ± 0.8‡</td>
<td>22.1 ± 1.2‡§</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Baseline corresponds to 2 mmol/L calcium concentration in the perfusate. Hearts were paced at 6 Hz.

*P < 0.05; †P < 0.005 vs wild type under equivalent workload conditions; ‡ P < 0.05; § P < 0.005 vs baseline.

Mitochondrial Function in Glucose- and Palmitate-Perfused Hearts

Mitochondrial analyses were repeated in fibers obtained from hearts that were perfused with glucose and palmitate at [2 mmol/L] Ca\(^{2+}\)/H\(_{100}\) to test the hypothesis that FA-induced mitochondrial uncoupling would further impair mitochondrial function in the hearts of ob/ob mice. Compared with glucose-perfused wild-type hearts, state 3 mitochondrial respiration rates and ATP production declined in permeabilized fibers obtained from palmitate-and-glucose–perfused wild-type hearts exposed to glutamate and pyruvate as substrates (Figures 2 and 4, respectively). These changes were most pronounced with pyruvate, for which state 3 was 19.77 ± 1.78 in glucose-perfused wild-type hearts versus 15.08 ± 0.89 nmol O\(_2\) · min\(^{-1}\)· mg dry weight\(^{-1}\) (P < 0.05) in glucose-and-palmitate–perfused wild-type hearts (Figure 4). ATP production and state 3 respiration rates were proportionally reduced, and thus, the ATP/O ratios were not different in glucose-

Figure 2. Mitochondrial respiratory parameters, ATP synthesis rates, and ATP/O ratios obtained from permeabilized fibers incubated with glutamate-malate. Open bars represent fibers obtained from glucose-perfused wild-type (WT) hearts (n=4); black bars, fibers obtained from glucose-perfused ob/ob hearts (n=4); diagonal cross-hatched bars, fibers obtained from glucose-and-palmitate–perfused wild-type hearts (n=4); and horizontal cross-hatched bars, fibers obtained from glucose-and-palmitate–perfused ob/ob hearts (n=4). State 2 (V\(_{\text{st}}\)) indicates respiration in the absence of ADP; state 3 (V\(_{\text{adp}}\)), ADP (1 mmol/L)-stimulated respiration; state 4 (V\(_{\text{oligomycin}}\)), oligomycin (1 μg/mL)-inhibited respiration; and RC, respiratory control ratio. Values are shown as mean ± SE. *P < 0.05, **P < 0.005 vs glucose-only wild type; † P < 0.05 vs glucose-and-palmitate–perfused wild type. ATP synthesis rates were significantly lower in glucose-and-palmitate ob/ob fibers than in similarly perfused wild-type fibers by unpaired t test (P < 0.05). mgdw indicates milligrams of dry weight.
versus glucose-and-palmitate–perfused wild-type hearts. In contrast, state 3 respirations tended to increase in ob/ob mitochondria obtained from palmitate-and-glucose–perfused hearts relative to ob/ob mitochondria obtained from hearts that were perfused with glucose alone. Thus, the reduction in respiration rates in ob/ob mitochondria that was apparent in glucose-perfused hearts was no longer seen (Figures 2, 3, and 4). State 4 (V_{oligomycin}) respirations that reflect uncoupled...
respiration were increased significantly in mitochondria obtained from ob/ob hearts when incubated with palmitoyl-carnitine \((P<0.05)\), and although a similar trend was observed in controls, that difference did not achieve statistical significance \((P=0.08)\). ATP levels were significantly lower in ob/ob mitochondria exposed to pyruvate and palmitoyl carnitine, and the calculated ATP/O ratios \((\text{ATP synthesis rates/state 3 respiration})\) were profoundly reduced in the ob/ob mitochondria obtained from hearts perfused with both glucose and palmitate relative to similarly perfused wild-type hearts.

**Mitochondrial Enzyme Activities and Protein Expression**

To elucidate the mechanisms responsible for diminished mitochondrial respiration in glucose-perfused ob/ob hearts, the activity of key enzymes involved in glucose oxidation and FA transport and oxidation, as well as the expression of various mitochondrial proteins, was examined. All assays, with the exception of UCP3 immunoblots, were performed in fresh hearts after rapid removal from deeply anesthetized mice. UCP3 immunoblots were performed on mitochondria that were isolated from hearts that were perfused with 1 mmol/L palmitate and 11 mmol/L glucose. As summarized in Table 3, total CPT, CPT 1, and CPT 2 activities were not different between ob/ob and control mice. However, both CS and \(\beta\)-hydroxyacyl-CoA dehydrogenase activities were slightly increased by 10% \((P=0.08)\) and 14% \((P=0.08)\), respectively, in obese mice. The active fraction of PDH was 21% lower in ob/ob animals than in control animals \((1.23\pm0.08\, \text{versus } 1.52\pm0.07\text{ mol} \cdot \text{min}^{-1} \cdot \text{mg tissue}^{-1} ; P<0.05)\), but total PDH activity was similar between the 2 groups. Complex I levels were reduced by 57%, and complex III and the \(\alpha\)-subunit of the ATP synthase were reduced by 30% and 33%, respectively, in ob/ob mice (Figure 5A). To evaluate whether increased expression of uncoupling proteins contributed to the enhancement of FA-induced uncoupling observed in ob/ob mitochondria, we examined the protein content of UCP2 and UCP3. UCP2 protein was undetectable in the hearts of both controls and ob/ob mice (data not shown). UCP3 levels were unchanged in ob/ob hearts compared with their age-matched controls (Figure 5B).

**Discussion**

This study demonstrated that obesity is associated with specific alterations in mitochondrial oxidative capacity and the coupling of oxygen consumption and ATP production.

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**Table 3. Mitochondrial Enzyme Activities**

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>ob/ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CPT, (\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}) ((n=4–5))</td>
<td>3.4±0.15</td>
<td>3.5±0.08</td>
</tr>
<tr>
<td>CPT 1, (\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}) ((n=4–5))</td>
<td>2.1±0.14</td>
<td>2.1±0.12</td>
</tr>
<tr>
<td>CPT 2, (\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}) ((n=4–5))</td>
<td>1.3±0.17</td>
<td>1.4±0.16</td>
</tr>
<tr>
<td>CS, (\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}) ((n=4–5))</td>
<td>110.9±3.85</td>
<td>122.7±9.32</td>
</tr>
<tr>
<td>HOAD, (\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}) ((n=4–5))</td>
<td>5.2±0.48</td>
<td>5.99±0.6</td>
</tr>
<tr>
<td>Total PDH, nmol (\cdot) \text{min}^{-1} \cdot \text{mg tissue}^{-1} ((n=9))</td>
<td>2.4±0.09</td>
<td>2.37±0.1</td>
</tr>
<tr>
<td>Active PDH, nmol (\cdot) \text{min}^{-1} \cdot \text{mg tissue}^{-1} ((n=9))</td>
<td>1.54±0.07</td>
<td>1.23±0.08*</td>
</tr>
<tr>
<td>% PDH active ((n=9))</td>
<td>64.23±2.29</td>
<td>52.24±3.42*</td>
</tr>
</tbody>
</table>

*Total CPT, CPTI, CPTII, CS, 3-hydroxyacylCoA dehydrogenase, and PDH activities are presented. Values are mean±SE.

*\(P<0.05\) vs wild-type.

![Figure 5](http://circ.ahajournals.org/figure5.png)
We found that mitochondria from glucose-perfused ob/ob hearts revealed marked impairments in respiratory capacity that were most pronounced in the presence of pyruvate and also correlated with decreased PDH activity. As a consequence, glucose-perfused ob/ob hearts were severely limited in their ability to increase MVo_{2} in response to increased workload when glucose was the sole substrate. This contrasts with the responses of ob/ob hearts in the presence of palmitate, in which MVo_{2} rose significantly at high workload. We therefore believe that the limitation in inotropic oxygen consumption in glucose-perfused hearts reflects impaired substrate flux via PDH and reduced oxidative capacity of mitochondria that is also due in part to depletion of key mitochondrial electron transport chain components. In contrast, in hearts perfused with glucose and palmitate, ob/ob hearts exhibited increased MVo_{2} under basal conditions in the absence of a concomitant increase in cardiac performance, which leads to reduced cardiac efficiency. Moreover, in response to an inotropic challenge, MVo_{2} increased further, but CE remained low. Furthermore, FA impaired the coupling of oxygen consumption and ATP generation in ob/ob mitochondria. Thus, the glucose-only perfusions highlighted the presence of global defects in mitochondrial respiratory capacity in ob/ob hearts, and the studies performed after glucose and palmitate perfusion revealed an additional defect, namely, FA-induced mitochondrial uncoupling. FAs therefore preferentially induce mitochondrial uncoupling in obese mouse hearts that is characterized by reduced ATP production that is not accompanied by a proportional reduction in oxygen consumption.

We have previously shown that in isolated working hearts perfused with palmitate and glucose, ob/ob mouse hearts demonstrated elevated rates of FA oxidation and MVo_{2} relative to controls and that increasing the FA concentrations caused a progressive rise in MVo_{2} in ob/ob but not in control hearts.6 These observations led us to hypothesize that FA preferentially uncoupled mitochondria from ob/ob mouse hearts. We therefore elected to initially study hearts that were perfused with glucose as the sole substrate to determine the phenotype of ob/ob hearts and their mitochondria in the absence of the potential contribution of exogenous FA-induced uncoupling. Intriguingly, we observed no difference in MVo_{2} in glucose-perfused ob/ob and wild-type hearts; however, their mitochondria were characterized by reduced rates of mitochondrial respiration and ATP generation, particularly in the presence of pyruvate as a substrate. We therefore reasoned that this reduction in mitochondrial function might reflect a limitation at the level of PDH. This was indeed confirmed by direct analysis of the active fraction of PDH, the activity levels of which were reduced in ob/ob hearts. To further demonstrate that limited PDH flux might impair myocardial performance, we determined MVo_{2} in glucose-perfused hearts that were subjected to an inotropic challenge. Interestingly, the inotropic response to calcium was attenuated in ob/ob mouse hearts, and MVo_{2} failed to increase. The lack of an increase in MVo_{2} despite a modest increase in contractility is unusual but not without precedent. McConville et al demonstrated that β-1-adrenergic stimulation of isolated rat hearts increased RPP in the absence of a concomitant increase in MVo_{2}. Our observations imply that the energy that fuels the increased E-C coupling that is induced by high calcium in ob/ob hearts might be coming from anaerobic glycolysis or from more rapid depletion of phosphocreatine pools. The present study design did not allow us to distinguish between these 2 possibilities. It is also possible that the reduction in MVo_{2} in glucose-perfused hearts simply reflects a reduction in work. We think that this is relatively unlikely, because the reduction in work was not proportional to the reduction in MVo_{2}, and in FA-perfused hearts, the relatively impaired inotropic response occurred despite an increase in MVo_{2}. Recent studies also suggest that the sensitivity of the E-C coupling machinery to calcium may be altered in hearts of ob/ob and db/db mice.3,21,22 Thus, the contribution of altered calcium sensitivity to the impaired inotropic response to calcium observed in the present study needs to be considered. It is unlikely, though, that differences in calcium sensitivity can completely account for the differences in the degree of the inotropic responses and changes in MVo_{2} that were observed in glucose- versus glucose-and-palmitate-perfused ob/ob hearts.

The present data suggest that there is a significant limitation in the ability of ob/ob mouse hearts to utilize glucose under high workload conditions and are supported by our earlier observations that glucose utilization is severely impaired in isolated working ob/ob hearts.6 Mitochondria from glucose-perfused ob/ob hearts that were exposed to glutamate and palmitoyl carnitine also demonstrated a significant reduction in state 3 respirations. Palmitoyl carnitine enters the mitochondria via CPT2 and then undergoes β-oxidation, which contributes reducing equivalents directly to the electron transport chain in addition to generating acetyl CoA. We observed no defects in the activity of CPT1 or CPT2 or in a representative enzyme of β-oxidation. Thus, the limitation in state 3 respiration likely represents reduced TCA flux or impaired electron transport chain activity. Glutamate enters the TCA cycle via conversion to α-ketoglutarate and then contributes its reducing equivalents to the electron transport chain. Thus, response to this substrate interrogates the TCA cycle and the electron transport chain. We observed no reduction in the activity of 1 of the rate-limiting enzymes of the TCA cycle, namely, CS. We cannot rule out the possibility that α-ketoglutarate dehydrogenase activity could be reduced; however, we did observe significant reductions in the content of various components of the electron transport chain. Taken together, these observations suggest that in addition to reduced PDH flux, mitochondrial energetics in obese mice is also limited by changes in electron transport chain flux or capacity.

Having demonstrated that mitochondria from ob/ob mice are defective, we then wanted to determine whether FA further impaired mitochondrial function by selectively uncoupling respiration from ATP generation in ob/ob hearts, by analyzing myocardial oxygen consumption and mitochondrial function in hearts that were perfused with glucose and palmitate. Consistent with our earlier observations in working hearts, the addition of FAs caused a preferential increase in MVo_{2} in ob/ob hearts that was not accompanied by any change in cardiac function. Furthermore, under high work-
load conditions, ob/ob hearts always exhibited higher MV\textsubscript{O2} than wild-type hearts, despite a blunted inotropic response. Why did MV\textsubscript{O2} increase? We believe that the increase in MV\textsubscript{O2} in part reflects mitochondrial uncoupling. The evidence for this includes the following. State 3 mitochondrial respirations were increased in glucose-perfused control hearts relative to glucose-perfused ob/ob hearts for all substrates. Perfusion of hearts with palmitate led to a reduction in state 3 respirations with pyruvate as substrate in control hearts. One mechanism for this may be an intact Randle cycle in control hearts. 23 Given the reduction of PDH activity in ob/ob hearts, we expected to see a pronounced effect of FA on reducing pyruvate respiration. In contrast, state 3 respirations tended to increase in ob/ob mouse hearts after palmitate perfusion. Despite this, ATP generation did not increase, and the ratio of ATP production to oxygen consumption declined. In the presence of palmitoyl-carnitine as a substrate, state 4 respirations were statistically increased in ob/ob hearts and tended to increase in control hearts that were perfused with palmitate. The increase in state 4 respirations is consistent with mitochondrial uncoupling in both groups, but the bioenergetic consequences of this were more severe in ob/ob mitochondria, as evidenced by the reduction in ATP generation and the reduction in ATP/O ratios relative to mitochondrial uncoupling from glucose-and-palmitate-perfused controls. Thus, in light of the reduced mitochondrial efficiency in FA-perfused ob/ob hearts, it would be expected that oxygen consumption would need to increase to maintain ATP generation.

To evaluate potential mechanisms for the preferential increase in mitochondrial uncoupling in the presence of FAs, we evaluated the possibility that uncoupling protein abundance was increased in the hearts of ob/ob mice. The UCP3 gene is regulated by PPAR-\alpha, and its expression levels can be regulated in the adult heart by synthetic PPAR-\alpha ligands and conditions that would increase ambient concentrations of free FAs. 13,24 Streptozotocin-induced diabetes is associated with increased expression of UCP3 mRNA in rat hearts, but changes in protein levels were not examined. 11 In contrast, UCP2 appears to be regulated by FA in neonatal cardiomyocytes but does not appear to be regulated in adults hearts. 25 Indeed, we detected minimal myocardial protein expression of UCP2 in the present study, which is consistent with previously published studies in the mouse. 26 Because few studies have actually examined changes in uncoupling protein content in the hearts of diabetic or obese rodents, we elected to measure UCP3 protein content by Western blot analyses and confirmed the specificity of our assay by confirming the absence of UCP3 in heart homogenates obtained from UCP3 KO mice. 27 To our surprise, we did not observe an increase in UCP3 protein content. Thus, the FA-induced mitochondrial uncoupling that we observed in ob/ob hearts appears to be independent of changes in UCP3 expression. Recent evidence suggests that both FA and superoxide can activate mitochondrial uncoupling. 28,29 We have previously demonstrated that rates of FA utilization are increased between 1.5- and 3-fold in isolated working ob/ob hearts depending on perfusion conditions. 6 Thus, increased mitochondrial FA flux could lead to or be associated with activation of UCP3. Increased FA oxidation, by increasing the delivery of reducing equivalents to the electron transport chain, ultimately increases the generation of superoxide, which could also increase mitochondrial uncoupling in obese hearts.

The present investigation was performed in animals that were significantly obese and insulin resistant. We have previously shown that at the age studied, these animals have relatively minor defects in glucose tolerance. 8 The present studies do not definitively establish whether the mitochondrial adaptations that we observed are specific to obesity or reflect independent effects of leptin deficiency. However, recent observations obtained in humans with obesity and insulin resistance are similar to those that we have observed in ob/ob mouse hearts. Our studies may therefore provide mechanistic insight into these human observations. In a study of women with morbid obesity, Peterson and colleagues 30 described increased rates of FA oxidation and uptake, increased MV\textsubscript{O2}, and decreased myocardial efficiency. Scheuermann-Freestone and colleagues 31 reported decreased myocardial phosphocreatine/creatinine ratios in individuals with type 2 diabetes. Moreover, myocardial energetics was inversely related to serum concentrations of free FAs. Taken together with our previously published observations of increased FA oxidation and increased MV\textsubscript{O2} in isolated working hearts from ob/ob mice and our current observations, we propose that myocardial energetics is reduced in obesity and insulin-resistant states on the basis of FA-induced mitochondrial uncoupling.

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References


**CLINICAL PERSPECTIVE**

Obesity is a risk factor for heart failure independent of underlying coronary artery disease. The mechanisms responsible for this association are partially understood. Studies in obese humans have revealed preserved myocardial function but increased oxygen consumption. To elucidate the mechanisms responsible for decreased myocardial efficiency in obesity, we measured myocardial oxygen consumption (MV˙O2), mitochondrial respirations, and ATP production in a mouse model of severe obesity and insulin resistance, the ob/ob mouse. Ob/ob and control hearts were perfused with glucose only or with glucose and palmitate. In glucose-perfused hearts, MV˙O2 was similar in ob/ob and control hearts under basal conditions; however, the rise in MV˙O2 in response to an inotropic challenge was blunted in ob/ob hearts, and mitochondria exhibited reduced respiratory capacity, decreased activity of pyruvate dehydrogenase, and reduced expression of mitochondrial complexes I, III, and V. In glucose-and-palmitate–perfused hearts, MV˙O2 was increased in ob/ob hearts at normal workload and remained elevated despite decreased performance at high workload. Mitochondria from ob/ob mice were uncoupled after exposure to fatty acids so that the amount of ATP generated per unit of oxygen consumed was reduced. These results indicate that mitochondrial dysfunction develops in the hearts of an animal model of obesity. There is defective respiratory capacity, particularly with glucose substrates and fatty acid–induced mitochondrial uncoupling. These mitochondrial changes may contribute to decreased myocardial energetics and efficiency in obesity and may potentially contribute to the increased risk of heart failure. Future studies will need to determine whether weight loss will reverse these mitochondrial abnormalities.
Reduced Mitochondrial Oxidative Capacity and Increased Mitochondrial Uncoupling Impair Myocardial Energetics in Obesity

Sihem Boudina, Sandra Sena, Brian T. O'Neill, Prakash Tathireddy, Martin E. Young and E. Dale Abel

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