Uncoupling protein 2 (UCP2) uncouples respiration from oxidative phosphorylation and may contribute to obesity through effects on energy metabolism. Because basal metabolic rate is decreased in obesity, UCP2 expression is predicted to be reduced. Paradoxically, hepatic expression of UCP2 mRNA is increased in genetically obese (ob/ob) mice. In situ hybridization and immunohistochemical analysis of ob/ob livers demonstrate that UCP2 mRNA and protein expression are increased in hepatocytes, which do not express UCP2 in lean mice. Mitochondria isolated from ob/ob livers exhibit an increased rate of H⁺ leak which partially dissipates the mitochondrial membrane potential when the rate of electron transport is suppressed. In addition, hepatic ATP stores are reduced and these livers are more vulnerable to necrosis after transient hepatic ischemia. Hence, hepatocytes adapt to obesity by up-regulating UCP2. However, because this decreases the efficiency of energy trapping, the cells become vulnerable to ATP depletion when energy needs increase acutely.

Obesity results from an imbalance between energy intake and energy expenditure (1). Uncoupling protein (UCP) homologues uncouple mitochondrial respiration from oxidative phosphorylation, increasing thermogenesis while reducing the efficiency of ATP synthesis (2). While UCP1 is expressed exclusively in brown fat, UCP2 and UCP3 are also expressed in white fat and skeletal muscle (3). The tissue distribution of UCP2 and UCP3 has provoked speculation that these two proteins may be important regulators of energy homeostasis in adults (4), a possibility that is supported by evidence that the UCP2-UCP3 gene cluster maps to regions of human and murine chromosomes that have been linked to obesity (5).

Because net energy expenditure is reduced in obese subjects, UCP2 and/or UCP3 expression or activity are predicted to be decreased. However, experimental evidence for this is relatively limited. A recent study of 6 lean and 6 obese,otherwise healthy, men demonstrated a slight, but consistent, reduction in UCP2 mRNA levels in the abdominal muscle of the obese subjects (6). Polymorphisms of UCP2, but not UCP3, have been associated with decreased basal metabolic rate in young Pima Indian men, although UCP2 mRNA levels in skeletal muscle were not influenced (5). In mice, resistance to obesity induced by feeding high fat diets has been associated with an early, selective induction of UCP1 and UCP2 in brown and white fat, respectively, but not with changes in UCP3 expression (7).

On the other hand, this evidence that decreased UCP2 may promote obesity is difficult to reconcile with observations that ob/ob and db/db obese mice have increased UCP2 mRNA levels in white adipose tissue (8), and that UCP2 mRNA levels in white fat are positively correlated with body mass index in humans (9). Also confusing are reports that caloric restriction, a situation that decreases resting energy expenditure, leads to increased UCP2-UCP3 mRNA expression in white fat and skeletal muscle in both obese and lean human subjects (9) and experimental animals (4, 10). Some explanation for these paradoxical findings may be provided by recent data that correlate circulating free fatty acid concentrations with UCP2 and UCP3 induction in white fat and skeletal muscle, respectively, suggesting that increased UCP2-UCP3 may represent a metabolic adaptation of these tissues to increased fatty acid supply (11).

Of the UCP isoforms that have been identified, UCP2 has the widest tissue distribution. Low levels of UCP2 mRNA have been detected by Northern blot analysis of many organs, including the liver (8). Tissues that express low levels of UCP2 transcripts constitutively are rich in macrophages, leading to speculation that the resident macrophage populations account for basal expression of UCP2 in these organs. This concept is supported by evidence that UCP2 mRNA was identified in macrophages (Kupffer cells), but not hepatocytes, that were isolated from the livers of healthy, lean rats (12). However, similar to adipocytes and myocytes, hepatocytes play a major role in regulating intermediary metabolism and energy homeostasis (13–15). Thus, UCP2 expression may be induced in these cells during obesity. The aim of the present study was to test this hypothesis and to evaluate the implications of altered UCP2 expression on hepatic mitochondrial function.

MATERIALS AND METHODS

In total, 38 adult (10–12 week), male ob/ob mice and 38 of their ?ob (lean control) litter mates were obtained from Jackson Laboratories (Bar Harbor, MD). Mice were housed in a temperature- and light-controlled environment with free access to food and water. All experiments were conducted in accordance with NIH and Johns Hopkins University guidelines for the humane use of laboratory animals.
Evaluation of Liver UCP2 Expression

Northern Blot Analysis—Using the method of Chomczynski and Sacchi (16), total RNA was isolated from aliquots of freeze-clamped livers obtained from 14 ob/ob mice and 15 lean control mice, as described (17). After RNA concentration and quality were confirmed, UCP2 mRNA expression from each mouse was evaluated by Northern blot analysis. Briefly, RNA (20 μg/μl) was fractionated by electrophoresis on agarose gels under non-denaturing conditions, transferred to nylon membranes by capillary blotting, and hybridized overnight at 42°C with 32P-UCP2 cDNA (provided by M. D. Lane). After staining the membranes with methylene blue to demonstrate lane-lane variations in 18 S RNA, bands were exposed to X-ray film to allow quantification. As negative controls, sections from some of these livers (n = 3 ob/ob mice and 3 lean control mice) were also evaluated for UCP2 by in situ hybridization and immunohistochemistry. Liver sections were fixed overnight in 3% buffered formalin, embedded in paraffin, sectioned, and placed on polylysine-coated glass slides. For in situ hybridization, the slides were put in a 60°C incubator for 30 min, deparaffinized in xylene twice for 5 min each, rehydrated in a series of graded ethanol washes of 100, 95, and 70%, and then rinsed in distilled water. The sections were digested with 20 μg/ml proteinase K (Boehringer-Mannheim, Indianapolis, IN) in 50 mM Tris-HCl (pH 7.6) at 55°C for 10 min, then the proteinase K was inactivated by washing 3 times in distilled water with agitation. The digestion procedure was followed by quenching of the endogenous peroxidase with 3% hydrogen peroxide at room temperature for 10 min. The biotin-labeled RNA probe for UCP2 was denatured at 95°C for 5 min and added to the slides, which were then covered with coverslips and incubated at 45°C overnight in a moisture chamber.

UCP2 signal amplification and development was done according to the manufacturer’s recommendations using the catalyzed signal amplification system from the DAKO Corp. (Carpinteria, CA). Briefly, after hybridization, the coverslips were removed by soaking in TBST solution (50 mM Tris-HCl, pH 7.6, 300 mM NaCl, 0.1% Tween 20). The slides were washed under stringent conditions by incubating in 0.1 × SSC (containing 15 mM NaCl and 1.5 mM sodium citrate) at 55°C for 20 min. Signal was amplified by applying streptavidin-horseradish peroxidase (1:500 dilution in the diluent) for 15 min, washing with TBST for 5 min 3 times, applying biotinyl-tyramide solution for 15 min, and again washing with TBST for 5 min 3 times. After these amplification cycles, secondary streptavidin-horseradish peroxidase was applied for 15 min. After three 5-min washes in TBST, UCP2 mRNA was detected using the avidin-biotin-complex (ABC) method. Controls were done with UCP2 sense (absence of an unrelated oligodeoxynucleotide). Mayer’s hematoxylin was used as counterstain before mounting.

Immunohistochemistry—Formalin-fixed, paraffin-embedded sections from the same livers that had been studied by in situ hybridization were incubated with primary goat antisera (1:50, v/v) to a conserved peptide from the UCP2 gene, which was then covered with coverslips and incubated at 45°C over-night in a moisture chamber.

Mitochondria from 1 mg/ml. Oxygen uptake was measured polarographically using a Rank Brothers oxygen electrode and membrane potentials were measured using a TPP+ electrode connected to a Cole-Parmer pH meter/ion detector. Membrane potential calculations were made assuming a mitochondrial volume of 1 μg/ml of protein and a mitochondrial TPP+ binding ratio (free TPP+/total TPP+) of 0.2 (20). Previous studies had demonstrated that these parameters were not significantly different between liver mitochondria from ob/ob and lean litter mates (21–23). The determination of membrane potential dependence of the proton leak activity in isolated mitochondria is based on the protocol described by Porter and Brand (24). The dependence of the proton leak rate on the membrane potential (detected simultaneously using a TPP+–sensitive electrode) shows a marked nonlinearity at high membrane potential, but is close to linear at lower membrane potential, giving a force-flow relationship that reflects the coupling efficiency of the mitochondrial membrane. Mitochondria were incubated at 37°C in the standard incubation medium in the presence of succinate (5 mM), rotenone (2 μM), and oligomycin (5 μM). The respiration rate was titrated with malonate, an inhibitor of succinate dehydrogenase, to generate a gradually decreasing energy supply. Malonate was added from a stock solution of 0.5 M in steps of 0.3–0.5 mM (final concentration) with full inhibition being obtained at 3.3 mM. The rate of O2 uptake at different malonate concentrations was compared with the change in membrane potential determined in the same incubation from the distribution of TPP+, using an ion-selective electrode. The force-flow relationship was determined from the slope of the regression line plotting the apparent (corrected for UCP2) proton leak activity in isolated mitochondria. Essentially similar results are obtained when other substrates or other methods to inhibit electron transport activity are used (see Ref. 24 and references therein).

Evaluation of Hepatic ATP Stores

In 9 ob/ob mice and 9 controls, liver ATP stores were evaluated in mid-morning (9–11 a.m.) either by phosphorous-NMR (n = 6 ob/ob mice and 6 lean controls) or by biochemical assay of freeze-clamped liver tissues obtained immediately after sacrifice (n = 3 ob/ob mice and 3 control mice).

Biochemical Assays—Frozen aliquots of liver were homogenized on ice in chilled perchloric acid and ATP concentration was measured by luminometer using commercial kits (from Sigma) according to the manufacturer’s instructions. ATP results (micrograms of ATP/mg of liver wet weight) were normalized for variations in the DNA concentrations in an equivalent amount of liver. Liver DNA was quantitated according to the method of Bliin and Stafford (25).

31P NMR Studies—Ob/ob mice (n = 6) or lean controls (n = 6) were lightly anesthetized with nembutal (50 mg/kg of body weight) and the right lobe of the liver was exteriorized through a midline laparotomy incision. A vessel loop was loosely positioned around the hilar arterial supply and a two-turn dual-tuned (to both 1H and 31P NMR frequency), 1-cm diameter, NMR surface coil was placed on the surface of the liver. A sealed vial containing a known concentration of phenylphosphonic acid, a 31P standard, was glued to the top of the surface coil to provide an intensity reference. The animal was then placed in the center of a GE Omega CSI 4.7 Tesla NMR magnet and the magnetic field homogeneity was optimized using the 1H NMR signal. Baseline 31P NMR spectra were recorded from the liver for 20 min; each spectrum consisted of an average of 400 acquisitions and took approximately 5 min to record. Following baseline spectra, the vessel loop was tightened to occlude vascular in-flow for 15 min, this ligature was then released for 60 min of reperfusion. 31P NMR spectra were recorded continuously throughout the ischemic and reperfusion periods normalized to readings from the adjacent standard in the same animal. The means (and standard deviations) of the normalized results from the entire groups of lean or ob/ob mice were calculated and analyzed by analysis of variance using computerized statistical programs.

Evaluation of Hepatic Injury

The response to two different, sublethal, acute stresses, i.e. hepatic ischemia/reperfusion and intraperitoneal injection of LPS, were compared in ob/ob mice and lean controls. The response to ischemia/reperfusion was evaluated in the same 6 ob/ob mice and 6 lean controls that had been studied by phosphorous-NMR. An additional 8 ob/ob mice and 8 lean control mice were injected intraperitoneal with 10 μg of LPS from Escherichia coli serotype B:0111:B4 (Sigma). In both

2 Mildeziene, V., Marcinkeviucyte, A., Hoek, J. B., and Kholodenko, B. (1998) in Biothermokinetics in the Post Genomic Era (Larson, C., Pahlman, I.-L., and Gustafsson, L., eds) Proceedings of the 8th International Meeting on Biothermokinetics, Gothenburg, Sweden, in press.
studies, serum activities of the liver-associated enzyme, alanine aminotransferase (ALT), and liver histology were obtained 24 h after the stress and used to measure liver injury. ALT determinations were performed by an autoanalyzer in the Clinical Chemistry Laboratory of The Johns Hopkins Hospital. Coded, formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin and inspected for hepatic necrosis and inflammation.

RESULTS

Body and Liver Weights and Liver Histology in Obese and Lean Mice—Genetically obese ob/ob mice have a spontaneous mutation in the OB gene that prevents synthesis of the appetite suppressing hormone, leptin (26). The resultant hyperphagia contributes to obesity. Thus, the ob/ob mice used in the subsequent studies weighed about twice as much as the lean controls (48 ± 3 versus 27 ± 6 g, p < 0.001). At baseline, these ob/ob mice exhibited no overt evidence of liver disease, such as jaundice, ascites, coagulopathy, or splenic congestion. However, the livers of ob/ob mice were heavier than those of lean mice (2.1 ± 0.4 versus 1.1 ± 0.1 g, p < 0.01) and histologic evaluation demonstrated that hepatocytes in adult ob/ob mice were engorged with large and small droplets of lipid, as reported previously (17). In contrast, the hepatocytes of the lean mice contained little lipid. Thus, like peripheral adiposity, fatty liver may be an adaptive response to overeating.

Interestingly, although ob/ob mice livers were larger and fattier than those of lean mice, liver weight normalized to body weight was similar in the two groups. The liver weight was 4.4% of the body weight in ob/ob mice and 4.1% of the body weight in lean mice. In addition, evaluation of hepatocellular DNA content in ob/ob and lean livers demonstrated that, when these results were expressed per g of liver wet weight, DNA concentrations in ob/ob mice (6.9 ± 0.6 mg of DNA/g of liver) were only 15% lower than in lean controls (8.1 ± 0.1 mg of DNA/g of liver). Furthermore, since ob/ob livers weighed almost twice as much as lean livers, the total amount of liver DNA and protein per animal was actually greater in ob/ob mice than in the lean controls. These findings suggested that ob/ob livers had adapted to the animal’s obese state, at least in part, by hyperplasia.

Effect of Obesity on Uncoupling Protein Expression in the Liver—Northern blot analysis was performed to compare the hepatic expression of UCP-2 mRNAs in ob/ob mice and lean controls. As shown in Fig. 1A, UCP2 was barely detectable in “resting” livers obtained from 3 lean mice. In contrast, the livers of 3 ob/ob mice expressed more UCP-2 mRNA at baseline. Results of the UCP-2 mRNA expression in 6 ob/ob mice and 6 lean controls is graphed in Fig. 1B and indicate that UCP-2 mRNA levels are 500–600% greater in ob/ob mice than in lean controls.

In situ hybridization confirmed these differences in UCP2 expression. No UCP2 transcripts were identified in hepatocytes in lean livers (Fig. 2, A and C). In contrast, in 3/3 ob/ob mice, expression of UCP2 mRNA was apparent in all acinar zones of the liver. A representative photomicrograph illustrating UCP2 transcripts in ob/ob liver is shown (Fig. 2B). Inspection of the sections at higher magnification indicated that the UCP2 signal was distributed in the cytoplasm of hepatocytes in ob/ob liver (Fig. 2D).

Immunohistochemistry confirmed that, similar to UCP2 mRNA levels, hepatocyte UCP2 protein expression was increased in all 3 ob/ob livers but could not be detected in any of the 3 lean livers that were examined. Representative photomicrographs that demonstrate the differences in UCP2 protein expression in lean (Fig. 3A) and ob/ob (Fig. 3B) mice are shown. Unlike UCP2, UCP1 and UCP3 could not be detected in the livers of ob/ob or lean mice by RNA analysis (data not shown). Therefore, an immunohistochemical evaluation of these UCP isoforms was not done.

Effect of Obesity on Liver Mitochondrial Function—Liver mitochondria were isolated from ob/ob and lean mice and studied to determine if obesity-related differences in hepatocyte UCP2 expression influenced mitochondrial function. Table I summarizes the respiratory activities and membrane potentials with two different substrate conditions (glutamate + malate or succinate + rotenone (a complex I inhibitor)), that activate the respiratory chain either at the level of NADH or at ubiquinone. As has been reported in previous studies (22), the ob/ob mitochondria had a markedly higher rate of succinate oxidation, both in State 3 (with ADP) and State 4 (no ADP). However, they maintained a normal membrane potential and the decrease in membrane potential after the addition of ADP was similar in preparations from ob/ob mice and lean controls. Furthermore, the RCI of ob/ob mitochondria was not decreased. These results indicate that ob/ob liver mitochondria generally exhibit an increased oxidation capacity, but maintain a similar degree of respiratory control.

However, very different results were obtained when mitochondria were incubated with substrate (succinate) and complex I inhibitor (rotenone) in the presence of an agent (oligomycin) which inhibits ATP synthase. Under these conditions, the O₂ uptake is entirely due to the proton leak across the mitochondrial membrane (since the ATPase is inhibited and there is no other ion transport), which is driven by the proton motive force (existing predominantly in the form of a membrane potential). A titration with increasing concentrations of malonate (an inhibitor of succinate dehydrogenase) suppresses the supply of electrons to the respiratory chain and decreases the membrane potential that the mitochondria can maintain. Since the dissipation of the membrane potential (measured as the O₂ uptake) is entirely due to the proton leak, this experiment allows measurement of the proton leak rates as a function of the membrane potential.
Under these experimental conditions, the lean mitochondria exhibited the standard relationship between membrane potential and proton leak (Fig. 4, left panel), at low membrane potential there was very little proton leak and the increase in proton leak with increasing membrane potential was very modest. Only when the membrane potential reached about 150 mV was there a dramatic rise in the rate of proton leak. As shown in Fig. 4 (right panel), the steepness of the plot in the membrane potential range of 100–160 mV was dramatically increased by 4–5-fold when ob/ob mitochondria were studied. However, the maximal potential was approximately similar to normal mitochondria. These findings suggest that ob/ob mitochondria have an increased rate of proton leak which partially dissipates their mitochondrial membrane potential when the rate of electron transport is suppressed. These results are consistent with the presence of an uncoupling agent in ob/ob mitochondria. However, the increased uncoupling is not due to the presence of excess free fatty acids in the mitochondrial

**FIG. 2.** *In situ* hybridization of UCP2 mRNA in the livers of lean mice (A and C) and ob/ob mice (B and D). Formalin-fixed liver sections were incubated with antisense probes for UCP2 as described under “Materials and Methods.” No signal was obtained when sections from control livers were incubated with UCP2 antisense probes (A and C) or when ob/ob sections were incubated in parallel with sense UCP2 probes or with unrelated oligonucleotide fragments (data not shown). However, in ob/ob livers, UCP2 transcripts are expressed throughout the lobule (B) and the speckle-typed expression of UCP2 mRNA is localized in hepatocytes (solid arrowheads) (D). Final magnifications = 200 × (A and B); 400 × (C and D).

**FIG. 3.** Immunohistochemical evaluation of UCP2 protein in lean (A) and ob/ob (B) liver. The photomicrographs shown are representative of results in 3/3 ob/ob livers and 3/3 livers from lean controls. UCP2 appears as brown staining in hepatocytes of ob/ob mice at low (× 220) and high (× 480) magnification but is not detected in livers of lean mice (× 220).
Conditions used were: mitochondria were isolated in a medium containing sucrose (250 mM), K-Hepes (10 mM), EGTA (0.1 mM), and fatty acid-free BSA (5 mg/ml for ob/ob mice, 2 mg/ml for lean littermates). All incubations were carried out at 37 °C in medium containing KCl (100 mM), sucrose (50 mM), K-Hepes (20 mM), MgCl₂ (1 mM), K-Pi (5 mM), TPP⁺ (0.33 μM), and the final pH was 7.2. Mitochondria were added to a protein concentration of 1 mg/ml, BSA was added over with the mitochondrial addition to a final concentration of 50 or 125 μg/ml for the lean litter mates and ob/ob mice, respectively. Substrate concentrations: glutamate, 5 mM; malate, 5 mM; succinate, 2 mM; rotenone, 2.5 μM; ADP was added at 0.33 mM (with glutamate/malate as substrate) or 0.17 mM (with succinate/rotenone as substrate) to initiate the State 4-State 3 transition. RCI, respiratory control index, ratio of O₂ uptake in State 3 and State 4. Data are mean ± S.E. obtained from three or four separate preparations.

| Substrate                  | Lean Glutamate + malate | Lean Succinate + rotenone | Obese Glutamate + malate | Obese Succinate + rotenone |
|----------------------------|-------------------------|---------------------------|--------------------------|---------------------------|
| O₂ uptake (nat O/min/mg)   | 172 ± 52                | 198 ± 14                  | 177 ± 31                 | 327 ± 47                  |
| ΔΨ (mV)                    | 147.8 ± 7.2             | 150.1 ± 3.6               | 157 ± 2.8                | 165 ± 3.4                 |
| O₂ uptake (nat O/min/mg)   | 28.5 ± 4.9              | 43.4 ± 10.5               | 34.8 ± 1.4               | 75.8 ± 4.5                |
| RCI (State 3)              | 5.9 ± 0.8               | 4.8 ± 0.4                 | 5.1 ± 0.7                | 4.3 ± 0.5                 |
| RCI (State 4)              | 4.5 ± 1.8               | 2.3 ± 0.6                 | 1.4 ± 1.2                | 1.4 ± 0.6                 |

**FIG. 4.** H⁺ permeability of isolated liver mitochondria from lean and ob/ob mice. The membrane potential dependence of the proton leak rate was determined in mitochondria oxidizing succinate (5 mM) in the presence of rotenone (2 μM). Oligomycin (5 μM) was added to inhibit ATP synthase activity. Under these conditions, O₂ uptake is attributable entirely to the proton leak across the inner membrane (20, 30, 48, 49). Simultaneous measurements of membrane potential and O₂ uptake were made (see "Materials and Methods") as the membrane potential was varied by inhibition of electron transport activity by titration of succinate oxidation with malonate (to a maximal concentration of 3.3 mM). Under the conditions used (i.e. in the presence of excess phosphate), there is no significant variation of ΔpH and ΔΨ was taken as an indicator of the proton motive force. Data points from three different pairs of ob/ob mice (open symbols) and lean controls are shown (closed symbols). In one experiment, mitochondria from ob/ob mice received an additional 30-min pretreatment with fatty acid-free BSA (10 mg/ml) on ice to remove free fatty acids, as recommended by Garlid et al. (35). This treatment did not significantly affect the relationship between membrane potential and proton leak rate (as reflected in O₂ uptake). The increased slope of the force-flow relationship at lower membrane potentials in mitochondria from ob/ob livers is consistent with uncoupling.
the animals hearts were beating. The ATP levels in these ob/ob mice (208 ± 3 μmol of ATP/mg of DNA) were slightly (i.e. about 15%), but consistently, lower than in the lean controls (253 ± 6 μmol of ATP/mg of DNA) when ATP results were expressed relative to liver DNA to correct for differences in hepatic steatosis (p, 0.05).

Hepatic 31P NMR spectroscopy was used to monitor changes in the ATP levels of each individual animal over time. To assure comparability among spectra obtained in 6 different ob/ob mice and 6 lean controls, all spectra were scaled to the intensity of the resonance from the phosphate standard, which was the same in all experiments. Baseline 31P NMR spectra from lean and ob/ob mice before (baseline), after 15 min occlusion of portal venous blood (end ischemia), and after 60 min of reperfusion (end reflow). ATP stores were evaluated by 31P NMR spectroscopy. The intensity of the β-ATP resonance has been normalized to the phosphorous spectra of an adjacent phenylphosphonic acid reference standard in each animal. Results shown are the mean ± S.E. in 6 lean, control mice and the all surviving ob/ob mice at each end point (n = 4–6). *, p < 0.0001 compared with lean; †, p = 0.003 compared with lean; ‡, p = 0.04 compared with lean. Note that in the obese group, end reflow is still significantly depressed compared with baseline (p = 0.01); in the lean group this is not significantly different (p = 0.2).

Obesity Induces Expression of Uncoupling Protein-2

Fig. 5. A, 31P NMR spectra from the liver of a single, representative lean mouse (left) and a representative ob/ob mouse (right) during the basal state (top panel), at the end of the ischemic period (middle panel), and at the end of reperfusion (bottom panel). The sharp signal at the beginning of each spectrum (S) is from the phenylphosphonic acid reference in the sealed glass bulb. The next cluster of peaks contains sugar and inorganic phosphates (P). The γ-, α-, and β-phosphate peaks of ATP follow. The intensity of the β-ATP resonance is directly proportional to the ATP content of the liver and is indicated by the large arrow above each spectrum. B, hepatic ATP stores in lean and ob/ob mice before (baseline), after 15 min occlusion of portal venous blood (end ischemia), and after 60 min of reperfusion (end reflow). ATP stores were evaluated by 31P NMR spectroscopy. The intensity of the β-ATP resonance has been normalized to the phosphorous spectra of an adjacent phenylphosphonic acid reference standard in each animal. Results shown are the mean ± S.E. in 6 lean, control mice and the all surviving ob/ob mice at each end point (n = 4–6). *, p < 0.0001 compared with lean; †, p = 0.003 compared with lean; ‡, p = 0.04 compared with lean. Note that in the obese group, end reflow is still significantly depressed compared with baseline (p = 0.01); in the lean group this is not significantly different (p = 0.2).
Differences in liver ATP stores after hepatic ischemia/reperfusion are illustrated in the spectra obtained from representative lean and ob/ob mice (Fig. 5A). In the lean mouse (left panels), liver ATP decreased at the end of ischemia and was near normal after reflow. Consistent with these results, in the lean mouse, the P1 peak increased by the end of ischemia and then returned toward baseline by the end of reflow. In contrast, in the ob/ob mouse (right panels), the ATP peak virtually disappeared by the end of ischemia and remained almost undetectable after reflow. In this animal, P1 increased during ischemia but did not fall appreciably after reflow. Thus, when animals are used as their own controls, it is even more apparent that lean and obese mice exhibited very different responses to ischemia/reperfusion. The lean mice tolerated this insult with minimal depletion of their hepatic ATP stores; the ob/ob mice became profoundly ATP depleted by the same brief ischemic episode. In fact, one-third of the ob/ob animals did not survive and in survivors, hepatic ATP levels remained consistently lower than pre-ischemic values after reperfusion.

Effect of Obesity on Hepatic Sensitivity to Acute Stress—The laparotomy wound was closed in all surviving mice; the animals were permitted to recover for 24 h and then sacrificed to evaluate the sequella of the hepatic ischemic episode. Serum activity of the liver-associated enzyme, ALT, was almost 10-fold greater in ob/ob mice than in lean controls (1598 ± 207 versus 163 ± 70 IU/liter, p < 0.001). Histologic assessment of the livers from the two groups of mice confirmed that this ALT elevation reflected massive hepatic necrosis in the ob/ob group.

We previously reported that obesity increases vulnerability to endotoxin (LPS)-mediated liver injury (17). Other groups have suggested that clamping the porta hepatis to cause hepatic ischemia leads to local increases in several LPS-inducible cytokines (27). Therefore, it is conceivable that endotoxemia or endotoxin-related cytokines may have contributed to the differences in liver injury that were observed after ischemia-reperfusion. Recently, Faggioni and colleagues (28) reported that LPS injection increased UCP2 expression in the livers of normal rats. Subsequently, we showed that hepatocytes are predominately responsible for the increase in liver UCP2 expression that follows LPS injection in rats (29). Because obesity increases hepatic sensitivity to LPS-mediated injury, LPS treatment may result in a greater induction of UCP-2 in ob/ob mice than lean controls. To evaluate this possibility, 8 ob/ob mice and 8 lean controls were injected intraperitoneal with a dose of LPS (10 µg/mouse) that produces little liver injury in healthy, lean mice. As previously reported (17), ob/ob mice experienced significantly more liver injury after this treatment than did lean controls, as evidenced by 10-fold higher ALT activities at 24 h after LPS exposure (data not shown). Notably, at this time point, UCP2 mRNA levels in the livers of ob/ob mice were 6 times greater than lean control mice that had been treated with an identical dose of LPS (Fig. 6).

**FIG. 6.** Northern blot analysis of hepatic UCP2 mRNAs in lean and ob/ob mice before (0) and 24 h after intraperitoneal injection of LPS (10 µg/mouse). A, a representative blot demonstrating UCP2 expression in 2 mice/group at each time point. Top panel, UCP2 mRNA. Bottom panel, the constitutively expressed 18 S RNA on the same blot. All lanes contain 20 µg of total RNA. B, PhosphorImager results from duplicate Northern blots (n = 8 ob/ob and 8 lean control mice). On each blot, UCP2 signal intensity was normalized to 18 S RNA expression in the same sample and expressed as a percentage of the untreated (time zero) lean control UCP2 expression on the same blot. Data are expressed as mean ± S.E. *, p < 0.05 for lean LPS(+) versus lean LPS(−); +, p < 0.001 for obese LPS(−) versus lean LPS(−); **, p < 0.01 for obese LPS(+) versus lean LPS(−).

**Discussion**

Obesity is a complex syndrome that results from imbalanced intake of energy substrates and energy utilization (1). The liver plays a major role in the regulation of energy homeostasis in mammals (13, 14) and, thus, is an important target organ in obesity. The combination of increased food (energy) consumption and hyperinsulinemia produce a state in which the supply of oxidizable substrates to hepatocytes is excessive. However, since this hormonal milieu also promotes the shunting of substrates into anabolic pathways (i.e., the synthesis and storage of fat), it is difficult to predict whether obesity increases or decreases the supply of substrate that is available for hepatic mitochondrial oxidation. Previous studies on mitochondrial function in livers of ob/ob mice demonstrated an increased electron transport activity in these animals (21–23), suggesting that the mitochondria experience an adaptive response that enhances the capacity for substrate oxidation. However, the implications of this adaptation for hepatic energy metabolism are poorly understood.

Mitochondria are the major sites of ATP production in the cell. During mitochondrial respiration, electrons derived from cellular substrates are transferred serially through the electron transport chain leading to the reduction of molecular O2. The energy derived from these redox reactions is conserved in the form of the proton electrochemical gradient, which drives the synthesis of ATP through the transmembrane ATP synthase complex (30). In the presence of an abundant energy supply, the rate of mitochondrial electron transport, and hence the rate of substrate oxidation, is generally matched to the cell's need for energy. Many of the mechanisms responsible for matching these processes in the intact cell are not yet well characterized. However, experimental conditions where the supply of mitochondrial ADP is restricted in the presence of saturating substrate supply (State 4) are characterized by a limited rate of electron transport, accompanied by a highly reduced state of NAD and other electron carriers of the electron transport chain, as well as a maximal proton electrochemical gradient (30). The mitochondrial electron transport chain is also one of the predominant sites of production of reactive oxygen species (ROS), such as superoxide (O2•−), which is generated by the one-electron reduction of O2 under conditions of highly reduced electron transport intermediates (31). The State 4 condition of high redox pressure combined with a limited rate of utilization of the proton electrochemical gradient in an oxygen-rich environment promotes the formation of O2•−. Mitochondri-
ally generated \( \text{O}_2^\cdot \) reacts with water to generate hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) in a reaction catalyzed by the manganese-dependent superoxide dismutase in the matrix. \( \text{H}_2\text{O}_2 \) may itself be a source of more highly reactive intermediates, such as the hydroxyl radical (\( \text{OH}^- \)), which may damage cellular constituents, or it may affect extramitochondrial redox-dependent signaling processes. Alternatively, mitochondrially generated \( \text{O}_2^\cdot \) can react with nitric oxide (NO) to generate peroxynitrite, which may cause covalent modification of proteins (32). Hence, a careful regulation of the formation of ROS through the mitochondrial electron transport chain is important for minimizing these and other potentially damaging consequences.

Among the mechanisms ostensibly developed by a variety of organisms to control unwanted generation of ROS under conditions of high substrate supply that exceeds its energy requirements, is the activation of alternative substrate oxidation pathways that are not efficiently coupled to ATP synthesis (33). Such alternative electron transport pathways are prominent in a variety of microorganisms and plants. During the past few years it has become apparent that a similar function may be fulfilled by uncoupling proteins in the inner mitochondrial membrane. Uncoupling proteins were first described in mitochondria from brown adipose tissue, where they are activated to meet the need for heat generation (2, 34). The brown adipose tissue uncoupling protein (UCP1) catalyzes the net transfer of \( \text{H}^+ \) across the mitochondrial membrane in the presence of appropriate activators (e.g., free fatty acids) leading to the dissipation of the electron transport-driven proton electrochemical gradient, at the expense of ATP synthesis (35). In recent years, other isoforms of uncoupling protein (UCP2 and UCP3) have been described that have a more widespread tissue distribution (8, 36–38). Interestingly, recent studies have linked the activation of the UCP2 protein in mitochondria isolated from rat liver to the suppression of \( \text{H}_2\text{O}_2 \), presumably reflecting the suppression of \( \text{O}_2^\cdot \) formation through the electron transport chain (39). However, these authors demonstrate that this effect of UCP2 protein was limited entirely to mitochondria derived from non-parenchymal cells of the liver and that mitochondria obtained from hepatocytes did not contain the UCP2 protein. In agreement with this report, others have demonstrated that UCP2 is expressed in Kupffer cells in the normal liver, but not in hepatocytes (12).

The present study provides in vivo evidence that hepatocytes induce UCP2 mRNA and protein expression during obesity. Thus, when confronted with an overly abundant substrate supply that exceeds cellular energy requirements, hepatocytes, similar to many other cells (2, 33), activate pathways that are not efficiently coupled to ATP synthesis. By decreasing the efficiency with which substrate oxidation generates ATP, these responses permit the utilization of excess substrate while helping to balance ATP supply with cellular energy requirements. Furthermore, if the model is correct that excess substrate supply to the mitochondrial electron transport chain increases the probability of ROS formation, induction of UCP2 to affect uncoupling of oxidative phosphorylation may diminish the redox pressure on the mitochondrial electron transport carriers and provide an added advantage by constraining mitochondrial ROS production (40). At this point, the molecular mechanisms that are involved in this obesity-related induction of UCP2 are unknown. However, it is intriguing that hepatocytes also up-regulate UCP2 after exposure to LPS (29), a situation that is known to induce the proinflammatory cytokine, tumor necrosis factor (TNF-\( \alpha \)), and increase hepatic ROS production (41, 42). Many of the hepatic responses to LPS, including the induction of mitochondrial oxidant production (43), are mediated by TNF-\( \alpha \) and TNF-inducible cytokines. Indeed, recent evidence demonstrates that overnight exposure of primary hepatocyte cultures to recombinant TNF-\( \alpha \) induces UCP2 mRNA expression by 200–300% (29). Adipose expression of TNF-\( \alpha \) mRNA and circulating levels of TNF-\( \alpha \) protein are increased in obese humans (44) and in two strains (ob/ob and db/db) of genetically obese mice (17, 45). The latter are known to express increased UCP2 mRNA in adipocytes (46). Thus, there is a growing body of evidence that UCP2 may be a TNF-inducible gene.

Up-regulation of UCP2 activity is predicted to decrease the efficiency of energy trapping (3) and, thus, has the potential for compromising the capacity to respond to acute energy needs of the cell in conditions of stress. The baseline 5-fold increase in UCP2 mRNA levels appears to be reasonably well tolerated by hepatocytes in obese mice because this is not accompanied by release of liver enzymes or histologic evidence of liver injury. It is conceivable that this benign outcome reflects the fact that UCP2 mRNA induction does not result in increased levels of UCP2 protein under normal conditions. However, the latter seems unlikely in the hepatocytes of ob/ob mice given the present hepatic immunohistochemistry results. On the other hand, studies with mitochondria isolated from ob/ob livers suggest that the consequences of increased UCP2 expression are influenced strongly by the cellular context in which this occurs. For example, changes in the proton motive force of mitochondria from ob/ob livers could only be demonstrated under experimental conditions that limited the supply of electrons to the respiratory chain. Similarly, in living ob/ob mice, the physiological implications of hepatic mitochondrial uncoupling appeared to depend on the balance between the availability of energy substrates and cellular energy requirements. The largest differences in the hepatic ATP stores of ob/ob mice and lean controls were observed after liver blood flow was interrupted transiently. This finding is consistent either with stress-related decreases in the efficiency of mitochondrial ATP synthesis or collapse of the mitochondrial membrane potential both of which may reflect increased uncoupling protein activity. However, because the effect of UCP2 on these parameters was not tested directly, potential contributions from other factors cannot be excluded. Intrinsic differences in hepatocyte vulnerability to injury induced by endotoxin or endotoxin-induced cytokines are particularly important to consider, because obstruction of portal perfusion increases hepatic exposure to these factors (27). Of interest, the present results confirm other reports that LPS induces UCP2 expression in normal liver (28) and indicate that ob/ob livers express more UCP2 mRNA than controls when challenged with endotoxin. Thus, relative overexpression of UCP2 (and consequent ATP depletion after exposure to an endotoxin challenge that acutely consumes liver ATP (47)) may also contribute to the obesity-related vulnerability to endotoxin liver injury that has been reported previously (17).

In summary, modulation of UCP2 activity may be a mechanism that hepatocytes employ to titrate the rates of mitochondrial ATP or oxidant production. Presumably, induction of UCP2 in hepatocytes conveys some adaptive advantage during obesity. However, the up-regulation of UCP2 may become maladaptive when the hepatic environment changes abruptly, emphasizing the importance of environmental conditions in dictating the ultimate fate of metabolically active cells. Adaptive responses, including the up-regulation of uncoupling proteins, that are beneficial when the supply of exogenous substrates is overly abundant, compromise cellular viability when substrate availability becomes limited acutely.

REFERENCES

1. Rosenbaum, M., Leibel, R. L., and Hirsch, J. (1997) N. Engl. J. Med. 337, 396–407
2. Nicholls, D. G., and Locke, R. M. (1984) Physiol. Rev. 64, 1–4
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3. Ricquier, D. (1998) Biochem. Soc. Trans. 26, 120–123
4. Samec, S., Seydoux, J., and Dulloo, A. G. (1998) FASEB J. 12, 715–724
5. Walder, K., Norman, R. A., Hansen, R. L., Schrauwen, P., Neverova, M., Jenkinson, C. P., Easlick, J., Warden, C. H., Pecqueur, C., Raimbaud, S., Ricquier, D., Silver, M. H. K., Shuldiner, A. R., Solanes, G., Lowell, B. B., Chung, W. K., Leibel, R. L., Fratley, R., and Ravussin, E. (1998) Hum. Mol. Genet. 7, 1431–1435
6. Nordfors, L., Hoffstedt, J., Nyberg, C., Thorne, A., Arner, P., Schalling, M., and Lonnqvist, F. (1998) Diabetologia 41, 935–939
7. Surwit, R. S., Wang, S., Petro, A. E., Sanchis, D., Raimbaud, S., Ricquier, D., and Collins, S. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 4061–4065
8. Gimeno, R. E., Dembski, M., Weng, X., Deng, N., Shyjan, A. W., Gimeno, C. J., Iris, F., Ellis, S. J., Wolfe, E. A., and Tartaglia, L. A. (1997) Diabetes 46, 900–906
9. Millet, L., Vidal, H., Andreelli, F., Larrouy, D., riou, J.-P., Ricquier, D., Lavill, M., and Langin, D. (1997) J. Clin. Invest. 100, 2665–2670
10. Boss, O., Samec, S., Dulloo, A., Seydoux, J., Muzzin, P., and Giacobino, J.-P. (1997) FEBS Lett. 412, 111–114
11. Boss, O., Bobbioni-Harsch, E., Assimacopoulos-Jeannet, F., Muzzin, P., Munger, R., Giacobino, J.-P., and Gelay, A. (1998) Lancet 351, 1933
12. Larrouy, D., Laharrague, P., Carrera, G., Viguier-Rascande, N., Levi-Meyrueis, C., Pecqueur, C., Nibbelink, M., Andre, M., Castella, L., and Ricquier, D. (1997) Biochem. Biophys. Res. Commun. 235, 760–764
13. van den Bergh, G. (1991) J. Inherited Metab. Dis. 14, 619–632
14. Wasserman, D. H., and Cherrington, A. D. (1991) Am. J. Physiol. 260, E811–E824
15. McCulloagh, A. J., and Tavill, A. S. (1991) Semin. Liver Dis. 11, 265–277
16. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–161
17. Yang, S. Q., Lin, H. Z., Lane, M. D., Clemens, M., and Diehl, A. M. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 2557–2562
18. Benkfalvi, A., Navabi, H., Rier, B., Becker, W., Jasani, B., and Schmid, J. W. (1994) J. Pathol. 174, 223–228
19. Walden-Dembski, E., Zapatona, J., Mohren, G., and Hoek, J. B. (1992) J. Biol. Chem. 267, 3701–3709
20. Brown, G. C., and Brand, M. D. (1988) Biochem. J. 252, 473–479
21. Brady, L. J., Brady, P. S., Romos, D. R., and Hoppel, C. L. (1985) Biochemistry 24, 439–444
22. Fraser, D. R., and Trayhurn, P. (1983) Biochem. J. 214, 163–170
23. Kryukov, S., and Howland, J. L. (1978) Arch. Biochem. Biophys. 188, 15–20
24. Porter, R. K., and Brand, M. D. (1993) Nature 362, 628–630
25. Blin, N., and Stafford, D. M. (1986) Nucleic Acids Res. 3, 2303–2308
26. Campfield, L. A., Smith, F. J., and Burn, P. (1996) Horm. Metab. Res. 28, 619–632
27. Camargo, C. A. J., Madden, J. F., Gao, W., Selvan, R. S., and Clavien, P. A. (1997) Hepatology 26, 1513–1520
28. Faggioni, R., Shigenaga, M., Moser, A., Feingold, K. R., and Grunfeld, C. (1998) Biochem. Biophys. Res. Commun. 244, 75–78
29. Cortez-Pinto, H., Yang, S. Q., Lin, H. Z., Costa, S., Hwang, C. S., Neverova, M., and Diehl, A. M. (1998) Biochem. Biophys. Res. Commun. 251, 313–319
30. Nicholls, D. G., and Ferguson, S. J. (1992) Bioenergetics: An Introduction to the Chemiosmotic Theory. 2nd Ed., Academic Press, New York
31. Chance, B., Sies, H., and Boveris, A. (1979) Physiol. Rev. 59, 527–605
32. Keller, J. N., Kindy, M. S., Holtsberg, F. W., St. Clair, D. K., Yen, H. C., Gernmeyer, A., Steiner, S. M., Bruce-Keller, A. J., Hutchinson, J. B., and Mattson, M. P. (1998) J. Neurosci. 18, 687–697
33. Skulachev, V. P. (1986) Qu. Rev. Biophys. 29, 169–202
34. Nedergaard, J., and Cannon, B. (1992) in Molecular Mechanisms in Bioenergetics (Ernster, L., ed) pp. 385–420, Elsevier, Amsterdam
35. Garlid, K. D., Orosz, D. E., Modriansky, M., Vandevoorde, V., Haegeman, G., and Flers, W. (1993) EMBO J. 12, 3095–3104
36. Lee, F. Y., Li, Y., Zhu, H., Yang, S. Q., Lin, H. Z., Trush, M. A., and Diehl, A. M. (1999) Hepatology, in press
37. Kern, P. A., Sahizahreh, M., Ong, J. M., Bosch, R. J., Deen, R., and Simesol, R. B. (1995) J. Clin. Invest. 95, 2111–2119
38. Yamakawa, T., Tanaka, S.-I., Yamakawa, T., Kiuchi, Y., Isoda, F., Kawamoto, S., Okuda, K., and Sekihara, H. (1995) Clin. Immunol. Immunopath. 73, 51–56
39. Blin, N., and Stafford, D. M. (1986) Nucleic Acids Res. 3, 2303–2308
40. Campfield, L. A., Smith, F. J., and Burn, P. (1996) Horm. Metab. Res. 28, 619–632
41. Camargo, C. A. J., Madden, J. F., Gao, W., Selvan, R. S., and Clavien, P. A. (1997) Hepatology 26, 1513–1520