Induction of Endogenous Uncoupling Protein 3 Suppresses Mitochondrial Oxidant Emission during Fatty Acid-supported Respiration*

Ethan J. Anderson‡§, Hanae Yamazaki†, and P. Darrell Neufer***

From the ‡John B. Pierce Laboratory and Cellular and Molecular Physiology, Yale University, New Haven, Connecticut 06519 and §Exercise and Sport Science and Physiology, East Carolina University, Greenville, North Carolina 27834

Uncoupling protein 3 (UCP3) expression increases dramatically in skeletal muscle under metabolic states associated with elevated lipid metabolism, yet the function of UCP3 in a physiological context remains controversial. Here, in situ mitochondrial H2O2 emission and respiration were measured in permeabilized fiber bundles prepared from both rat and mouse (wild-type) gastrocnemius muscle after a single bout of exercise plus 18 h of recovery (Ex/R) that induced a ∼2–4-fold increase in UCP3 protein. Elevated uncoupling activity (i.e. GDP inhibitable) was evident in Ex/R fibers only upon the addition of palmitate (known activator of UCP3) or under substrate conditions eliciting substantial rates of H2O2 production (i.e. respiration supported by succinate or palmitoyl-L-carnitine/malate but not pyruvate/malate), indicative of UCP3 activation by endogenous reactive oxygen species. In mice completely lacking UCP3 (ucp3−/−), Ex/R failed to induce uncoupling activity. Surprisingly, when UCP3 activity was inhibited by GDP (rats) or in the absence of UCP3 (ucp3−/−), H2O2 emission was significantly (p < 0.05) higher in Ex/R versus non-exercised control fibers. Collectively, these findings demonstrate that the oxidant emitting potential of mitochondria is increased in skeletal muscle during recovery from exercise, possibly as a consequence of prolonged reliance on lipid metabolism and/or altered mitochondrial biochemistry/morphology and that induction of UCP3 in vivo mediates an increase in uncoupling activity that restores mitochondrial H2O2 emission to non-exercised, control levels.

Despite intense interest, the physiological function of mitochondrial uncoupling protein 3 (UCP3) remains unresolved and a matter of considerable debate. UCP3 is expressed almost exclusively in skeletal and cardiac muscle and shares sequence identity with UCP1, the canonical uncoupling protein of the inner mitochondrial membrane. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: UCP, uncoupling protein; ROS, reactive oxygen species; RG, red gastrocnemius; WG, white gastrocnemius; Ex/R, 2-h exercise/18-h recovery; SS, subsarcolemmal; IMF, intermyofibrillar; MES, 4-morpholineethanesulfonic acid; FFA, free fatty acid.

*To whom correspondence should be addressed: Dept. of Physiology, Brody School of Medicine, East Carolina University, Greenville, NC 27834. Tel.: 252-744-2780; E-mail: neuferp@ecu.edu.
1 The abbreviations used are: UCP, uncoupling protein; ROS, reactive oxygen species; RG, red gastrocnemius; WG, white gastrocnemius; Ex/R, 2-h exercise/18-h recovery; SS, subsarcolemmal; IMF, intermyofibrillar; MES, 4-morpholineethanesulfonic acid; FFA, free fatty acid.
Endogenous UCP3 Suppresses Mitochondrial Oxidant Emission

in vivo due to the extremely dynamic nature of the reticular morphology of mitochondria in intact cells (30). In skeletal muscle, the mitochondrial reticulum is intra- and inter-connected among two distinct populations of mitochondria (i.e. subsarcolemmal and intermyofibrillar), representing an integrated system that is thought to facilitate the distribution of energy conversion, membrane potential, and antioxidant defenses (31–35). In the present study we employed a permeabilized fiber approach to investigate the impact of an increase in endogenous UCP3 expression (induced by acute exercise plus recovery) on the control of mitochondrial function. Our findings reveal that skeletal myofibers during recovery from exercise display an overall increase in mitochondrial oxidant-emitting potential under basal state 4 conditions. This increase in the potential for free radical generation is offset by an increase in the expression of UCP3, which when activated by fatty acid and/or mitochondrial ROS, mediates an increase in uncoupling activity that suppresses H₂O₂ emission to levels comparable with controls.

MATERIALS AND METHODS

Animals and Reagents—Male Sprague-Dawley rats were obtained from Charles River Laboratory (Wilmington, MA). Mice lacking UCP3 (ucp3−/−), originally developed by Vidal-Puig et al. (10), were purchased from The Jackson Laboratory (Bar Harbor, ME). All rodents were housed in temperature (22 °C)- and light-controlled rooms and were given free access to food and water. Animals were introduced to treadmill exercise (modified Exer3; Columbus Instruments) several days before the study. This familiarization period consisted of three 5-min bouts of exercise (rats 20 m/min; mice 10 m/min) every other day. At the time of the experiments, all animals were 7–8 weeks of age. Rat experiments were conducted at The Pierce Laboratory and Yale University, whereas mice experiments were performed at East Carolina University. All three institutes are Assessment and Accreditation of Laboratory Animal Care-accredited. Procedures for both rats and mice were approved by the Animal Care and Use Committees of each institute. Amplex Red Ultra reagent was obtained from Molecular Probes. Horse-radish peroxidase was obtained from Fluka Biochemika, and all other chemicals were purchased from Sigma-Aldrich.

Study Design—To induce an increase in endogenous UCP3 expression in skeletal muscle, animals completed a bout of treadmill running at moderate intensity (~50% maximal O₂ uptake (VO₂ max), rats 20 m/min, mice 13 m/min) for 2 h or until exhaustion (unable to keep pace and support body weight), whichever occurred first. Red (RG) and white (WG) (rats) or mixed (mice) portions of the gastrocnemius muscle were obtained from anesthetized animals (100 mg/kg ketamine/xylazine intraperitoneal) either before exercise (controls), immediately following exercise (0 h), or at fixed time points thereafter (4, 8, 12, 18, 24, and 48 h). Animals studied from 0 to 18 h were food-restricted, whereas 24- and 48-h rats were provided free access to standard chow at 12 h post-exercise. At the aforementioned time points, portions of RG, WG, or mixed muscle (~300 mg) were either flash-frozen in liquid N₂, used for mitochondrial isolation, or used for preparation of permeabilized muscle fibers (~10 mg) (see below). After surgery, animals were sacrificed by cervical dislocation while anesthetized.

RNA Isolation and Reverse Transcription-PCR of UCP3 mRNA—Total RNA was isolated from 150–200 mg of tissue by a modified guanidinium thiocyanate-phenol-chloroform extraction method as described previously (36). RNA was resuspended in 50 μl of nuclease-free H₂O containing 0.1 mM EDTA. Reverse transcription of total RNA samples was performed using the Superscript II RNase H⁻ system (Invitrogen) as previously described (14), and mRNA content was determined by fluorescence-based real-time PCR (TaqMan) technology (ABI PRISM 7700 sequence detection system; Applied Biosystems). For real-time PCR, primer pairs and TaqMan probes were designed for UCP3 and β-actin from rat-specific sequence data (Entrez, NIH) using computer software (Primer Express; Applied Biosystems). For each of the genes, a Blast Search revealed that sequence homology was obtained only for the target gene of interest. TaqMan probes were labeled with minor groove binding 5’-6-carboxyfluorescein and 3’-non-fluorescent quencher. Optimal primer concentration and probe concentration was determined for each oligonucleotide set. PCR amplification was performed in triplicate according to the verification of the efficiency of the amplification, as previously reported (37). The reaction mixture consisted of diluted template, TaqMan probe, forward and reverse primers as determined from prior optimization, 2×TaqMan Universal Master-Mix (Applied Biosystems), and nuclease-free H₂O. An identical cycle profile was used for all genes: 50 °C for 2 min + 95 °C for 10 min + (94 °C for 15 s + 60 °C for 1 min) ×40 cycles. To perform relative quantification, a standard curve was established by preparing serial dilutions of a reference sample. Each dilution was run through real-time PCR, and the critical threshold cycle number was determined for each dilution and plotted against the log of the dilution. All standard curves and experimental samples were assayed simultaneously using a 384-well platform to ensure uniformity and to allow relative comparison of mRNA expression among all samples.

Mitochondrial Isolation and Immunoblot of UCP3 Protein—Mitochondria were isolated from muscle using differential centrifugation as described elsewhere (38). The resulting pellet was resuspended in Mito Buffer B containing, 200 mM sucrose, 50 mM KH₂PO₄, 50 mM K₂HPO₄, 10 mM EGTA, 5 mM NaF, 2 mM sodium orthovanadate, 2 sodium pyrophosphate, and Complete® protease inhibitor mixture (1 tablet/50 ml, Roche Diagnostics) and kept frozen at ~20 °C until protein analysis. Mitochondrial protein in each preparation was separated using SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with antibodies specific for UCP3 (Calbiochem/Novabiochem) or UCP2 (Abcam, Inc). The amount of UCP3 in each preparation was normalized to mitochondrial yield for that preparation by probing for pyruvate dehydrogenase content with a custom specific antibody for the E1α subunit (BIOSOURCE International).
Preparation of Permeabilized Muscle Fibers—The technique is partially adapted from established methods (39, 40) and has been previously described (41). Briefly, small portions (~10 mg) of RG, WG, or mixed muscle were dissected and placed in ice-cold buffer X containing 60 mM K-MES, 35 mM KCl, 7.23 mM K2EGTA, 2.77 mM CaK2EGTA, 20 mM imidazole, 0.5 mM dithiothreitol, 20 mM taurine, 5.7 mM ATP, 15 mM phosphocreatine, 6.56 mM MgCl2·6H2O (pH 7.1, 295 mosm). The muscle was trimmed of connective tissue and cut down to fiber bundles (~2 x 7 mm, 2–3 mg wet weight). Using a pair of needle-tipped forceps under a dissecting microscope, fibers were gently separated from one another to maximize surface area of the fibers in the bundle. To permeabilize the myofibers, each fiber bundle was placed in ice-cold buffer X containing 50 μg/ml saponin and incubated on a rotator for 30 min at 4 °C. The permeabilized fiber bundles were then washed in ice-cold buffer Z containing 110 mM K-MES, 35 mM KCl, 1 mM EGTA, 5 mM K2HPO4, 3 mM MgCl2·6H2O, 0.5 mg/ml bovine serum albumin, 0.05 mM pyruvate, and 0.02 mM malate (pH 7.1, 295 mosm) and remained in buffer Z on a rotator at 4 °C until analysis (<45 min) without any deterioration in mitochondrial function (respiratory control ratios of 5–7 were routinely observed using pyruvate/malate; not shown). Directly before assay, fiber bundles were washed in ice-cold buffer Z plus 5 mM pyrophosphate to deplete the fibers of endogenous nucleotides (42) and to prevent Ca2+-independent contraction of the fibers during the assay.

H2O2 Production in Permeabilized Fibers—Fluorescence of Amplex Red oxidation (index of H2O2 production) was measured continuously (ΔF/min) using a Spex Fluoromax 3 (Jobin Yvon, Ltd.) spectrofluorometer with temperature control and magnetic stirring at 37 °C (41). After establishing background ΔF (de-energized permeabilized fiber bundle in the presence of 10 μM Amplex Red, 1 unit/ml horseradish peroxidase, 10 μg/ml oligomycin, 10 μM atracyloside, 25 units/ml superoxide dismutase), the reaction was initiated by the addition of substrate. H2O2 production rate was calculated from the slope of ΔF/min after subtracting background from a standard curve established for each reaction condition. At the conclusion of each experiment, permeabilized fiber bundles were dried overnight on glass at 100 °C or freeze-dried in a lyophilizer (Labconco) overnight. H2O2 production was expressed as pmol·min⁻¹·mg dry weight⁻¹.

Mitochondrial Respiration in Permeabilized Fibers—State 4 respiration was measured at 37 °C using a modified Clark-type O2 electrode (Hansatech Instruments) in buffer Z with 10 μg/ml oligomycin and 10 μM atracyloside. After establishing a background rate of O2 consumption in the presence of a de-energized permeabilized fiber bundle, substrates were added to initiate respiration. The rate of O2 consumption was calculated in 2-min increments and expressed as nmol O2 consumed·min⁻¹·mg dry weight⁻¹.

Plasma-free Fatty Acid and Glucose—Plasma glucose and FFA concentrations were determined using standard commercially available kits according to the manufacturer instructions (Wako Chemicals).

RESULTS

Plasma Glucose and Free Fatty Acids (FFAs) during Recovery from Exercise—Plasma glucose decreased from control (non-exercised, fed rat) (p < 0.001) by 53% after 2 h of moderate-intensity treadmill exercise and remained ~30% below control through 18 h of recovery (rats food restricted 0–18 h; Fig. 1). Plasma FFA concentration was significantly (p < 0.05) elevated over control immediately after exercise (62%) and continued to rise through 8 h of recovery (141%). As expected, resumption of food intake at 12 h of recovery returned plasma glucose and FFA to near control concentrations by 24 h after exercise.

Skeletal Muscle UCP3 mRNA and Protein Expression during Recovery from Exercise—UCP3 mRNA increased (p < 0.05) in response to exercise, peaking 4 h after exercise at ~8-fold over control in RG and ~20-fold over control in WG (Fig. 2A). In both RG and WG, UCP3 mRNA remained significantly elevated through 18 h of recovery but returned to near control levels after 24 h of recovery (ad libitum food intake resumed at 12 h for the 24- and 48-h groups). Fig. 2B shows representative Western blots of mitochondrial protein extracts analyzed for both UCP3 and pyruvate dehydrogenase protein from both RG and WG muscle. UCP3 protein expression (normalized to pyruvate dehydrogenase) increased (p < 0.05) steadily during recovery, peaking 18 h after exercise at 1.7- and 3.7-fold over control in RG and WG, respectively (Fig. 2C). UCP2 protein content was unaffected by exercise in both rats and mice (Fig. 2D).

Palmitate Stimulates a GDP-inhibitable Increase in Succinate-supported State 4 Respiration in RG- and WG-permeabilized Muscle Fibers—A feature common to all mitochondrial uncoupling proteins is their ability to catalyze in the presence of fatty acids, a net increase in basal proton conductance (i.e. state 4 respiration) that is strongly inhibited by physiological concentrations of purine nucleotides (for review, Refs. 43–46). To determine whether the exercise-induced increase in UCP3 expression translates to increased UCP3 activity, fatty acid-stimulated state 4 respiration was measured in permeabilized muscle.
Malate-supported Respiration—Recent studies have shown that both exogenous (9, 21) and endogenous (23) superoxide as well as its liperoxyl derivatives (24, 25) activate UCP3-mediated proton conductance. In particular, succinate-generated superoxide, as a result of reverse electron flow through complex I, was shown to be a potent activator of UCP3 (23). To determine whether the elevated UCP3 expression evident 18 h after exercise is associated with an increase in superoxide-induced uncoupling activity, H₂O₂ emission and respiration were measured in permeabilized RG and WG fibers during respiration supported by succinate. Parallel experiments were conducted in the absence or presence of GDP to determine the role of UCP3. In control animals, maximal rates of succinate-generated mitochondrial state 4 H₂O₂ emission were identical in the absence or presence of GDP in both RG and WG. In 18-h Ex/R rats, however, maximal rates of H₂O₂ emission were significantly (p < 0.05) higher in the presence of GDP in both RG and WG as compared with experiments conducted in the absence of GDP (see Fig. 4A, Table 1), indicating the presence of UCP3-mediated uncoupling activity. Interestingly, overall rates of mitochondrial H₂O₂ emission were significantly (p < 0.01) greater in Ex/R (in the presence of GDP) as compared with controls, implying that the recovery period after exercise is associated with altered mitochondrial function that either increases the propensity for superoxide formation and/or decreases H₂O₂ scavenging efficiency. In the absence of GDP (i.e. activated UCP3), H₂O₂ emission rates were either partially (RG) or fully (WG) restored to control levels. In all experiments, maximal H₂O₂ emission was attenuated by 80–90% after the addition of rotenone, identifying and confirming the primary source of superoxide as reverse electron flow through mitochondrial complex I.

Elevated UCP3 Expression in Ex/R Fibers Suppresses Mitochondrial H₂O₂ Emission during Succinate- but Not Pyruvate/
lipid metabolism. To test this hypothesis, \( \text{H}_2\text{O}_2 \) emission and respiration rates were measured in both control and Ex/R-permeabilized fibers supported by 25 \( \mu \text{M} \) palmitoyl-l-carnitine. As with succinate, \( \text{H}_2\text{O}_2 \) emission rates in control fibers supported by palmitoyl-l-carnitine were identical in the absence or presence of GDP. However, in both RG and WG from Ex/R rats, \( \text{H}_2\text{O}_2 \) emission rates in the presence of GDP (UCP activity inhibited) were significantly \( (p < 0.05) \) higher than in controls (Fig. 5A, Table 1), similar to the findings with succinate-supported respiration, again providing evidence that mitochondria during the recovery period after exercise may be particularly susceptible to conditions favoring \( \text{H}_2\text{O}_2 \) emission. When experiments were conducted in the absence of GDP, \( \text{H}_2\text{O}_2 \) emission rates in both RG and WG fibers from Ex/R rats were well below that observed in controls, indicative of elevated UCP3-mediated proton leak.

Consistent with the \( \text{H}_2\text{O}_2 \) emission data, mitochondrial state 4 respiration supported by palmitoyl-l-carnitine was significantly affected by GDP (Table 1). In contrast with succinate, respiration and \( \text{H}_2\text{O}_2 \) emission supported by pyruvate/malate was insensitive to GDP across all experimental conditions (Table 1), consistent with the idea that UCP3 is inactive in the absence of elevated rates of superoxide formation.

Mitochondrial \( \text{H}_2\text{O}_2 \) Emission Rates Are Higher During Respiration Supported by Palmitoyl-carnitine Versus Pyruvate—Recovery from exercise is one of a number of metabolic states (fasting, high fat feeding, hyperthyroidism, etc.) in which both the reliance of skeletal muscle on lipid metabolism and expression of UCP3 are elevated (1, 11–14, 47). To determine whether state 4 respiration supported by lipid metabolism is associated with elevated ROS production, mitochondrial \( \text{H}_2\text{O}_2 \) emission rates were compared in control-permeabilized fibers during respiration supported by 25 \( \mu \text{M} \) palmitoyl-l-carnitine versus 2.5/1.0 \( \mu \text{M} \) pyruvate/malate. \( \text{H}_2\text{O}_2 \) emission rates were \( \sim 2.5-5 \)-fold and \( \sim 6-9 \)-fold higher \( (p < 0.05) \) during respiration supported by palmitoyl-l-carnitine versus pyruvate/malate in RG and WG fibers, respectively (Table 1).

Elevated UCP3 Expression Suppresses Mitochondrial \( \text{H}_2\text{O}_2 \) Emission during Respiration Supported by Lipid Metabolism—The higher rate of \( \text{H}_2\text{O}_2 \) emission generated by fatty acids (Table 1 and Ref. 29) raises the possibility that induction of UCP3 \textit{in vivo} may represent a natural means of limiting ROS emission when state 4 respiration is largely supported by (\( p < 0.05 \)) greater in both RG and WG fibers from Ex/R rats than controls. Interestingly, in the presence of GDP, overall state 4 respiration remained slightly elevated \( (p < 0.05) \) in WG fibers from Ex/R compared with controls, indicating a possible additional non-UCP-mediated proton leak. In control fibers, palmitoyl-l-carnitine-supported respiration was not affected by the absence or presence of GDP (Table 1).

Elevated UCP3 Expression Induced by Ex/R Is Associated with Reduced Mitochondrial \( \text{H}_2\text{O}_2 \) Emission/O2 Consumption—The ratio of mitochondrial \( \text{H}_2\text{O}_2 \) emission/O2 consumption provides an index of overall mitochondrial oxidant-emitting potential and is shown in Fig. 6. During respiration supported by succinate to stimulate high rates of superoxide production, GDP-inhibitable uncoupling activity is evident only in permeabilized fibers from Ex/R rats and was sufficient to restore the ratio of \( \text{H}_2\text{O}_2 \) emitted/O2 consumed to control (RG) or below (WG) control levels. During respiration supported by the more physiological substrate palmitoyl-l-carnitine, GDP-inhibitable uncoupling activity again was evident only in fibers from Ex/R rats, lowering the ratio of \( \text{H}_2\text{O}_2 \) emitted/O2 consumed by \( > 50\% \) in RG and \( > 75\% \) in WG relative to controls.

\( \text{H}_2\text{O}_2 \) Emission Remains High in \textit{ucp3}\(^{-/−}\) Mice during Ex/R—To further examine whether UCP3 mediates the Ex/R-induced increase in uncoupling activity, wild-type and \textit{ucp3}\(^{-/−}\) mice were studied before and 18 h after an exercise/recovery proto-
Endogenous UCP3 Suppresses Mitochondrial Oxidant Emission

col. Similar to rats, Ex/R in wild-type mice induced an increase in muscle UCP3 protein (Fig. 7A) that was associated with a restoration of H2O2 emission and state 4 respiration to basal rates; i.e. succinate-supported H2O2 emission was significantly \((p < 0.05)\) elevated, and state 4 respiration was significantly \((p < 0.05)\) reduced in the presence of GDP but nearly identical to unexercised controls in the absence of GDP (Fig. 7, B and C), consistent with a ROS-activated UCP3-mediated increase in proton leak. Conversely, in \(ucp3^{-/-}\) mice after Ex/R, H2O2 emission rates were significantly \((p < 0.05)\) higher, and O2 respiration rates were significantly \((p < 0.05)\) lower both in the absence and presence of GDP as compared with unexercised \(ucp3^{-/-}\) and wild-type mice in the absence of GDP. In the non-exercised condition, fibers from \(ucp3^{-/-}\) mice displayed a significantly lower rate of state IV respiration, consistent with previous data from isolated mitochondria (10). Taken together, these data demonstrate that the induction of endogenous UCP3 during Ex/R mediates a ROS-activated increase in proton leak that restores H2O2 emission and O2 respiration rates to control levels.

**DISCUSSION**

The results from the present study demonstrate that a physiological increase in endogenous UCP3 expression induced by acute exercise/recovery attenuates mitochondrial H2O2 emission and accelerates state 4 respiration in skeletal muscle of both rats and mice. Importantly, UCP3-mediated uncoupling activity was evident only in the presence of palmitate (a known activator of UCPs) or under substrate conditions known to generate substantial rates of mitochondrial ROS production (i.e. during respiration supported by succinate or palmitoyl-L-carnitine, but not pyruvate), consistent with the purported activation of UCP3 by superoxide and/or downstream lipid oxidation/peroxidation products (22–25). Interestingly, when endogenous UCP3 activity was inhibited or in the complete absence of UCP3 (\(ucp3^{-/-}\)), mitochondrial H2O2 emission was markedly higher in Ex/R than non-exercised controls, providing evidence that the recovery period after exercise is associated with a change in mitochondrial structure/function that increases the propensity for ROS production/emission. Collectively, these data demonstrate that a physiological increase in UCP3 expression and subsequent activation by fatty acids and/or elevated ROS production mediates an increase in proton conductance that restores mitochondrial H2O2 emission to levels com-

---

**FIGURE 4.** Representative experimental traces of mitochondrial state 4 H2O2 emission and O2 consumption supported by succinate in permeabilized RG (left column) and WG (right column) fibers prepared from CTL and Ex/R rats. Permeabilized, de-energized muscle fibers (de-en FB) were added to assay buffer (see “Materials and Methods”) maintained at 37°C in a thermal-jacketed cell. A basal rate of oxidation was established upon which 2.5 and 1 \(\mu\)M pyruvate/malate (pyr/mal), 0.75 and 3 \(\mu\)M succinate (succ), and 10 \(\mu\)M rotenone were added successively at the points indicated. A, typical fluorimeter traces measuring mitochondrial state 4 H2O2 emission in permeabilized RG and WG muscle fibers prepared from Ex/R rats in the presence (dark line) and absence (light line) of 1 mM GDP. Because CTL fibers (dashed line) were insensitive to GDP, one representative trace is given for both conditions. B, typical polarography traces showing state 4 respiration in paired fibers prepared from RG and WG under identical assay conditions as above. Rotenone was not added to the assay buffer during the respiration measurements. AU, absorbance units.

**TABLE 1**

State 4 respiration and H2O2 emission in permeabilized AG and WG fibers prepared from CTL and Ex/R rats

Values are the means \(\pm\) S.E. of maximal state 4 O2 consumption rates (nmol/min/100 mg dry wt\(^{-1}\)) and H2O2 emission rates (pmol/min/100 mg dry wt\(^{-1}\)). Both O2 consumption and H2O2 emission were measured in a water-jacketed cell maintained at 37°C. GDP was present at 1 mM where indicated. For substrate concentrations and all other assay conditions, see “Materials and Methods.”

| Substrate               | Muscle type | \(H_2O_2\) emission | \(O_2\) consumption |
|-------------------------|-------------|---------------------|---------------------|
|                         |             | Control \(-\)GDP     | Exercise/Recovery \(+\)GDP | Control \(-\)GDP | Exercise/Recovery \(+\)GDP |
| Pyruvate + malate       | RG          | 3.7 ± 0.4           | 2.7 ± 0.4           | 4.4 ± 1.1 | 2.8 ± 0.3 |
|                        | WG          | 2.0 ± 0.5           | 1.4 ± 0.2           | 1.8 ± 0.4 | 1.8 ± 0.3 |
| Succinate + pyruvate/malate | RG       | 43.8 ± 4.1          | 47.3 ± 1.1          | 69.9 ± 3.7 | 90.3 ± 7.1
d | 9.40 ± 0.55 | 9.40 ± 0.49 |
|                        | WG          | 58.1 ± 4.3          | 58.6 ± 6.0          | 51.9 ± 4.7 | 76.5 ± 6.0
d | 4.54 ± 0.40 | 4.15 ± 0.26 |
| Palmitoyl-L-carnitine + malate | RG       | 9.7 ± 0.4           | 11.4 ± 0.9          | 6.6 ± 1.8 | 13.6 ± 0.4
d | 1.77 ± 0.03 | 1.74 ± 0.07 |
|                        | WG          | 11.5 ± 0.7          | 12.3 ± 0.4          | 4.1 ± 1.0 | 13.4 ± 0.8
d | 0.60 ± 0.02 | 0.57 ± 0.05 |

\(\text{a} \) Significant \((p < 0.05)\) difference in rate between Ex/R and CTL for that condition.

\(\text{b} \) Significant \((p < 0.05)\) difference from \(-\)GDP.
parable with control conditions (resting, fed state). Furthermore, basal respiration supported by fatty acids generates substantial rates of ROS production (Table 1 and Refs. 29 and 48) raising the possibility that the induction of UCP3 in vivo may represent a natural means of limiting ROS emission during metabolic states in which the reliance of skeletal muscle on lipid metabolism is increased.

The archetypal uncoupling protein, UCP1, is constitutively expressed at very high levels in the mitochondria of brown adipose tissue (~10% of total mitochondrial membrane protein) (49). Under normal circumstances, UCP1 uncoupling activity is inhibited by purine nucleotides. However, in response to cold exposure, fatty acids released due to adrenergic stimulation of lipolysis override this inhibition, activating UCP1-mediated proton conductance to uncouple respiration from ATP synthesis (4, 5). The purine nucleotide binding domain of UCP1 is highly conserved in UCP3 (3), which has led to the extensive use of GDP inhibition as well as fatty acid-induced activation as criteria for the identification of UCP3-mediated proton conductance. Deciphering the physiological function of UCP3, however, has remained elusive and controversial due in part to the low concentrations of the protein present in muscle under basal conditions (~0.01–0.1% of total mitochondrial membrane protein) (50). The expression of UCP3 increases dramatically in response to various metabolic challenges (e.g. exercise, fasting, ROS exposure, etc.) (51–53), suggesting that the functional significance may lie in the transient nature of its regulation. As shown in Fig. 3, the increased presence of UCP3 in myofibers from Ex/R rats was associated with a marked increase in O2 consumption upon the addition of palmitate. More importantly, this palmitate-stimulated increase in respiration was completely inhibited by GDP, indicating an uncoupling protein-mediated effect. Although both adenine nucleotide translocase (ANT) and UCP2 represent two additional proteins that may mediate proton leak, adenine nucleotide translocase activity was blocked by inclusion of atracyloside in all experiments, whereas UCP2 was expressed at low levels in muscle and was largely unresponsive.
Endogenous UCP3 Suppresses Mitochondrial Oxidant Emission

FIGURE 7. Quantified rates of succinate-supported oxidant emission (A) and respiration (B) in permeabilized RG fibers prepared from wild-type and ucp3−/− mice in CTL and Ex/R conditions. Rates of succinate-supported mitochondrial state 4 H2O2 emission (A) and O2 consumption (B) in permeabilized RG fibers prepared from ucp3−/− and ucp3+/+ (indicated at the bottom) mice in both control (Ctl, white bars) and Ex/R (black bars) conditions and expressed in C as the ratio of H2O2 emitted/O2 consumed. The presence or absence of 1 mM GDP is as indicated at the bottom. Data are the means ± S.E. of maximal state 4 H2O2 emission (pmol-min−1·mg dry wt−1) and O2 consumption (nmol-min−1·mg dry wt−1) rates; n = 3–5 for each condition. * indicates a significant (p < 0.05) difference between Ex/R and control in the same genotype. † indicates a significant (p < 0.05) difference in rate from −GDP. ‡ indicates a significant (p < 0.05) difference in rate from +/+ for that particular condition.

to exercise (12, 47, 54). In addition, activation of uncoupling by palmitate was greater in WG than RG, consistent with the greater UCP3 induction in WG muscle.

Aside from direct activation by fatty acids, there is a growing body of evidence suggesting that UCP3 is specifically activated by mitochondrial superoxide or, more likely, lipid peroxide products derived from superoxide (8, 24). By catalyzing an increase in proton conductance, it has been suggested that activation of UCP3 serves as a feedback mechanism, relieving pressure on the electron transport system when membrane potential is high and, thus, preventing continued high rates of ROS production (24, 55, 56). Much of the evidence in support of this hypothesis has been derived from in vitro studies of isolated mitochondria using succinate, a complex II substrate that generates both a high membrane potential and a high rate of superoxide production at complex I due to reverse electron flow. In the present study increased UCP3 expression after Ex/R in both rats and wild-type mice was associated with attenuated rates of mitochondrial H2O2 emission and accelerated rates of O2 consumption during succinate-supported respiration, both of which were partially to fully sensitive to inhibition by GDP (Fig. 4, Table 1). Parallel experiments conducted in ucp3−/− mice confirmed that the increase in proton leak after Ex/R was indeed mediated by UCP3 (Fig. 7). Importantly, these experiments were conducted in the absence of added palmitate and in the presence of fatty acid-free bovine serum albumin, demonstrating that ROS-induced UCP3-mediated uncoupling does not require the presence or metabolism of fatty acids.

It was anticipated that permeabilized fibers from control and Ex/R rats would display similar high rates of H2O2 emission during succinate-supported respiration when UCP3 was inhibited by GDP. Remarkably, however, mitochondrial H2O2 emission was significantly higher in both RG and WG fibers from Ex/R versus control rats (Fig. 3, Table 1). Similarly, H2O2 emission was significantly higher (independent of GDP) in permeabilized fibers from Ex/R versus non-exercised ucp3−/− mice (Fig. 7). Together, these findings indicate that the recovery period after exercise is associated with a change in skeletal muscle mitochondrial biochemistry and/or morphology that increases the oxidant emitting potential (i.e. either accelerates the rate of H2O2 production or decreases the efficiency of H2O2 scavenging). Changes in the stoichiometry of electron transport proteins or membrane fatty acid composition can influence the intrinsic redox circuitry of the respiratory system (57, 58). There is also some evidence to suggest that mitochondrial morphology may be altered under different cellular conditions (59, 60). In skeletal muscle the extensive interconnected tubular structure of mitochondria is thought to facilitate the transmission of membrane potential as well as the distribution of antioxidative activities throughout the fiber (35). The mitochondrial reticulum is also a highly dynamic structure, regulated by a balance between protein-mediated mitochondrial fission and fusion (30). Whether fragmentation of the mitochondrial reticulum, which decreases the efficiency of ROS scavenging (59, 60), accounts for the increase in H2O2 emission in Ex/R permeabilized fibers will require further study.

Skeletal muscle transitions to an increased reliance on lipid metabolism during the recovery period after a bout of exercise (61, 62), similar to other metabolic states associated with increased UCP3 expression (1, 11–14, 47). To determine the effect of increased UCP3 expression under more physiological conditions, mitochondrial function was examined in permeabilized fibers supported by palmitoyl-L-carnitine, an activated fatty acid that bypasses carnitine palmitoyltransferase (CPT)-I and serves as a direct substrate for CPT-II and β-oxidation. Initial studies conducted in control fibers (−GDP) revealed that palmitoyl-L-carnitine-supported respiration generates ~2.5 to >5-fold higher rates of H2O2 emission in RG and WG, respectively, as compared with respiration supported by pyruvate (Table 1). St-Pierre et al. (29) have also reported higher rates of H2O2 release in isolated mitochondria supported by palmitoyl-L-carnitine, leading to speculation that UCP3 may be induced in vivo to attenuate ROS production when the contribution of β-oxidation to resting skeletal muscle metabolism is increased. The findings in the present study suggest that an increased reliance on fatty acid metabolism may have more than an acute affect on mitochondrial function; similar to the findings with succinate, H2O2 emission rates were again signifi-
Endogenous UCP3 Suppresses Mitochondrial Oxidant Emission

Significantly higher in permeabilized fibers from Ex/R versus control rats when UCP3 activity was inhibited, consistent with an apparent increase in the oxidant emitting potential of the mitochondrial reticulum in response to exercise/recovery. Importantly, in the absence of GDP (activated UCP3), palmitoyl-1-carnitine-supported H$_2$O$_2$ emission was reduced ~40% in Ex/R RG and ~75% in Ex/R WG compared with control RG and WG, lending support to the notion that the increase in expression and activity of UCP3 counteracts both the change in oxidant emitting potential and the increased rate of ROS production associated with elevated fatty acid oxidation.

Using an adenoviral approach in L6 myotubes, MacLellan et al. (20) recently reported that a ~2.5-fold increase in UCP3 protein, similar to that observed in the present study, significantly increased fatty acid oxidation. Importantly, basal O$_2$ uptake and mitochondrial membrane potential were unaffected, indicating that mild UCP3 overexpression did not induce artificial uncoupling as found in previous UCP3 overexpression studies (7, 48). Unfortunately, the potential influence of fatty acids on UCP3-mediated proton leak was not assessed, as measures of both respiration and membrane potential were made in the absence of palmitate and under low glucose conditions, likely limiting fatty acid and/or ROS-mediated activation of UCP3 uncoupling. Indeed, under high glucose conditions, UCP3 overexpression was found to decrease ROS production (20).

Only one previous study has examined the impact of increased endogenous UCP3 on mitochondrial function. Silvestri et al. (63) demonstrated that isolated mitochondria from skeletal muscle of thyroid hormone-treated (hyperthyroid) rats supported by succinate plus rotenone display a GDP-inhibitable increase in respiration and loss of membrane potential in response to titration of palmitoyl-carnitine, a response that was absent in euthyroid rats and, thus, attributed to the increased UCP3 expression in the hyperthyroid rats. However, in contrast to the present study, elevated UCP3 expression was not associated with a greater proton leak in isolated mitochondria during respiration supported by succinate. These data were interpreted as evidence against a link between mitochondrial superoxide generation and UCP3-mediated proton conductance, suggesting rather that UCP3 was exporting surplus fatty acid anions from the mitochondrial matrix to facilitate acyl-CoA oxidation. However, nigericin, which eliminates the pH gradient across the inner membrane (64), was used in the proton leak experiments. Because superoxide production at complex I is dependent on the pH gradient and substantially reduced by nigericin (65), it is unlikely that sufficient levels of superoxide were present to activate UCP3 in those experiments.

Consistent with our previous findings (41), the rate of H$_2$O$_2$ emission per O$_2$ consumed (i.e. index of mitochondrial oxidant emitting potential) in control rats was considerably higher in white as compared with red permeabilized myofibers. The extent to which inherent differences in redox circuitry influencing H$_2$O$_2$ production and/or differences in mitochondrial morphology and cyto-architecture affecting the efficiency of H$_2$O$_2$ removal may account for the apparent higher oxidant emitting potential in white myofibers is unknown. Of further consideration is the nature of the reticular network established by the two distinct mitochondrial subpopulations in muscle: subsarcolemmal (SS) and intermyofibrillar (IMF). SS mitochondria occupy the cytosolic space immediately under the plasma membrane and as such are exposed to a greater physiological O$_2$ tension than IMF mitochondria, which occupy the core of the cell along the acto-myosin filaments. SS mitochondria in fact are characterized by a higher oxidized state and greater state 4 respiration than IMF mitochondria under control conditions (33), consistent with a theory put forward recently by Skulachev (35), suggesting that SS mitochondria may act as a natural “antioxidant barrier,” protecting the core parts of the cell from reactive oxidants. The extent to which basal UCP3 activity may contribute to this protection is unclear (34, 51, 66, 67). However, arguing against a protective role for UCP3, at least under basal conditions, was the finding that basal rates of respiration and H$_2$O$_2$ emission were not affected by inhibition of UCP3 in fibers from non-exercised control rats or wild-type mice (i.e. no effect of GDP, Table 1 and Fig. 7). Nevertheless, the theory is intriguing considering that WG muscle fibers, which contain less SS mitochondria than RG fibers (68), show a much greater induction of UCP3 during recovery from exercise (Fig. 2). Moreover, SS mitochondria display a greater increase in UCP3 protein in response to fasting than IMF mitochondria (34, 51). The implication is that WG myofibers may have a greater propensity to emit ROS during metabolic states when the reliance on fatty acid metabolism is elevated, thereby requiring a greater induction of UCP3 as a compensatory mechanism to attenuate ROS emission.

In conclusion, the data in the present study suggest that the induction and activation of UCP3 in skeletal muscle is a physiological response that counteracts both the increased fatty acid-supported H$_2$O$_2$ emission and increased oxidant-emitting potential of the mitochondria during periods in which the muscle is predominantly reliant on lipid metabolism. The resulting increased mitochondrial uncoupling activity appears to function as a negative feedback mechanism to restore mitochondrial ROS emission to basal levels under this metabolic condition.

REFERENCES
1. Gong, D. W., He, Y., Karas, M., and Reitman, M. (1997) J. Biol. Chem. 272, 24129–24132
2. Matsuda, J., Hosoda, K., Itoh, H., Son, C., Doi, K., Tanaka, T., Fukunaga, Y., Inoue, G., Nishimura, H., Yoshimasa, Y., Yamori, Y., and Nakao, K. (1997) FEBS Lett. 418, 200–204
3. Vidal-Puig, A., Soriano, G., Grujic, D., Flier, J. S., and Lowell, B. B. (1997) Biochem. Biophys. Res. Commun. 235, 79–82
4. Locke, R. M., Rial, E., Scott, I. D., and Nicholls, D. G. (1982) Eur. J. Biochem. 129, 373–380
5. Rial, E., Poussett, A., and Nicholls, D. G. (1983) Eur. J. Biochem. 137, 197–203
6. Rolfe, D. F., and Brand, M. D. (1996) Am. J. Physiol. 271, C1380–C1389
7. Cadenas, S., Echtay, K. S., Harper, J. A., Jekabsons, M. B., Buckingham, J. A., Grau, E., Abuin, A., Chapman, H., Clapham, J. C., and Brand, M. D. (2002) J. Biol. Chem. 277, 2773–2778
8. Echtay, K. S., Esteves, T. C., Pakay, J. L., Jekabsons, M. B., Lambert, A. J., Portero-Otin, M., Pamplona, R., Vidal-Puig, A. J., Wang, S., Roe buck, S. J., and Brand, M. D. (2003) EMBO J. 22, 4103–4110
9. Echtay, K. S., Roussel, D., St-Pierre, J., Jekabsons, M. B., Cadenas, S., Stuart, J. A., Harper, J. A., Roe buck, S. J., Morrison, A., Pickering, S., Clapham, J. C., and Brand, M. D. (2002) Nature 415, 96–99
