High-sucrose diet increases ROS generation, FFA accumulation, UCP2 level, and proton leak in liver mitochondria

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Submitted 3 December 2010; accepted in final form 7 September 2011

Ruiz-Ramírez A, Chávez-Salgado M, Peñeda-Flores JA, Zapata E, Masso F, El-Hafidi M. High-sucrose diet increases ROS generation, FFA accumulation, UCP2 level, and proton leak in liver mitochondria. Am J Physiol Endocrinol Metab 301: E1198–E1207, 2011. First published September 13, 2011; doi:10.1152/ajpendo.00631.2010.—Obesity, a risk factor for insulin resistance, contributes to the development of type 2 diabetes and cardiovascular diseases. The relationship between increased levels of free fatty acids in the liver mitochondria, mitochondrial function, and ROS generation in rat model of obesity induced by a high-sucrose diet was not sufficiently established. We determined how the bioenergetic functions and ROS generation of the mitochondria respond to a hyperlipidemic environment. Mitochondria from sucrose-fed rats generated H$_2$O$_2$ at a higher rate than the control mitochondria. Adding fatty acid-free bovine serum albumin to mitochondria from sucrose-fed rats significantly reduced the rate of H$_2$O$_2$ generation. In contrast, adding exogenous oleic or linoleic acid exacerbated the rate of H$_2$O$_2$ generation in both sucrose-fed and control mitochondria, and the mitochondria from succrose-fed rats were more sensitive than the control mitochondria. The increased rate of H$_2$O$_2$ generation in sucrose-fed mitochondria corresponded to decreased levels of reduced GSH and vitamin E and increased levels of Cu/Zn-SOD in the intermembrane space. There was no difference between the levels of lipid peroxidation and protein carbonylation in the two types of mitochondria. In addition to the normal activity of Mn-SOD, GPX and catalase detected an increased activity of complex II, and upregulation of UCP2 was observed in mitochondria from sucrose-fed rats, all of which may accelerate respiration rates and reduce generation of ROS. In turn, these effects may protect the mitochondria of sucrose-fed rats from oxidative stress and preserve their function and integrity. However, in whole liver these adaptive mechanisms of the mitochondria were inefficient at counteracting redox imbalances and inhibiting oxidative stress outside of the mitochondria.

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ABDOMINAL OBESITY AND HIGH LEVELS of circulating free fatty acids (FFAs) have been indicated as primary contributors to acquired insulin resistance and hypertension because they induce oxidative stress that affects insulin signaling and nitric oxide availability (30, 11). Insulin resistance leads to continuous lipolysis within adipocytes, releasing FFAs into the local circulation, where they are transported into the liver. In the liver, FFAs can be incorporated into triglycerides that accumulate and lead to nonalcoholic fatty liver disease, the primary hepatic complication of obesity (13, 57). Oxidative stress and mitochondrial dysfunction have been shown to play a critical role in the initiation and progression of fatty liver to the more serious condition of nonalcoholic steatohepatitis in obesity models (15, 21, 38). Several studies using an animal model of fatty liver induced by a high-fat diet suggest that chronic exposure to a high-fat diet negatively affects the bioenergetics of the liver (39, 58). However, recent studies of high-fat diet-induced insulin resistance in rodents have reported fatty acid accumulation and oxidative stress in the liver without significant effects on mitochondrial function (9, 55). Tissues and cells affected by metabolic diseases such as diabetes have been described as developing an adaptive mechanism to defend against oxidative stress and mitochondrial dysfunction (1). One putative mechanism is a mild physiological uncoupling of oxidative phosphorylation that would reduce superoxide generation by reducing the mitochondrial membrane potential (6, 32).

The overexpression of mitochondrial uncoupling protein (UCP2) in fatty hepatocytes of genetically obese mice has been suggested to be an adaptation of mitochondria to obesity-related oxidative stress (60). Because the primary function of UCP2 is to mediate proton leak, the overexpression of UCP2 in fatty liver may compromise ATP biosynthesis (8). In a model of abdominal obesity induced by a high-sucrose diet, the increased FFA level in both plasma and the liver (17–19) suggests mitochondrial dysfunction accompanied by high reactive oxygen species (ROS) generation and oxidative stress in the liver. Generation of ROS in response to a hyperlipidemic environment may also contribute to the maintenance of a normal or altered redox status of the liver in this model of abdominal obesity. We hypothesized that mitochondria might adapt to prolonged exposure to increased concentrations of FFA in vivo by altering the activity levels of antioxidant enzymes and enzymes that can affect oxidative phosphorylation, such as UCP2.

We explored these questions by examining the relationship between H$_2$O$_2$ generation, endogenous FFA accumulation, and proton leak in isolated mitochondria from sucrose-fed rats (SFR). We also examined the redox status of liver homogenate and isolated mitochondria from SFR by determining the activities of some antioxidant enzymes and the levels of antioxidant molecules such as reduced glutathione, vitamin E, and reduced coenzyme Q.

MATERIALS AND METHODS

Chemicals. Solvents were purchased from Sigma Chemical (St. Louis, MO) and were of HPLC grade. Butylated hydroxytoluene (BHT), fatty acid-free bovine serum albumin (BSA), tetraethoxypropane (TEP), reduced glutathione (GSH), vitamin E, ubiquinone-9, and ubiquinone-10 were also purchased from Sigma Chemical. Ubiquinol-9 (CoQ$_9$) and ubiquinol-10 (CoQ$_{10}$) were prepared by reducing the corresponding quinones with sodium borohydride (Sigma Chemical), as described by Takada et al. (52).

Animals. The animal experiments were approved by the Laboratory and Animal Care Committee of the National Institute of Cardiology...
Male Wistar rats, aged 28 days and weighing ~45 ± 2 g, were housed with a 12:12-h light-dark cycle and randomly separated into two groups of eight to 10 animals: group 1 (control) rats, given tap water for drinking; and group 2 (SFR) rats, given drinking water containing 30% sucrose for ~20 wk. All animals were fed ad libitum with commercial rat chow (LabDiet 5008; PMI Nutrition International, Richmond, IN).

Plasma and tissue sampling. After overnight fasting, the animals were decapitated. The blood was immediately collected in a tube containing EDTA (0.1%) and centrifuged at 600 g for 20 min at 4°C. The plasma was supplemented with 0.005% BHT as an antioxidant and stored at ~70°C until the lipid analysis could be performed.

The liver was homogenized in 50 ml of cold buffer (250 mM sucrose, 10 mM Tris, 1 mM EDTA, adjusted to pH 7.4 with KOH) and kept on ice. The homogenate was centrifuged at 600 g for 5 min at 4°C, and the supernatant was withdrawn and stored at ~70°C until required for lipid analysis.

Intra-abdominal fat, an important characteristic of metabolic syndrome, a marker of insulin resistance, and a source of free fatty acids, was dissected from the retroperitoneal cavity and around both kidneys and immediately weighed. Visceral and duodenal fat were not included in this procedure.

Biochemical analyses of plasma samples. The plasma triglyceride (TG) concentration was measured according to the method described by Nägele et al. (40). The plasma insulin level was determined by radioimmunnoassay (Coat-a-Count; Diagnostic Products, Los Angeles, CA).

Measurement of liver triglycerides. For determination of hepatic triglycerides, liver tissue (50 mg) was homogenized in 2 ml of isopropanol with a Polytron disrupter. The homogenate was centrifuged at 2,000 g for 10 min, and the supernatant was collected. Supernatant triglycerides were measured with a Triglyceride Kit (Wako Diagnostics), as described elsewhere (5).

Preparation of mitochondria. Mitochondria were isolated from rat liver tissue by conventional differential centrifugation. The tissue was homogenized in 30–40 ml of cold buffer (250 mM sucrose, 10 mM Tris, 1 mM EDTA, adjusted to pH 7.4 with KOH) and kept on ice. The homogenate was centrifuged at 600 g for 10 min at 4°C. The pellet, containing cell debris and no homogenized tissue, was discarded, and one aliquot of liver homogenate was frozen for later analysis of antioxidant enzyme activities and FFA content. The rest of the supernatant was centrifuged at 8,000 g for 10 min at 4°C to pellet the mitochondria. The mitochondria were washed with a buffer containing 0.1% fatty acid-free BSA and finally resuspended in the same buffer without BSA. Prior to experimentation, the mitochondria were stored on ice at a final concentration of 30–40 mg/ml protein. The protein concentration was determined by the modified method of Lowry et al. (37). Mitochondria prepared in this way were active for 5–6 h, as determined by their ability to maintain transmembrane potential in the presence of oxidizable substrates.

Mitochondrial oxygen uptake and respiratory complex enzyme activities. Mitochondrial respiratory rates were measured with a Clark-type O2 electrode by incubating 0.50 mg protein/ml of fresh mitochondria at 30°C in an air-saturated medium containing 125 mM KCl, 10 mM HEPES (pH adjusted to 7.4 with KOH), 10 mM EGTA, 2 mM K2HPO4, 5 mM MgCl2 and 5 mM succinate (oxidative substrate) in the presence of rotenone.

The activities of NADH-dehydrogenase (CI), succinate dehydrogenase (CII), succinate-cytochrome c oxidoreductase (CIII), and cytochrome c oxidase (CIV) were determined as described previously (56). Briefly, the activity of CI was measured by monitoring the oxidation of NADH in a solution containing 35 mM potassium phosphate buffer (pH 7.5), 0.2 mM NADH, and 1.7 mM potassium ferricyanide. Absorbance changes were followed at 340 nm, using an extinction coefficient of 6.22 mM/cm for NADH.

The activity of CII was measured by monitoring the absorbance of dichlorofluoromethylhydroxylamine in a solution containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM KCN, 0.05 mM dichlorofluoromethylhydroxylamine, and 16 mM succinate. Absorbance changes were followed at 600 nm, using an extinction coefficient of 19.1 mM/cm for dichlorofluoromethylhydroxylamine.

The activity of CIII was measured by following the reduction of cytochrome c in a solution containing 0.1 mM cytochrome c, 0.5 mM KCN, 3 mM succinate, and potassium phosphate buffer (pH 7.5).

The activity of CIV was determined by following the oxidation of cytochrome c in a solution containing 0.1% reduced cytochrome c and 10 mM potassium phosphate buffer (pH 7.0). Absorbance changes were followed at 550 nm, using an extinction coefficient of 18 mM/cm for cytochrome c.

Proton leak. Oxygen consumption and the inner transmembrane potential were recorded in parallel using a Clark-type oxygen electrode and the fluorescence of tetrathionylhydroxylamine methyl ester (TMRM), respectively. The transmembrane potential was estimated from the fluorescence quenching of the cationic dye TMRM (47), which accumulated and was quenched inside energized mitochondria, in the solution described above for the measurement of oxygen uptake. The excitation wavelength was 548 nm (6-nm slit), and the emission wavelength was 573 nm (6-nm slit). The final concentration of the dye was 0.25 μM. Fluorescence measurements were performed with a PerkinElmer LS50B spectrofluorometer, using a stirred cuvette maintained at 30°C. Liver mitochondria (1 mg) were incubated in 2 ml of reaction medium containing 2 μM oligomycin, 3 μM rotenone, and 80 ng/ml of nigericin (added to abolish ΔΨ so that the total proton motive force would be equal to ΔΨ). The reaction was started by adding succinate (5 mM). Oxygen consumption and the membrane potential were progressively inhibited by sequential additions of malonate in increasing amounts (final concentrations: 0.5, 1, 2, 3, 4, and 5 mM), which inhibited succinate dehydrogenase, thereby decreasing electron availability in the system and creating a range of membrane potentials. At the end of each experiment, carbonyl cyanide p-trifluoromethoxyphenylhydrazone was added to dissipate the ΔΨ. In some experiments, 0.5 mM GDP (a known UCP2 inhibitor) and 5 μM oleic acid [a UCP2 activator (10 nmol/mg protein)] were added to the incubation medium before the malonate additions to study the participation of UCP2 in the proton leak.

The calibration procedure was performed as described elsewhere (47). The partitioning of the dye was calculated from the equation

\[
\text{TMRM}^t = \text{Ki} \times \text{TMRM}^m + \text{Ko} \times \text{TMRM}^o,
\]

where (TMRM)^t corresponds to the total TMRM accumulated by energized mitochondria, (TMRM)^m is the amount of dye inside the matrix (nmol/μl), and (TMRM)^o is the amount of dye outside the matrix (nmol/μl). To determine (TMRM)^t, mitochondria that had been incubated with TMRM in the presence and absence of substrate were centrifuged, and the fluorescence of the supernatant with substrate was subtracted from the initial fluorescence of the supernatant without substrate. To determine (TMRM)^o, the mitochondria were energized, and carbonyl cyanide p-trifluoromethoxyphenylhydrazone was added to attain a target membrane potential. After a steady state was reached, the fluorescence of the medium was recorded, and the contents of the cuvette were centrifuged to separate the mitochondria. The value of (TMRM)^o was calculated from the fluorescence of the supernatant. The value of (TMRM)^m was calculated using the equation described above with Ko = 88 (μl/mg) and Ki = 33 (μl/mg) at 30°C. ΔΨ was estimated from the Nernst equation [ΔΨ = 60.1 log (TMRM)^o/(TMRM)^m].

Measurement of H2O2 generation by the mitochondria. The fluorescence of oxidized dichlorofluoromethylhydrazide (DCF) was measured with a Perkin Elmer LS50B spectrofluorometer, using a stirred cuvette maintained at 30°C. The mitochondria (0.5 mg/ml) were incubated in the solution described above for the measurement of oxygen uptake, with the addition of 0.1 μM DCF and 1 U/ml horseradish peroxidase. The fluorescence signal was amplified by electron transfer from...
peroxidase to DCF during the reduction of H$_2$O$_2$, which is produced by superoxide anion dismutation. The forward electron pyruvate mode was assessed by energizing the mitochondria with pyruvate/malate (5 mM/3 mM). The oxidation of DCF was detected using excitation/emission wavelengths of 484 nm (6-nm slit)/525 nm (6-nm slit). The rate of H$_2$O$_2$ generation was calibrated by constructing standard curves from known concentrations of H$_2$O$_2$ in solutions consisting of the standard incubation buffer, the appropriate dye, horseradish peroxidase, and mitochondria. The effect of ADP (50 μM), GDP (0.5 mM), and various concentrations of oleic or linoleic acid on the generation of H$_2$O$_2$ was examined using the same buffer described above for the measurement of oxygen uptake and membrane potential, with the addition of 2 μM oligomycin and 80 ng/ml nigericin.

**Western blotting of UCP2.** Mitochondrial proteins (100 μg) were separated by electrophoresis on a 12.5% polyacrylamide gel and then electrotransferred to an immobilon-P membrane (Millipore) and blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 for 2 h at room temperature. The blocked membrane was incubated overnight at 4°C in the same buffer containing goat polyclonal antibody to the NH$_2$-terminal domain of UCP2 (1/4000) or goat polyclonal anti-adenine nucleotide translocase (ANT; 1/1,000) for the primary membrane (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were rinsed with Tris-buffered saline containing 0.1% Tween-20 and then incubated with horseradish peroxidase-coupled anti-goat IgG as a secondary antibody (1/5,000) (Santa Cruz Biotechnology). The bands were visualized using enhanced chemiluminescence detection reagent (Millipore) and exposed on Kodak Biomax ML scientific image film for 5–10 min.

**Lipid extraction and analysis of FFA composition.** FFAs were extracted from 10 mg of mitochondrial protein or from 10 mg of protein from liver homogenate in the presence of 10 and 100 μg of heptadecanoic acid (internal standard), respectively, using chloroform-methanol (2:1, vol/vol) containing 0.002% BHT, as described by Folch et al. (23). The obtained lipid residue was dissolved in 1 ml of methanol containing 0.1 ml of 2,2-dimethoxypropane and 0.01 ml of concentrated H$_2$SO$_4$ to esterify FFAs to their corresponding methyl esters at room temperature for 15 min, as described by Tserng et al. (54) and modified in our laboratory (18). These reaction conditions are necessary to avoid the esterification of fatty acids from phospholipids, cholesterol esters, and triglycerides. This method was compared with the method using thin-layer chromatography to separate FFAs from the other lipid components of total lipid extract, and no significant difference was found between the two methods. The method used in this work has the advantage of limiting the time of lipid extract exposure to autoxidation. The concentration and composition of FFA methyl esters were evaluated by gas liquid chromatography, as described previously (17).

**Oxidative stress markers.** For lipid peroxidation, plasma, liver homogenate, and mitochondrial fraction were treated at 90°C for 30 min with 0.8% thiobarbituric acid (TBA) in 20% acetic acid (pH 2.5). The tubes were then placed in an ice bath for 10 min before extraction with n-butanol. The amount of TBA-reactive substances (TBARS) in each sample was calculated from the fluorescence intensity at an excitation wavelength of 513 nm and an emission wavelength of 533 nm, using malondialdehyde (MDA) derived from TEP as standard (16).

For protein carbonylation assay, the carbonyl level was determined in 5 mg of liver homogenate protein and 5 mg of mitochondria protein using the 2,4-dinitrophenylhydrazine to derive the carbonyl function according to the method described elsewhere (36). The extension coefficient used to evaluate the concentration of carbonylated protein was 22,000 M/cm at a wavelength of 390 nm.

**HPLC analysis of mitochondrial vitamin E and CoQ.** Reduced CoQ and vitamin E were simultaneously extracted from liver mitochondria and analyzed by HPLC, as described elsewhere (51). Five milligrams of mitochondrial protein, in the presence of 25 μg BHT (to prevent autoxidation), was mixed in a glass tube with 4 ml of cold methanol for 1 min, using a vortex mixer, and then 3 ml of cold petroleum ether was added to the mixture. The resulting solution was centrifuged at 1,200 × g, and the petroleum ether layer was collected and dried under nitrogen. The residue was dissolved in 1 ml of methanol-ethanol (1:1) and filtered through a nylon membrane with 0.22-μm pore size. It was then separated on reverse-phase C18 HPLC column (Nova Pack, 3.9 × 150 mm, 5 μm; Waters), using a methanol-ethanol mixture (1:1 by vol) containing 50 mM sodium acetate and 250 mM acetic acid as a mobile phase at a flow rate of 1 ml/min. Analysis was carried out by a digital electrochemical detector equipped with glassy carbon as a working electrode and a hydrogen electrode as a reference. The working electrode was set at 550 mV. The concentration of ubiquinol and vitamin E was estimated by using a calibration curve with standard solutions of known concentration.

**Mitochondrial and liver homogenate GSH content.** Acid extraction of GSH was performed using 1 and 10 mg of mitochondrial and liver homogenate protein, respectively, in 1 ml of solution containing 200 mM metansulfonic acid and 5 mM diethylenetriaminepentaacetic acid (34). The mixture was agitated in a vortex for 1 min and centrifuged at 21,000 × g for 30 min at 4°C. Five hundred microliters of the supernatant was diluted 1:1 with the mobile phase (50 mM NaH$_2$PO$_4$, 0.05 mM octanesulfonic acid adjusted to pH 3 with phosphoric acid); this extract was filtered through a membrane of 45-μm pore size; 50 μl was analyzed by HPLC equipped with the same column as that described above and an electrochemical detector with a glassy carbon as a working electrode (+700 mV). The analysis was performed at a mobile phase flow rate of 1 ml/min. The concentration of GSH was estimated by comparison of the peak area with those of standard solutions of known concentration.

**Superoxide dismutase activity staining.** The technique using the native polyacrylamide gel is 10 times more efficient than the spectrophotometric method. It can assay the activity of a single superoxide dismutase (SOD) isoform on the polyacrylamide gel and excludes the interference coming from non-SOD molecules in the crude tissue extract, which is not possible to discard when the spectrophotometric method is used. SOD activity in mitochondria preparation was assessed as described (22). Briefly, 10 μl of each sample (containing 2 mg/ml total protein) was loaded onto 5% stacking an 8% native polyacrylamide gel at 10 μl × Tris-glycine buffer (pH 8.3), and the supernatant was diluted 1:1 with the mobile phase (50 mM NaH$_2$PO$_4$, 0.05 mM octanesulfonic acid adjusted to pH 3 with phosphoric acid); this extract was filtered through a membrane of 45-μm pore size; 50 μl was analyzed by HPLC equipped using the same column as that described above and an electrochemical detector with a glassy carbon as a working electrode (+700 mV). The analysis was performed at a mobile phase flow rate of 1 ml/min. The concentration of GSH was estimated by comparison of the peak area with those of standard solutions of known concentration.

**Catalase and glutathione peroxidase activities.** Catalase activity was determined using 50 μg of liver homogenates and mitochondria by measuring the exponential decay of 10 mM H$_2$O$_2$ (Δε$_{340}$ = 39.4 M/cm) in 50 mM phosphate buffer, pH 7.0 monitored at 240 nm, and the activity was expressed as nanomoles per minute per milligram of protein, as described previously (60).

For glutathione peroxidase (GPx), 50 μg of protein from isolated liver mitochondria from each animal was suspended in 1 ml of buffer (50 mM Tris-HCl, pH 7.6, 5 mM EDTA, 1 mM GSH, 0.22 mM NADPH, and 0.4 U of glutathione reductase), and the reaction was initiated by adding tert-butyl hydroperoxide to a final concentration of 0.22 mM. NADPH consumption was monitored continuously over 3 min at 340 nm in a spectrophotometer. Final results were expressed as nanomoles per minute per milligram of mitochondrial protein.

**Statistical analysis.** All values are presented as means ± SE. Between-group differences for selected variables were calculated...
using one-way ANOVA. Differences were considered statistically significant at $P < 0.05$, calculated by a statistical and graphic system (SigmaPlot; Jandel).

**RESULTS**

Changes in insulin, TGs, FFAs, and intra-abdominal fat accumulation. In the SFR model, obesity was induced by supplementing the animals’ drinking water with sucrose. Both the body weight and the amount of intra-abdominal fat, an important characteristic of metabolic syndrome, a marker of insulin resistance, and a source of FFAs, increased in SFR. The levels of fasting plasma insulin, TGs, and FFAs also significantly increased in SFR relative to the control rats (CR), as described previously (18). However, the level of plasma glucose in SFR was not significantly different from that of CR (Table 1). The concentration of TG in the liver homogenate of SFR was significantly higher than that of CR.

Mitochondrial oxygen uptake and respiratory complex activity. The mitochondria from SFR exhibited significantly higher rates of oxygen uptake while oxidizing succinate/rotenone in the presence (state III) and absence (state IV) of ADP than the mitochondria from CR (Table 2). Although SFR mitochondria exhibited increased rates of oxygen uptake in both states, the ratio between the rates in state III and IV was not significantly different from that of the CR mitochondria.

The activities of the mitochondrial respiratory complexes CI, CIII, and CIV (nmol·min$^{-1}$·mg protein$^{-1}$) from SFR did not differ significantly from those of CR. The activity of CII increased significantly in SFR mitochondria relative to CR mitochondria (Table 2).

Proton leak. Figure 1 illustrates the relationship between the respiration rate and membrane potential in liver mitochondria. SFR mitochondria consume more oxygen than CR mitochondria but generate the same $\Delta\Psi$ ($P < 0.01$). This result suggests that SFR mitochondria have a higher respiration rate and a lower membrane potential than CR mitochondria (Fig. 1A).

Oleic acid (2.5 μM) increased the respiration rate and decreased the membrane potential of both types of mitochondria (Fig. 1B and C). The stimulation of proton leak by oleic acid appears to be larger ($P < 0.01$) in SFR mitochondria (Fig. 1C) than in CR mitochondria (Fig. 1B).

We examined the possibility that UCP2 might be involved in the effect of oleate on mitochondrial respiration and membrane potential by adding GDP, an inhibitor of UCP2. GDP decreased the uncoupling effect of oleic acid on SFR mitochondria (Fig. 1C) and had no effect on CR mitochondria (Fig. 1B).

Table 1. General characteristics of animals

<table>
<thead>
<tr>
<th>Variables</th>
<th>CR</th>
<th>SFR</th>
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<tbody>
<tr>
<td>Body mass, g</td>
<td>440 ± 29</td>
<td>526 ± 57*</td>
</tr>
<tr>
<td>Intra-abdominal fat, g</td>
<td>2.9 ± 0.8</td>
<td>15.3 ± 5.2**</td>
</tr>
<tr>
<td>Plasma triglycerides, mM</td>
<td>0.5 ± 0.1</td>
<td>1.2 ± 0.3**</td>
</tr>
<tr>
<td>Liver triglycerides, μmol/mg</td>
<td>65.5 ± 5.5</td>
<td>223.7 ± 30.6**</td>
</tr>
<tr>
<td>Plasma glucose, mM</td>
<td>6.2 ± 0.1</td>
<td>5.6 ± 0.5</td>
</tr>
<tr>
<td>Plasma insulin, pM</td>
<td>106.8 ± 17.4</td>
<td>285.2 ± 53.5***</td>
</tr>
<tr>
<td>Plasma FFA, mM</td>
<td>0.67 ± 0.04</td>
<td>1.26 ± 0.08**</td>
</tr>
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</table>

Values are expressed as means ± SE ($n = 7$ different animals). CR, control rats; SFR, sucrose-fed rats; FFA, free fatty acids. Triglycerides in liver are expressed in μmol/mg protein of liver homogenate. The values for all variables were obtained at the end of the treatment period. **Significantly different from CR group ($P < 0.001$); ***$P < 0.01$; *$P < 0.05$.

Table 2. Mitochondria oxygen uptake and respiratory complex enzyme activities

<table>
<thead>
<tr>
<th></th>
<th>CR</th>
<th>SFR</th>
</tr>
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<tbody>
<tr>
<td>RC</td>
<td>7.2 ± 0.5</td>
<td>7.4 ± 1.06</td>
</tr>
<tr>
<td>State III</td>
<td>110.9 ± 9.2</td>
<td>133.4 ± 9.2*</td>
</tr>
<tr>
<td>State IV</td>
<td>153.5 ± 0.7</td>
<td>197.2 ± 2.7*</td>
</tr>
<tr>
<td>NADH-DH (CI)</td>
<td>619.2 ± 36.4</td>
<td>683.5 ± 64.9</td>
</tr>
<tr>
<td>SDH (CII)</td>
<td>22.6 ± 1.5</td>
<td>32.9 ± 4.5*</td>
</tr>
<tr>
<td>SCCR (CIII)</td>
<td>38.5 ± 3.14</td>
<td>40.7 ± 4.8</td>
</tr>
<tr>
<td>COX (CIV)</td>
<td>297.5 ± 29.4</td>
<td>330.2 ± 29.15</td>
</tr>
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</table>

Values are expressed as means ± SE ($n = 6$ different animals). RC, respiratory control; NADH-DH (CI), NADH-dehydrogenase (complex I); SDH (CII), succinate dehydrogenase (complex II); SCCR (CIII), succinate-cytochrome c oxidoreductase (complex III); COX (CIV), cytochrome c oxidase (complex IV). The rate of $O_2$ consumption was expressed as nmol·min$^{-1}$·mg protein$^{-1}$, and it was performed in the presence of 5 mM of succinate + rotenone. RC corresponds to the ratio of state III to state IV. Complex enzyme activities were expressed as nmol·min$^{-1}$·mg protein$^{-1}$. *$P < 0.05$, CR vs. SFR. State IV respiration is defined as oxygen consumption by isolated mitochondria on a particular substrate in the absence of ADP or any metabolic poisons or inhibitors. State III respiration is defined as ADP-stimulated respiration.

This result suggests that the effect of GDP on the coupling efficiency in mitochondria from SFR may be mediated by an uncoupling protein such as UCP2.

Generation of ROS by the mitochondria. The mitochondrial electron transport chain produces the superoxide anion, which is converted by mitochondrial SOD to $H_2O_2$, a more stable and more lipid-soluble metabolite than the superoxide anion. $H_2O_2$ can be detected by the oxidation of DCF in the presence of peroxidase. In the absence of ADP (state IV), the SFR mitochondria that metabolized pyruvate/malate generated $H_2O_2$ at a significantly greater rate than the CR mitochondria (2.70 ± 0.05 vs. 0.8 ± 0.05 pmol·min$^{-1}$·mg$^{-1}$ protein; Fig. 2). In the presence of ADP (state III), SFR mitochondria generated much less $H_2O_2$ than in state IV (0.29 ± 0.07 vs. 2.04 ± 0.05 pmol·min$^{-1}$·mg$^{-1}$), and no statistically significant difference was observed between the SFR and CR mitochondria (0.29 ± 0.07 vs. 0.21 ± 0.06 pmol·min$^{-1}$·mg$^{-1}$).

Precinubcation of the mitochondria with 0.5 mM GDP (an inhibitor of UCP2) resulted in increased ROS generation in SFR mitochondria but not in CR mitochondria. Adding GDP stimulated $H_2O_2$ generation in SFR mitochondria under both conditions with and without ADP.

The addition of BSA to the incubation medium reduced the rate at which the mitochondria generated $H_2O_2$ (Fig. 3A). At low concentrations of BSA (0.2 and 0.4 mg/ml), the rate of $H_2O_2$ generation decreased faster in SFR mitochondria than in CR mitochondria. Increasing the concentration of BSA slowly decreased the generation of $H_2O_2$ in SFR mitochondria but did not affect the CR mitochondria. These results show that BSA effectively traps mitochondria-associated FFA and reduces $H_2O_2$ generation in CR mitochondria. BSA significantly affected the rate of ROS generation in SFR mitochondria but did not completely recover the rate of ROS generation observed in CR mitochondria. Therefore, the addition of BSA partially protected CR mitochondria against the generation of ROS.

The addition of low concentrations of oleic acid (2.5 and 5 nmol/mg protein) did not affect the rate of ROS generation in the mitochondria of either group (Fig. 3B). At higher concentrations (10 nmol to 40 nmol/mg protein), oleic acid addition...
increased the rate of ROS generation in both types of mitochondria. The increased slope of the SFR curve at high concentrations of oleic acid suggests that the SFR mitochondria were more sensitive to oleic acid than the CR mitochondria. Similarly, linoleic acid addition, at the same concentrations, to

the mitochondria increased H$_2$O$_2$ generation in both the SFR and CR mitochondria (Fig. 3C). At concentrations of linoleic acid with $<30$ nmol/mg protein, this increase was more pronounced in the SFR than in the CR mitochondria. At concentrations $\geq 30$ nmol/mg protein, the rate of H$_2$O$_2$ generation plateaued in both types of mitochondria. Linoleic acid stimulated the rate of H$_2$O$_2$ generation to a much greater extent and at lower concentrations than oleic acid in both types of mitochondria (compare Fig. 3, B and C).

Western blot of UCP2. The expression level of UCP2 was higher in SFR mitochondria than in CR mitochondria, where it was very low (Fig. 4). No difference in the expression of ANT was observed between SFR and CR. Therefore, the level of ANT was used as a loading control.

Liver homogenate and mitochondria FFA composition. The total FFA concentration was increased significantly in both liver homogenate and mitochondria from SFR compared with those from CR (Table 3). Both tissues also exhibited a change in FFA composition. In SFR mitochondria, the concentrations of palmitic ($P < 0.01$), palmitoleic ($P < 0.001$), and oleic acid ($P < 0.01$) increased significantly; the concentration of arachidonic acid ($P < 0.05$) decreased, and the concentrations of stearic and linoleic acids did not change significantly. The concentrations of palmitic, palmitoleic, and oleic acid increased significantly in SFR liver homogenate, whereas the concentrations of polyunsaturated fatty acids, such as linoleic and arachidonic acids, did not change in either group of animals. The difference between the total FFA concentrations of the liver homogenate from SFR and CR animals was more important than the difference between the total FFA concentrations of the mitochondria of the two groups. This discrepancy might result from the mitochondria preparation proce-

increased the rate of ROS generation in both types of mitochondria. The increased slope of the SFR curve at high concentrations of oleic acid suggests that the SFR mitochondria were more sensitive to oleic acid than the CR mitochondria. Similarly, linoleic acid addition, at the same concentrations, to

the mitochondria increased H$_2$O$_2$ generation in both the SFR and CR mitochondria (Fig. 3C). At concentrations of linoleic acid with $<30$ nmol/mg protein, this increase was more pronounced in the SFR than in the CR mitochondria. At concentrations $\geq 30$ nmol/mg protein, the rate of H$_2$O$_2$ generation plateaued in both types of mitochondria. Linoleic acid stimulated the rate of H$_2$O$_2$ generation to a much greater extent and at lower concentrations than oleic acid in both types of mitochondria (compare Fig. 3, B and C).

Western blot of UCP2. The expression level of UCP2 was higher in SFR mitochondria than in CR mitochondria, where it was very low (Fig. 4). No difference in the expression of ANT was observed between SFR and CR. Therefore, the level of ANT was used as a loading control.

Liver homogenate and mitochondria FFA composition. The total FFA concentration was increased significantly in both liver homogenate and mitochondria from SFR compared with those from CR (Table 3). Both tissues also exhibited a change in FFA composition. In SFR mitochondria, the concentrations of palmitic ($P < 0.01$), palmitoleic ($P < 0.001$), and oleic acid ($P < 0.01$) increased significantly; the concentration of arachidonic acid ($P < 0.05$) decreased, and the concentrations of stearic and linoleic acids did not change significantly. The concentrations of palmitic, palmitoleic, and oleic acid increased significantly in SFR liver homogenate, whereas the concentrations of polyunsaturated fatty acids, such as linoleic and arachidonic acids, did not change in either group of animals. The difference between the total FFA concentrations of the liver homogenate from SFR and CR animals was more important than the difference between the total FFA concentrations of the mitochondria of the two groups. This discrepancy might result from the mitochondria preparation proce-

increased the rate of ROS generation in both types of mitochondria. The increased slope of the SFR curve at high concentrations of oleic acid suggests that the SFR mitochondria were more sensitive to oleic acid than the CR mitochondria. Similarly, linoleic acid addition, at the same concentrations, to
dure, which includes incubation step with 0.1% BSA to stabilize the mitochondrial membrane and remove any excess fat from the external membrane.

Plasma, liver, and mitochondrial oxidative stress parameters. The levels of lipid oxidation products, as measured by the TBARS assay, were significantly higher in both plasma \((P < 0.05)\) and liver homogenate \((P < 0.01)\) from SFR than in samples from CR. In contrast, no statistically significant change in the concentration of TBARS was observed in the mitochondria from either group (Table 4). The protein oxidation level, measured as protein carbonylation, was significantly greater \((P < 0.05)\) in the liver homogenate of SFR than in the homogenate of CR, whereas no difference was observed between the mitochondria of the two groups.

Mitochondrial vitamin E, ubiquinols, and reduced glutathione. The concentration of vitamin E was significantly smaller \((P < 0.01)\) in SFR mitochondria than in CR mitochondria (Table 5). The levels of reduced CoQ9 and CoQ10 were both significantly lower \((P < 0.05)\) in SFR mitochondria. The amount of GSH decreased by \(60\%\) in SFR liver homogenate and by \(28\%\) in SFR mitochondria relative to CR tissues \((P < 0.05); \text{Table 5} \). Activity of GPx, catalase, and mitSOD. The activity of GPx in both total liver homogenate and mitochondria from SFR was

### Table 3. FFA composition of mitochondria and liver homogenate

<table>
<thead>
<tr>
<th>FFA</th>
<th>Mitochondria</th>
<th>Liver Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic</td>
<td>14.5 ± 0.7</td>
<td>21.8 ± 1.1**</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>0.6 ± 0.5</td>
<td>3.2 ± 0.8***</td>
</tr>
<tr>
<td>Stearic</td>
<td>13.8 ± 0.8</td>
<td>11.7 ± 0.4</td>
</tr>
<tr>
<td>Oleic</td>
<td>3.4 ± 0.2</td>
<td>10.8 ± 0.7**</td>
</tr>
<tr>
<td>Linoleic</td>
<td>6.7 ± 1.1</td>
<td>5.2 ± 1.1</td>
</tr>
<tr>
<td>Arachidonic</td>
<td>7.0 ± 1.1</td>
<td>4.9 ± 0.9*</td>
</tr>
<tr>
<td>Total FFA</td>
<td>55.1 ± 2.7</td>
<td>62.6 ± 2.3*</td>
</tr>
</tbody>
</table>

Data are means ± SE and represent the concentration in \(\mu\)mol/mg protein in liver homogenate and in nmol/mg protein in mitochondria \((n = 7 \text{ different animals})\). **Significantly different from CR group \((P < 0.001)\); ***Significantly different from CR group \((P < 0.001); **P < 0.01; *P < 0.05. 

Fig. 3. Effects of fatty acid-free BSA (A), oleic acid (B), and linoleic acid (C) on \(H_2O_2\) generation. All experiments were performed in the presence of 2 \(\mu\)M oligomycin and 80 ng/ml nigericin. The mitochondria from CR and SFR rats were energized with a mixture of pyruvate and malate (5 and 3 mM each, respectively) and treated with different concentrations of BSA or FFA. Data correspond to the mean ± SE of 6 different animals.

Fig. 4. Western blotting of UCP2. The membrane was incubated with goat polyclonal antibody to the NH2-terminal domain of UCP2 and with goat polyclonal anti-adenine nucleotide translocase (ANT; 1/1,000) after stripping for control load, as described in MATERIALS AND METHODS. Data correspond to means ± SE of 6 different mitochondria preparations from each animal. *P < 0.001. The Western blot is representative of 1 experiment, and each band corresponds to 1 mitochondria preparation from each animal. OD, optical density.
Mitochondria

Table 5. Antioxidant activities in liver homogenate and mitochondria

<table>
<thead>
<tr>
<th>Variables</th>
<th>CR</th>
<th>SFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH, nmol/mg protein</td>
<td>Liver homogenate</td>
<td>0.64 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>1.5 ± 0.086</td>
</tr>
<tr>
<td>Protein carbonyls, nmol/mg protein</td>
<td>Liver homogenate</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td>0.5 ± 0.02</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE (n = 6 different animals). GSH, glutathione peroxidase. The values for all variables were obtained at the end of the treatment period. **Significantly different from CR group (P < 0.01); *P < 0.05.

Fig. 5. Superoxide dismutase (SOD) activity staining in the mitochondria from SFR (black bars) and CR (open bars). A total of 10 µl of each sample (containing 2 mg/ml total protein) was loaded onto a 10% native polyacrylamide gel. The activity of SOD was detected as described in MATERIALS AND METHODS. The gel was scanned with a Gel Doc scanner (Bio-Rad), and the bands were quantified with Image Quant software (GE Healthcare). One hundred percent corresponds to the mean of pixels in the Mn-SOD activity band from control animals. Data correspond to means ± SE of 6 different mitochondria preparations from each animal. *P < 0.05, CR Zn/Cu-SOD vs. SFR Zn/Cu-SOD. The gel is representative of 1 experiment. Each band corresponds to 1 mitochondria preparation from each animal. The band corresponding to 100 ng of Bovine Cu/Zn-SOD, purchased from Sigma, is considered as a positive control of SOD activity.

DISCUSSION

Oxidative stress markers in fatty liver. Supplementing the animals’ drinking water with sucrose resulted in increased levels of FFAs and TGs in both plasma and the liver, as described previously (18). The accumulation of TGs in the liver could arise from the esterification of fatty acids taken up in the circulation, from de novo lipogenesis, or both, as reviewed elsewhere (41). In our model, the accumulation of FFAs and TGs in the liver could result from insulin resistance, which is reflected in the accumulation of fat in the intra-abdominal cavity. It has been suggested that FFAs secreted into the local circulation by accumulated intra-abdominal adipose tissue play a major role in the development of fatty liver (59).

TGs and FFAs have been shown to induce ROS production and cellular necrosis (2, 48). The increased levels of lipid peroxidation (TBARS) and protein carbonylation that we observed in SFR liver homogenate can be attributed to increased levels of FFAs, such as palmitic acid and oleic acid. Palmitic acid may induce hepatic insulin resistance by stimulating the generation of superoxide anions by NADPH oxidase (24). The generation of ROS via the NADPH oxidase system has been shown to contribute to oxidative stress in several pathologies. Nevertheless, further study is needed to clarify the contribution of this system to the development of oxidative stress in the liver in the context of our model of abdominal obesity.

The high availability of TGs and FFAs as energetic substrates for oxidation makes the mitochondria one of the major sources of ROS, which can then contribute to the oxidative stress observed in the liver homogenate. FFAs can induce superoxide anion generation by directly interacting with mitochondrial complexes I or III, inhibiting their activities and affecting the mitochondrial electron transport chain (48, 49).

Mitochondrial ROS generation and FFAs. The mitochondria isolated from SFR generated ROS at a higher rate than the CR mitochondria when pyruvate and malate were used as oxidizable substrates (forward electron transport mode). The difference in the rate of H2O2 generation between isolated mitochondria from SFR and CR may arise from the increased levels of FFAs found in SFR mitochondria. Accordingly, the
addition of BSA to the mitochondria incubation medium reduced the H$_2$O$_2$ generation rate of the SFR mitochondria more than that of the CR mitochondria. BSA may bind FFAs and reduce their ability to stimulate the rate of ROS generation in SFR mitochondria. In this research, BSA was included during the preparation of mitochondria but not in the media used for the experimental analyses. The presence of BSA in the assay of mitochondrial function may obscure the contribution of FFAs to pathological states. In several studies of mitochondria isolated from ischemic liver tissue, the inclusion of BSA reversed mitochondrial dysfunction entirely (4, 20). The increased sensitivity of the SFR mitochondria to BSA indicates the presence of higher levels of FFAs than in the CR mitochondria. Analysis of the FFA composition revealed increased amounts of palmitic, palmitoleic, and oleic acids in SFR mitochondria. Thus, the difference in the rate of ROS generation between the two groups of mitochondria may arise from differing levels of FFA accumulation in the mitochondria. FFAs have been shown to increase the rate of ROS generation by the forward mode of electron transport when pyruvate and malate are used as substrates (49). In our experiments, the minimal concentration required for oleic acid to stimulate the rate of H$_2$O$_2$ generation was similar to that found in isolated mitochondria from SFR rats (~10 nmol/mg protein). Linoleic acid stimulated the rate of H$_2$O$_2$ generation at much lower concentrations than oleic acid in both types of mitochondria. In general, mitochondria from both SFR and control animals were more sensitive to exogenous linoleic than oleic acid in generating ROS. This difference in sensitivity may arise from the difference in the number and position of double bonds present in the carbon chain of each fatty acid. The mechanism by which the number and position of double bonds of FFA may affect mitochondrial ROS generation is not known. Any explanation of fatty acid interaction with mitochondrial respiratory complex proteins will necessarily be speculative because studies on the interaction of these protein structures with FFAs are not yet available. For a given FFA chain length, the aqueous solubility of a fatty acid increases exponentially with the level of unsaturation in the chain (53). Richieri et al. (45) reported that there is little difference between the binding affinities of oleic and linoleic acids for liver fatty acid-binding protein. Thus, the increasing aqueous solubility of fatty acids with increasing carbon chain unsaturation may indicate that water solubility plays an important role in determining the difference between the effects of oleic and linoleic acids on mitochondrial ROS generation. Furthermore, other studies have shown that arachidonic acid, a polyunsaturated FFA, stimulates ROS generation in the mitochondria from the liver and heart more strongly than oleic and palmitic acids (10).

**Function of mitochondria and UCP2.** SFR mitochondria exhibited increased ROS generation despite simultaneously overexpressing UCP2. Although the function of UCP2 is still unclear, it has been linked to the accumulation of FFAs in hepatocytes and muscle cells (12, 46) and to mitochondrial ROS production (43). The mild uncoupling mediated by UCP2 may accelerate respiration rates and reduce the generation of ROS.

The increased proton leak rate exhibited by isolated mitochondria from SFR may arise from the upregulation of UCP2 and the increased FFA levels in the mitochondria. In SFR mitochondria, the proton leak was associated with increases in both state III and state IV respiration rates, although the ratio corresponding to the respiratory control (for RC see Table 2) did not differ between the two groups of mitochondria. This result indicates that mitochondria from the SFR liver, which consume oxygen at a high rate, are as well coupled to the phosphorylation of ADP to produce ATP as the mitochondria from CR liver despite the overexpression of UCP2. This phenomenon is not unique to our system. The mitochondrial oxygen uptake of heart and muscle tissue from diabetic animals has been shown to be more coupled to ATP synthesis in the presence of ADP than from control animals despite an approximately two- to fourfold increase in UCP3 content. These results suggest that the expression of UCPs and respiration are not completely interconnected (28).

**Oxidative stress and activity of mitochondrial respiratory complexes.** The normal activities of mitochondrial respiratory complexes I and III and the increased activity of complex II suggest that SFR mitochondria have developed an adaptive mechanism to protect the mitochondrial respiratory complexes from oxidative stress. The mitochondrial respiratory complex activities have been shown to be highly susceptible to oxidative stress (56).

This protection can be attributed to normal activity levels of mitochondrial antioxidant enzymes such as catalase, GPx, and Mn-SOD, which prevent the accumulation of H$_2$O$_2$ and oxidative stress that may damage respiratory complex proteins. In mice given a high-fat diet, overexpression of catalase in muscle mitochondria attenuated H$_2$O$_2$ generation and completely preserved insulin resistance (2).

In several pathologies, such as diabetes, vascular and neurodegenerative diseases, and aging, the inactivation of mitochondrial respiratory complexes or alterations of critical subunits are also thought to produce ROS (31, 35, 44). We did not observe any significant difference in the activities of mitochondrial respiratory complexes I and III (the main source of superoxide anion generation) between the two types of mitochondria. This result suggests that mitochondrial respiratory electron transfer did not accumulate electrons, which are transferred to oxygen by monovalent reduction to form a superoxide anion. This observation is consistent with an association between FFA accumulation and increased H$_2$O$_2$ generation in SFR mitochondria.

**Redox status of mitochondria and liver homogenate.** The increased rate of H$_2$O$_2$ generation could also result from the higher activity of Cu/Zn-SOD in the intermembrane space of the mitochondria. The increased activity of this enzyme may be a response to prevent the accumulation of oxidative damage in mitochondria. Paradoxically, increased activity of Cu/Zn-SOD has also been suggested to boost the production of toxic H$_2$O$_2$ in the intermembrane space of liver mitochondria (26). The excess H$_2$O$_2$ may affect the redox state of the entire liver and decrease the concentration of reduced GSH, as reviewed elsewhere (7). In our model of high-sucrose diet-induced obesity, the amount of reduced GSH decreased by ~60% in the liver homogenate, but it decreased by only 28% in the mitochondria. The decreased level of GSH is a good indicator of altered redox status in the liver of SFR animals. The elevated activity of Cu/Zn-SOD in the intermembrane space of the mitochondria and the reduced activity of catalase in the liver homogenate may contribute to the accumulation of H$_2$O$_2$. H$_2$O$_2$ can be degraded to hydroxyl radical (HO) by the Fenton reaction,
ultimately producing high levels of MDA (a final product of lipid peroxidation) and protein carbonylation in liver homogenate from SFR. Furthermore, the decreased levels of reduced CoQ10, vitamin E, and reduced glutathione could also contribute to the increased rate of H2O2 by SFR mitochondria. Reduced glutathione, vitamin E, and CoQ10 are primary intracellular antioxidants; reduction of the intracellular content of or depletion of the levels of these compounds has been shown to reflect the generation of ROS (25, 27).

Summary. The high-sucrose diet-induced accumulation of fat in the liver did not affect the oxidative phosphorylation capacity of the mitochondria despite the increased levels of FFAs in the mitochondria and the overexpression of UCP2. In addition, the mitochondria from SFR did not exhibit increased levels of oxidative markers such as MDA and protein carbonylation despite displaying an increased rate of H2O2 generation in vitro. The increased rate of H2O2 generation could also result from decreased levels of reduced CoQ10, vitamin E, and reduced glutathione and elevated activity of Cu/Zn-SOD in the mitochondrial intermembrane space. The excess H2O2 in the intermembrane space may cross the external mitochondrial membrane and affect the redox state of the entire liver by decreasing the concentration of reduced GSH. Additionally, the reduced activity of catalase observed in the SFR liver homogenate may contribute to increased levels of lipid peroxidation and protein carbonylation in whole liver cells.

Together, our observations indicate that although mitochondria develop an internal adaptive mechanism against oxidative stress and hyperlipidemia, they are inefficient at countering redox imbalances and inhibiting FFA-induced extramitochondrial oxidative stress in whole fatty liver cells challenged by obesity resulting from long-term exposure to a high-sucrose diet.

ACKNOWLEDGMENTS

We greatly appreciate the partial support we received from the doctoral program in Biological Sciences of the National Autonomous University of Mexico to Angélica Ruiz-Ramírez.

GRANTS

This work was supported in part by the Institute of Science and Technology of the Federal District, Mexico City (ICyTDF), Mexico (Grant No. PICDS08-67).

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

A.R.R., M.C.S., J.A.P.F., E.G.Z., F.R.M., and M.E.H. performed the experiments; A.R.R., M.C.S., J.A.P.F., F.R.M., and M.E.H. analyzed the data; A.R.R. and M.E.H. interpreted the results of the experiments; A.R.R. and M.E.H. prepared the figures; A.R.R., M.C.S., J.A.P.F., E.G.Z., F.R.M., and M.E.H. approved the final version of the manuscript; M.E.H. did the conception and design of the research; M.E.H. drafted the manuscript; M.E.H. edited and revised the manuscript.

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