UCP1 and Defense against Oxidative Stress
4-HYDROXY-2-NONENAL EFFECTS ON BROWN FAT MITOCHONDRIA ARE UNCOUPLING PROTEIN 1-INDEPENDENT

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Uncoupling proteins have been ascribed a role in defense against oxidative stress, particularly by being activated by products of oxidative stress such as 4-hydroxy-2-nonenal (HNE). We have investigated here the ability of HNE to activate UCP1. Using brown fat mitochondria from UCP1+/+ and UCP1−/− mice to allow for identification of UCP1-dependent effects, we found that HNE could neither (re)activate purine nucleotide-inhibited UCP1, nor induce additional activation of innately active UCP1. The aldehyde nonenal had a (re)activating effect only if converted to the corresponding fatty acid by aldehyde dehydrogenase; the presence of a carboxyl group was thus an absolute requirement for (re)activation. The UCP1-dependent proton leak was not increased by HNE but HNE changed basal proton leak characteristics in a UCP1-independent manner. In agreement with the in vitro results, we found, as compared with UCP1+/+ mice, no increase in HNE/protein adducts in brown fat mitochondria isolated from UCP1−/− mice, irrespective of whether they were adapted to thermoneutral temperature (30 °C) or to the cold (4 °C). The absence of oxidative damage in UCP1−/− mitochondria was not due to enhanced activity of antioxidant enzymes. Thus, HNE did not affect UCP1 activity, and UCP1 would appear not to be physiologically involved in defense against oxidative stress. Additionally, it was concluded that at least in brown adipose tissue, conditions of high mitochondrial membrane potential, high oxygen tension, and high substrate supply do not necessarily lead to increased oxidative damage.

A generally accepted view of the molecular mechanism of UCP1 function and regulation has not been achieved (reviewed in Refs. 1–4). Roles for purine nucleotides as inhibitors and for fatty acids as (re)activators are generally accepted, but the issue has been raised as to whether alternative physiological activators exist and/or whether certain cofactors are necessary for UCP1 activity. These discussions may also be relevant concerning the phylogenetically related mitochondrial membrane proteins UCP2 and UCP3 (for review, see Refs. 5–8).

Particularly, to explain the lack of constitutive uncoupling activity of uncoupling proteins reconstituted in liposomes, or the lack of basal uncoupling (inhibitable by GDP) in skeletal muscle mitochondria expressing UCP3 (9), it has been suggested that non-fatty acid cofactors are required for activation, ubiquinone (10) and superoxide (11) being those originally suggested. Although the necessity for additional activators has been questioned in reconstituted systems (12), in isolated mitochondria (13) and in animals (14), several other cofactor candidates have been discussed. A series of compounds that are formed downstream of superoxide interaction with the mitochondrial membrane have been proposed to be the true activators of UCPs (or to be directly involved in the uncoupling mechanism). These include carbon-centered radicals (15), hydroperoxy fatty acids (16, 17), and lipid peroxidation products, e.g. 4-hydroxy-2-nonenal (HNE) (3, 6, 24). The question of a cofactor necessity for UCP function has thus been linked to the earlier suggestion that UCP activity can control the formation of reactive oxygen species (ROS) (18). A much discussed (8) scheme has been proposed where conditions leading to increased oxygen stress lead to superoxide production, which results in the formation of compounds such as HNE that could activate UCPs. This should lower the mitochondrial membrane potential and, consequently, diminish the risk of oxidative damage (3, 6).

Due to the broad implications of this hypothesis for pathological processes, we have here studied the proposed interaction between HNE and the originally identified uncoupling protein UCP1. The choice of UCP1 was made because it is uncontroversial that UCP1 functions as an uncoupling protein; the proton leak associated with UCP1 is thus easily observable. We have used the availability of UCP1-ablated mice (19) to enable us to dissociate the UCP1-dependent effects of HNE from UCP1-independent effects, which may occur in the mitochondria.

We conclude that the reported HNE effects on brown fat mitochondria are UCP1-independent. In vivo studies confirm that no protective effect of UCP1 can be identified physiologically. Our results may have significance for the understanding of the regulation not only of UCP1 but also of the other uncoupling proteins and may also contribute to the identification of physiological conditions associated with the increased risk of oxidative damage.

EXPERIMENTAL PROCEDURES

Animals—UCP1-ablated mice (progeny of those described in Ref. 19) were backcrossed to C57Bl/6 mice for 10 generations and after intercrossing were maintained as UCP1−/− and UCP1+/+ strains. The mice were fed ad libitum (R70 Standard Diet, Lactamin), had free access to water, and were kept on a 12:12-h light:dark cycle, routinely at normal (24 °C) animal house temperature. Adult (8–12-week-old) male mice were routinely used for the experiments.

For experiments on warm-acclimated animals, UCP1−/− and UCP1+/+ adult male mice were divided into age- (7–8-week-old) and body weight (23–24 g)-matched groups, one per cage, and acclimated at 30 °C (i.e.

3 The abbreviations used are: HNE, 4-hydroxy-2-nonenal; UCP, uncoupling protein; ROS, reactive oxygen species; BSA, bovine serum albumin; DHE, dihydroethidium; SOD, superoxide dismutate; MDA, malonyl dialdehyde; Tes, 2-[2-hydroxyethyl]-1-bis(hydroxyethyl)aminomethane sulfonic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

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mitochondrial preparations from wild-type mice were used as control. 

**Mitochondrial Preparations**—Brown fat mitochondria were prepared principally as described (21) with some modifications (22). Mitochondrial protein concentration was measured using the fluorescamine method (23), and the suspensions were diluted to stock concentrations of 25 mg of mitochondrial protein/ml of 125 mM sucrose with 0.2% (w/v) in experiments with warm-acclimated mice) fatty acid-free bovine serum albumin (BSA).

**Oxygen Consumption**—Isolated mitochondria, at final concentrations of 0.5 or 0.3 mg (indicated in figure legends) of mitochondrial protein/ml, were added to 1.1 ml of a continuously stirred incubation medium consisting of 125 mM sucrose, 20 mM K+-Tes (pH 7.2), 2 mM MgCl2, 1 mM EDTA, 4 mM potassium P, and 1.3 μM of oligomycin/ml. The substrates were 5 mM pyruvate plus 3 mM malate or 5 mM glycerol 3-phosphate in the presence of 2 μg of rotenone/ml. UCP1+/+ and UCP1−/− brown fat mitochondria exhibit identical oxidative capacities, estimated as maximal rates of FCCP-stimulated respiration (22). The final mitochondrial concentration of fatty acid-free BSA was adjusted to 0.1% (w/v) for oxygen consumption measurements performed in mitochondria respiring on pyruvate plus malate. This was increased to 1% (w/v) in experiments where glycerol 3-phosphate was used as substrate (as indicated (i.e. similarly to Ref. 24). Oxygen consumption rates were monitored with a Clark-type oxygen electrode (Yellow Springs Instrument Co.) in a sealed chamber at 37 °C, as described (22). Data for GDP concentration-response curves were analyzed with the general fit option of the KaleidaGraph application for Macintosh for adherence to simple Michaelis-Menten kinetics, \( V(x) = V_{max} - \Delta V_{max} \cdot x / (K_m + x) \), where \( x \) is the concentration of GDP.

**Mitochondrial Membrane Potential**—Measurements were performed in brown fat mitochondria with the dye safranin O (25). Mitochondria were incubated with 2 μg/ml oligomycin, 5 μM safranin, 0.1% (w/v) fatty acid-free BSA, and 5 mM glycerol 3-phosphate in the presence of 2 μg of rotenone/ml. The changes in absorbance of safranin O were followed at 37 °C in an Amino DW-2 dual-wavelength spectrophotometer at 511–533 nm with a 3-nm slit. Signals were recorded every 0.5 s via a PowerLab/ADInstrument. The data were stored and analyzed using the Chart version 5.1.1 program (PowerLab/ADInstrument). Calibration curves were analyzed using the Chart 4.1.1 program (PowerLab/ADInstrument). The absorbance readings were used to calculate the membrane potential (mV) by the Nernst equation according to: \( \Delta \psi_m = 61 \cdot mV \cdot \log (K_{in}^{+}/K_{out}^{+}) \). To determine the basal proton leak, mitochondrial membrane potential and oxygen consumption measurements were performed in parallel with the same media and conditions, but in the presence of increasing amounts of antimycin A, as indicated.

**Western Blotting**—For HNE-adduct detection, aliquots of freshly isolated mitochondrial suspension were stored under nitrogen at −80 °C after supplementation with protease inhibitor mixture (Complete Mini, Roche). Protein concentrations of the thawed mitochondrial samples were quantified using the Lowry method. Equal amounts of mitochondrial protein were loaded on SDS-polyacrylamide gel. After electrophoresis, proteins were transferred by electroblotting to a polyvinylidene difluoride membrane. HNE protein adducts were detected with polyclonal antibodies from Alpha Diagnostics (HNE12-S, dilution 1:1000). After incubation with horseradish peroxidase-conjugated secondary antibodies, the membrane was incubated with detection reagent (ECL, Amersham Biosciences) and the chemiluminescence signal was detected with a CCD camera (Fuji). Quantifications were performed with the Image Gauge 3 software.

For UCP1 determination, the membrane used for detection of the HNE adducts was stripped and blotted with UCP1 polyclonal antibodies (prepared in rabbit from the C-terminal peptide of mouse UCP1), dilution 1:3000. For cytochrome oxidase subunit 1 determination, the membrane used for detection of HNE adducts and UCP1 was blotted, after stripping, with cytochrome oxidase subunit 1-monomoclonal antibodies (Molecular Probes), diluted 1:2000.

**Superoxide Measurement**—Net superoxide release rates were assessed directly in isolated brown fat mitochondria by fluorescence measurements with the dye dihydroethidium (DHE) (Molecular Probes), the conversion of which to ethidium is superoxide-induced (14, 27). The fluorescence emitted by the ethidium formed was followed on a spectrophotometer (Sigma ZES II) at 37 °C using an excitation wavelength of 495 nm and collecting the emission via a cutoff filter at 580 nm. The data were acquired, stored, and analyzed using the Chart 4.1.1 program (PowerLab/ADInstrument). Chemical and biological validations of this method were reported in Ref. 14: generation of superoxide by the xanthine plus xanthine oxidase system or by mitochondria in the presence of DHE resulted in a significant increase in ethidium-emitted fluorescence, which was blocked by addition of recombinant SOD (14). When estimated by this method, superoxide generation was diminished in brown fat mitochondria from hSOD2−/− mice (i.e. mitochondrial superoxide dismutase-overexpressing mice) as compared with wild-type mice (14). The assay thus detected mitochondrial superoxide release.

**Aconitase and Citrate Synthase Activities**—Aconitase activity was measured spectrophotometrically as NADPH formation, monitored at 340 nm using a coupled assay (28). The frozen mitochondrial samples (the same as those used for Western blotting) were rapidly thawed immediately prior to assay, and 2 μl of 10 times diluted sample were added to 500 μl of assay buffer (50 mM Tris-HCL, pH 7.4, 0.6 mM MnCl2, 5 mM sodium citrate, 0.2 mM NADP+, 0.1% (v/v) Triton X-100, and 0.4 units/ml isocitrate dehydrogenase (Sigma)) pre-equilibrated to 30 °C. Each sample was assayed in duplicate; readings were taken at 15-s intervals over 7 min, and the resulting linear slopes were averaged to give a measurement of aconitase activity for that sample.

Superoxide inactivates the Krebs cycle enzyme aconitase, whereas citrate synthase, another Krebs cycle enzyme, is insensitive to superoxide; therefore, the aconitase/citrate synthase ratio is a convenient measure of oxidative damage in mitochondria (28). Citrate synthase activity was determined as in Ref. 29. To validate the assay and to induce excessive oxidative damage, brown fat mitochondria were also exposed to a superoxide-generating system (370 μM xanthine plus 23 μg/ml xanthine oxidase) for 15 min at 30 °C. Confirmation that this system provides a high level of superoxide was obtained in Ref. 14.

**Lipid Peroxidation**—Interscapular brown adipose tissue was rapidly dissected from UCP1+/− and UCP1+/+ mice and immediately frozen in

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*H. Feldmann, B. Cannon, and J. Nedergraas, unpublished observations.*
liquid nitrogen. The wet weight of brown adipose tissue was measured and the left lobe of tissue was used for the lipid peroxidation assay and the right lobe for the determination of antioxidant enzyme activities.

The amount of peroxidative reactants was estimated as the formation of thiobarbituric acid-reactive substances (mainly the lipid peroxidation product, malonyl dialdehyde (MDA)) (30). Brown adipose tissue homogenates were prepared in 50 mM Tris-HCl buffer (pH 7.4) in a 1:10 ratio (w/v) and sonicated three times at 100 watts for 20 s with 10-s pauses in a Bronson model B-12 sonicator. The samples were then centrifuged at 20,000×g for 60 min. Supernatants were used for the determination of antioxidant enzyme activities. Inducible peroxidative reactions were stimulated by addition of 312.5 μM ascorbic acid and 6.25 μM FeSO₄ (final concentrations) and incubation at 37 °C for 1 h. Spontaneous peroxidative reactions were analyzed in samples incubated in parallel without addition of ascorbic acid and FeSO₄.

**Antioxidant Enzyme Assays**—The right lobe of interscapular brown adipose tissue was homogenized in buffer containing 250 mM sucrose, 50 mM Tris-HCl, and 1 mM EDTA (pH 7.2), in a 1:10 ratio (w/v) and sonicated three times at 100 watts for 20 s with 10-s pauses in a Bronson model B-12 sonicator. The samples were then centrifuged at 20,000×g in an Eppendorf centrifuge for 60 min. Supernatants were used for determination of manganese-containing mitochondrial superoxide dismutase 2 (Mn-SOD), CuZn-containing cytosolic superoxide dismutase 1 (CuZn-SOD), and catalase.

SOD activity was measured by the adrenaline method (31). 100 μl of acidified adrenaline solution was added to 3 ml of alkaline carbonate buffer (50 mM Na₂CO₃, 0.1 mM EDTA, pH 10.2), and absorbance of adrenochrome at 480 nm was monitored for 4 min. The decrease in the rate of change of the absorbance caused by the samples was followed. One unit of SOD was defined as the amount of enzyme reducing the rate of autoxidation of adrenaline by 50%. Mn-SOD-specific activity was obtained by inhibiting CuZn-SOD for 20 min at room temperature with 4 mM KCN (final concentration) before the dismutase assay. CuZn-SOD activity was calculated by subtracting Mn-SOD activity from the total SOD activity. Catalase activity was measured spectrophotometrically by a method (32) based on the rate of hydrogen peroxide degradation by the catalase contained in the examined samples.

**Chemicals**—Fatty acid-free bovine serum albumin, fraction V, was from Roche Diagnostics GmbH (Germany). HNE was from Cayman Chemical (Ann Arbor, MI). Nonanoic acid and fluorescamine (4-phenyl spiro-[furan-2(3H),1-phthalan]-3,3′-dione) were from Fluka Chemie GmbH. 2-Nonenoic acid was from CHEMOS GmbH (Regensburg). Other chemicals were all from Sigma. GDP was dissolved in 20 mM Tes (pH 7.2) and the pH of the solution readjusted. FCCP was dissolved in 95% ethanol and diluted in 50% ethanol; oligomycin and rotenone were dissolved in 95% ethanol. Nonanoic acid, 2-nonenoic acid, 2-nonenal, all-trans-retinal, and HNE were dissolved in 95% ethanol, divided into small aliquots, and stored under nitrogen at −80 °C. Ethanol in a final concentration of 0.1% did not in itself have any effects on the parameters measured.

**Statistics**—All data are expressed as mean ± S.E. Statistical analysis for the comparison of two groups was performed using Student’s t test.
The presence of a carboxyl group is an absolute requirement for compounds (re)activating UCP1—HNE, 2-nonenal, and all-trans-retinal have been suggested as general UCP activators (24). We have examined their effects on UCP1, i.e., the uncoupling protein found in brown fat mitochondria and the only uncoupling protein with a verified physiological uncoupling ability.

We recently demonstrated a role for fatty acids as kinetically competitive (re)activators of UCP1 in GDP-inhibited brown fat mitochondria (22). Our interpretation of those experiments was that fatty acids only function to overcome the GDP inhibition, in our hands in a kinetically simple competitive manner, and that fatty acids do not participate in the uncoupling function of UCP1 as such. We therefore initially examined the ability of HNE to reactivate GDP-inhibited UCP1, i.e., to have a mechanism of action similar to that of fatty acids. To identify the UCP1-dependent effects, we examined the effect of HNE in brown fat mitochondria isolated from both UCP1+/+ and UCP1−/− mice.

For comparison of activating efficiency, we first determined the effects of the corresponding fatty acids 2-nonenic and nonanoic acid. In brown fat mitochondria without UCP1, small uncoupling effects of these fatty acids were observed (Fig. 1, A–C, thin lines), indicating a minor UCP1-independent uncoupling mediated by these fatty acids. Both fatty acids were able to (re)stimulate oxygen consumption (pre-inhibited by GDP) in UCP1+/− mitochondria (Fig. 1, B and C, thin lines).

The UCP1-dependent effect was estimated as the difference between the fatty acid effects in the two mitochondrial preparations (UCP1+/+ and UCP1−/−) (Fig. 1D). As seen, the efficiency and apparent affinity were essentially identical for 2-nonenic and nonanoic acid. The (re)activation ability was thus independent of the presence or absence of a double bond in the fatty acid.

The activator candidate, the aldehyde HNE, had practically no effect in UCP1−/− mitochondria (Fig. 1, A–C, heavy lines). Similarly but notably, no ability to stimulate oxygen consumption was found in UCP1+/− mitochondria (Fig. 1, B and C, heavy lines). Thus, there was no UCP1-dependent effect of HNE at all (Fig. 1D). HNE cannot (re)activate GDP-inhibited UCP1. Consequently, in contrast to the corresponding fatty acids, aldehydes were not able to reactivate GDP-inhibited UCP1 in brown fat mitochondria. Because this inhibited state of UCP1 probably mimics the UCP1 state in unstimulated brown fat cells, it is not evident how HNE could activate UCP1 in situ.

Oxidized aldehyde can activate UCP1—Mammalian mitochondria possess several highly active pathways for HNE metabolism, and one, aldehyde dehydrogenase, has as its product hydroxy-nonenonic acid (33). Because a fatty acid is thus a major product of HNE metabolism, we investigated the effect of supplying isolated UCP1+/+ mitochondria with aldehyde dehydrogenase (plus NAD+) when studying the effect of the aldehyde 2-nonenal (Fig. 2). Nonenal did not in itself reactivate GDP-inhibited UCP1 (Fig. 2A). The presence of aldehyde dehydrogenase...
ase plus NAD\(^+\) dramatically altered the response of the mitochondria to the aldehyde. Addition of 2-nonenal now stimulated UCP1-dependent oxygen consumption to the same extent as did 2-nonenolic acid (Fig. 2, B and C). This demonstrated that the aldehyde has a stimulatory effect only if converted to a fatty acid.

Thus, HNE (Fig. 1), 2-nonenal (Fig. 2), or all-trans-retinal (data not shown) could not stimulate oxygen consumption in an UCP1-dependent manner in brown fat mitochondria. Conversion of the aldehyde into a fatty acid led to stimulation of oxygen consumption in UCP1\(^{-/-}\) mitochondria. The presence of a carboxyl group is thus an absolute requirement for compounds (re)activating UCP1.

**No HNE Activation of Uninhibited UCP1**—Although the above experiments demonstrate that HNE cannot (re)activate GDP-inhibited UCP1, it would be possible that HNE could function as an activator for (or enhance the activity of) UCP1 when UCP1 is in its uninhibited state. Indeed, it has been reported by Echtay et al. (24) that HNE stimulates a proton conductance inhibitable by GDP in brown fat mitochondria. The inhibition by GDP has been interpreted to indicate that the HNE effect is on UCP1 activity. To ensure that HNE activation of UCP1 (or enhancement of activity) would be manifest in our experiments we used experimental conditions similar to those used by Echtay et al. (24). We again utilized UCP1-ablated mice to identify...
possible UCP1-independent effects of HNE, allowing us to clearly identify the contribution of UCP1 to the response to HNE in UCP1-possessing brown fat mitochondria.

For experiments examining possible augmentation of UCP1 activity, it is necessary to investigate brown fat mitochondria, which have a low initial degree of uncoupling, i.e. brown fat mitochondria where the innate respiratory rate is clearly limited by UCP1 activity and not by total respiratory chain activity. To be certain of this, we used brown fat mitochondria from warm-acclimated mice (Fig. 3) (also similar to Echtay et al. (24)). In such mitochondria from UCP1+/− mice, glycerol 3-phosphate-supported respiration was rather low and could be increased severalfold by FCCP (Fig. 3A, thin line). In these mitochondria lacking UCP1, there was, as expected, no effect of HNE (Fig. 3A, heavy line). In UCP1+/− mitochondria, the basal rate of glycerol 3-phosphate oxidation was twice as high as in UCP1−/− mitochondria (Fig. 3A and B), principally as expected because of the presence and activity of UCP1. As a further stimulation by FCCP could be observed (Fig. 3B), the initial respiration was limited by the level of activity of UCP1. Thus, if this activity was limited by the lack of activator, any activator effect of HNE should be easily manifest. However, even here, where UCP1 activity was rate-limiting for respiration and no GDP was present, HNE was without stimulatory effect (Fig. 3B). Thus, HNE could not enhance uninhibited UCP1 activity.

We further analyzed whether brown fat mitochondria treated with HNE became less sensitive to GDP (physiologically this would mean being more resistant to the inhibition by the high purine nucleotide concentration that normally occurs in the cell). There was no GDP effect and no HNE modulation in UCP1−/− mitochondria (Fig. 3C). The expected GDP inhibitory effect was observed in the UCP1+/− mitochondria (Fig. 3D), decreasing the respiratory rate to that observed in UCP1−/− mitochondria. Notably, the degree of inhibition was not changed by the presence of HNE in UCP1+/− mitochondria (Fig. 3D). The GDP concentration-response curve was also unaffected by HNE (Fig. 3E). Thus, HNE was neither able to augment the activity of uninhibited UCP1 nor to modulate the sensitivity of UCP1 to GDP inhibition.

Effect of HNE on Proton Leak Is UCP1-independent—The activating effects of HNE have generally been demonstrated as an increase in proton leak parameters and not only as respiratory rates, as performed above (although the classical bioenergetic prediction would be that an increased proton leak should also be observable as an increased respiratory rate under state 4 conditions). We therefore characterized proton leak in the UCP1-limited system examined above. We measured the relation between mitochondrial membrane potential and oxygen consumption under well established standard conditions enabling characterization of basal proton leak kinetics (Fig. 4), here by successive inhibition of respiration with antimycin A (Fig. 4A). Basal respiration (as expected and also seen in Fig. 3) was higher in UCP1+/− than in UCP1−/− mitochondria (Fig. 4A), but the respiration in both types of mitochondria was inhibited by antimycin with approximately the same EC50 (Fig. 4A, thin lines). HNE did not significantly influence the sensitivity of glycerol 3-phosphate respiration to antimycin A (Fig. 4A).

The initial membrane potential was higher in UCP1+/− than in UCP1−/− mitochondria (Fig. 4B), as expected and shown earlier (34), but as the amount of UCP1 present here was low, the difference in potential between UCP1−/− and UCP1+/− was only about 20 mV. With increasing antimycin A concentrations, the successively inhibited respiration could not counteract the proton leak and maintain a high membrane potential, and the membrane potential consequently decreased to
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120 mV in both types of mitochondria (Fig. 4B, thin lines). There was no effect of HNE at low concentrations of antimycin but as the antimycin concentration was successively increased, a more pronounced decrease of membrane potential was observed in the HNE-treated mitochondria (Fig. 4B, heavy line). Unexpectedly, this was observed not only in UCP1−/− mitochondria but also in brown fat mitochondria without UCP1 (Fig. 4B).

These data (Fig. 4, A and B) are plotted to show classical proton leak kinetics in Fig. 4C. As expected, proton leak was higher in UCP1−/− mitochondria than in UCP1+/− mitochondria (Fig. 4C, thin lines). The proton leak thus ascribable to UCP1 can be identified by subtraction (UCP1+/− − UCP1−/−) and is shown in Fig. 4D (thin line). In the non-treated brown fat mitochondria, the UCP1-mediated leak is thus characterized by a virtually pseudo-ohmic current with a slope of 4.4 nmol of O$_2$·min$^{-1}$·mg$^{-1}$ per mV (pseudo-ohmic because, in a diode-like fashion, it is not opened until ≈120 mV).

When plotted as a proton leak, there were also clear effects of the presence of HNE (Fig. 4C, heavy lines). However, the effect of HNE on leak current characteristics seemed similar in UCP1+/− mitochondria and in UCP1−/− mitochondria (Fig. 4C). An activating effect of HNE on UCP1 would be expected to increase the proton leak ascribable to UCP1. However, the proton leak ascribable to UCP1 in HNE-treated mitochondria was not increased (Fig. 4D); if anything, it was slightly lower (2.5 nmol of O$_2$·min$^{-1}$·mg$^{-1}$ per mV) than that observed in the absence of HNE (4.4). Thus, HNE did not increase the proton leak that can be ascribed to UCP1.

No UCP1-dependent Formation of HNE Adducts in Brown Adipose Tissue Mitochondria—HNE is a major product of endogenous lipid peroxidation (35) and expected to be formed under conditions of increased oxidative stress. In the established view, superoxide production from the respiratory chain is increased when membrane potential is high and respiration is low (i.e., in coupled mitochondria), when there is also a high oxygen tension and a high concentration of mitochondrial sites capable of generating superoxide (36). Under such conditions, superoxide should be formed, leading to the generation of HNE. HNE is a highly reactive compound that can react with several functional groups in mitochondrial proteins to form thioesters and Michael adducts (37–39). In contrast, in uncoupled mitochondria, superoxide production should be low, HNE should not be formed and thus no HNE/protein adducts should be generated.

The HNE/protein adducts are stable compounds, and post-mortem analysis of adduct density in the proteins of isolated mitochondria
should therefore reveal the intensity of the oxidative stress that had occurred in situ. We therefore analyzed whether the expected superoxide overproduction, due to lack of UCP1, resulted in an increased content of HNE adducts in brown fat mitochondria in UCP1-ablated mice. Protein modification by HNE was determined by immunoblotting with an antibody against the sulf-HNE-adduct (37, 38, 40).

We examined the presence of HNE adducts in brown fat mitochondria isolated from UCP1+/+ and UCP1−/− mice that had been living at 24 °C (Fig. 5A, right side lanes). As expected, the antibody detected several bands at different molecular weights. One protein at about 50 kDa was dominantly labeled; in the heart it has been indicated that this protein is the β-subunit of ATP-synthase (41). This should increase the risk of lipid peroxidation (43) and thus potentially lead to increased formation of HNE and HNE/protein adducts. However, cold acclimation did not result in higher levels of HNE/protein adducts in mitochondria from UCP1-ablated mice (Fig. 5A), and quantification of the total labeling of the gel verified this (Fig. 5B).

To examine conditions expected to further increase the risk of oxidative stress and lipid peroxidation, we analyzed the effect of cold acclimation on the formation of HNE/protein adducts. In UCP1-ablated mice, cold acclimation leads to an increased polyunsaturation of mitochondrial membrane fatty acids5 (as it does in wild-type mice (42)). This should increase the risk of lipid peroxidation (43) and thus potentially lead to increased formation of HNE and HNE/protein adducts. However, cold acclimation did not result in higher levels of HNE/protein adducts in mitochondria from UCP1-ablated mice (Fig. 5, A and B).

To investigate whether conditions with coupled brown fat mitochondria due to the absence of UCP1, and coupled brown fat mitochondria due to the absence of physiological stimulation, resulted in different degrees of oxidative damage, we compared mitochondria from wild-type and UCP1-ablated mice, living either at thermoneutrality (where UCP1 would not be physiologically stimulated) or at 4 °C. Again, we found no evidence for alterations in the levels of HNE/protein adducts (Fig. 5C). Thus, taken together, we could not establish an essential role of UCP1 in the defense against oxidative damage in brown fat mitochondria, as there was no increased oxidative damage in UCP1−/− brown fat mitochondria, despite the expected higher generation of superoxide and the concurrent absence of the proposed UCP1-mediated elimination of superoxide in these animals (3, 44).

No Formation of HNE/UCP1 Adducts—We compared the Western blots of HNE adducts with the Western blot of UCP1 (Fig. 5) in an attempt to identify bands of HNE adducts that matched the UCP1 molecular mass of 32 kDa, or bands with another molecular mass that were present in UCP1+/+ but absent in UCP1−/− samples (adduction may change the apparent molecular mass) (Fig. 5, A and C). None of the HNE adduct bands had an apparent molecular weight of UCP1 protein or were related to the presence/absence of UCP1 (Fig. 5A, A and C).

Treatment of UCP1+/+ brown fat mitochondria with increasing concentrations of HNE in vitro led to formation of HNE adducts with mitochondrial proteins (as well as with the albumin present in the incubation medium; the band of 69 kDa) in a concentration-dependent manner (Fig. 5D). However, although UCP1 is one of the most abundant proteins in brown fat mitochondria, we found no evidence for specific formation of HNE/protein adducts in the 32-kDa region; if anything, there was remarkably little HNE adduct formation at this molecular size (Fig. 5D). Thus, we found no evidence for a specific, perhaps activating, HNE modification of UCP1.

UCP1+/+ and UCP1−/− Brown Fat Mitochondria Exhibit Equal Rates of Net Superoxide Release—The absence of increased levels of HNE adducts in UCP1−/− mitochondria could have two explanations:

superoxide generation by the respiratory chain is not reduced by UCP1 activity, even though UCP1 activity leads to low membrane potential (this possibility contrasts with the effect on superoxide production earlier proposed (3, 44)), or superoxide generation is normally reduced by active UCP1, but in the UCP1−/− mice, antioxidant systems are compensatorily up-regulated and successfully eliminate excess ROS. Either of these possibilities would explain the absence of increased oxidative stress in the UCP1−/− animals. We therefore examined these possibilities.

To analyze the net superoxide release in isolated brown fat mitochondria, we used the dye DHE, which is converted to ethidium by superoxide; the formation of superoxide can therefore be followed as fluorescence (14, 27). We first examined UCP1+/+ brown fat mitochondria in the absence of GDP, i.e. the mitochondria were uncoupled. When these mitochondria were incubated with glycerol 3-phosphate in the presence of rotenone there was a linear increase in fluorescence (Fig. 6A). Antimycin A was then added as a positive control to enhance mitochondrial superoxide generation. This resulted in a pronounced increase in fluorescence that was inhibited by addition of recombinant superoxide dismutase (Fig. 6A). Thus, although UCP1 is uninhibited in these mitochondria, resulting in a low membrane potential (Fig. 4) and a high respiration rate (flux) (Figs. 3 and 4), mitochondrial ROS production was not totally abolished. We then examined under identical conditions brown fat mitochondria from UCP1-ablated mice, i.e. brown fat mitochondria with high membrane potential (Fig. 4) and low respiration (Figs. 3 and 4). These conditions have been proposed to lead to high production of mitochondrial ROS (3, 44). Surprisingly, we found that the coupled, low-flux UCP1−/− brown fat mitochondria exhibited equal rates of net superoxide release as did the UCP1-containing, uncoupled brown fat mitochondria (Fig. 6B).

Our second approach to estimate superoxide release in brown fat mitochondria was to measure the activity of the mitochondrial matrix enzyme aconitase. Aconitase activity is readily and specifically impaired by free radicals (28) and therefore widely used as a marker of oxidative damage of mitochondria (13, 14, 45). Brown fat mitochondrial aconitase was, as expected, sensitive to superoxide, as its activity was dramatically decreased in the presence of the superoxide-generating system xanthine plus xanthine oxidase (Fig. 6C). However, we could see no effect of the absence of UCP1 on aconitase activity (Fig. 6C) and no UCP1-dependent difference in the ability of exogenous superoxide to inhibit the activity (Fig. 6C). Thus, all results obtained from UCP1−/− mitochondria: no extensive formation of HNE/protein adducts (Fig. 5), no enhanced superoxide release (Fig. 6B), and no lowered aconitase activity (Fig. 6C), as compared with wild-type mitochondria, are contradictory to a protective role of UCP1 in oxidative stress.

Tolerance to Oxidative Agents and Activities of Antioxidant Enzymes Are Not Enhanced in Brown Adipose Tissue from UCP1-ablated Mice—The second possible explanation for the absence of increased levels of HNE-adducts in UCP1−/− mitochondria is that the absence of UCP1 in UCP1−/− mitochondria may induce compensatory mechanisms leading to an increased mitochondrial tolerance to oxidative agents and/or enhanced activity of antioxidant systems.

Possible recruited mechanisms in protection against lipid peroxidation were analyzed by measurement of the rates of lipid peroxidation, estimated as the formation of the lipid peroxidation product, MDA, under basal, spontaneous, and stimulated conditions of oxidative stress in brown adipose tissue from UCP1−/− and UCP1+/+ mice (Fig. 6D). Basal levels of MDA in brown adipose tissue were very low (hardly measurable) and were not different in UCP1+/+ and UCP1−/− mice. Levels of spontaneous lipid peroxidation were higher compared with basal but were not different between UCP1+/+ and UCP1−/− brown

5 A. Ocloo, I. G. Shabalina, J. Nedergaard, and M. D. Brand, unpublished observations.
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![Image](image_url)

**FIGURE 6.** Superoxide measurements and antioxidative parameters in brown fat mitochondria and brown adipose tissue of UCP1+/+ and UCP1−/− mice. A, traces of superoxide measurement in brown fat mitochondria isolated from UCP1+/+ and UCP1−/− mice. Mitochondria (Mit), 0.5 mg, were incubated in 3 ml of assay buffer with the same composition, under the same conditions, and with the same substrate (5 mM glycerol 3-phosphate in the presence of 2 μg/ml rotenone) as for oxygen consumption (Fig. 3) (i.e. in the absence of GDP), followed by addition of 50 μM DHE. Further additions of 1.2 μg/ml antimycin A and 30 units/ml recombinant SOD were made. B, quantification of the change in fluorescence signal as exemplified in A. The rate of net superoxide release was calculated as the change in fluorescence intensity during the linear response after addition of DHE. The values represent the mean ± S.E. of 4 (UCP1+/+) or 2 (UCP1−/−) independent mitochondrial preparations, each analyzed in triplicate. C, aconitase activity in brown fat mitochondria isolated from UCP1+/+ and UCP1−/− mice. Aconitase activity was expressed as units per unit of citrate synthase activity. Values represent the mean ± S.E. of seven UCP1+/+ and seven UCP1−/− independent mitochondrial preparations. X/O indicates the activity after the addition of the superoxide-generating system xanthine plus xanthine oxidase. D, MDA formation in brown adipose tissue of UCP1+/+ and UCP1−/− mice kept at 24 °C. MDA was expressed as nmol of MDA/mg of protein and represents the mean ± S.E. of 3 UCP1+/+ and 5 UCP1−/− animals. The three incubation conditions are defined under “Experimental Procedures.” E, Mn-SOD, CuZn-SOD, and catalase activities. Values are expressed as units per mg of protein and represent the mean ± S.E. of three UCP1+/+ and five UCP1−/− animals. *, p < 0.05 versus UCP1+/+ mice.

adipose tissue. Further incubation of brown adipose tissue homogenate with oxidative agents led to a dramatic increase in MDA formation (Fig. 6D); however, again, UCP1+/+ and UCP1−/− brown adipose tissues were equally sensitive (Fig. 6D). Thus, there was no indication that the lack of difference in HNE/protein adduct formation between UCP1+/+ and UCP1−/− mitochondria (Fig. 5) could be explained by differences in lipid peroxidation capacities.

We also measured antioxidant enzyme activities in brown adipose tissue isolated from UCP1−/− and UCP1+/+ mice (Fig. 6E). The mitochondrial Mn-SOD activity was not increased but rather was significantly lower in UCP1+/+ than in UCP1−/− mice (Fig. 6E). No statistically significant difference in CuZn-SOD activity was found in the brown adipose tissue of UCP1+/+ versus UCP1−/− mice (Fig. 6E). As the product of superoxide dismutase action is hydrogen peroxide (which in itself may be a precursor of hydroxyl radicals), we also measured catalase activity; again no difference was found (Fig. 6E). Thus, there was no indication that the means to eliminate mitochondrially produced superoxide had been compensatorily elevated in UCP1−/− brown adipose tissue.

Thus, we found no indications that the lack of evidence for increased oxidative stress in UCP1-ablated mice should be due to the recruitment of any compensatory mechanisms. Rather it would seem that the outcome of the studies in Figs. 1–4, implying an inability of HNE to activate UCP1 in an isolated system (isolated brown fat mitochondria), is in accordance with the results in Figs. 5 and 6 showing an apparent absence of increased oxidative stress in UCP1-ablated mice in vivo.

**DISCUSSION**

Uncoupling proteins have been ascribed a role in protection against oxidative stress. Particularly, metabolites resulting from oxidative stress have been suggested to be activators of uncoupling proteins. In the present investigation, we have examined the effect of one such metabolite, HNE, on one of the uncoupling proteins, UCP1. In experiments.
with isolated brown fat mitochondria, we found that HNE was unable to (re)activate UCP1 when it was inhibited by purine nucleotides. HNE was also unable to augment the activity of uninhibited UCP1 as it did not increase the proton leak ascribable to UCP1 (but it did increase the proton leak of the mitochondria in a UCP1-independent way). In the intact animal, we found no indications of increased oxidative stress-related damage due to the absence of UCP1. These results thus question the suggested protective role of UCP1 and may have implications for the suggested function of the other uncoupling proteins and for the understanding of the nature of the physiological conditions associated with oxidative stress in general.

**HNE Cannot (Re)activate UCP1**—In the cell, uncoupling proteins, including UCP1, are exposed to cytosolic purine nucleotides, mainly ATP and ADP. UCP1 activity is thought to be chronically inhibited by these nucleotides, and the ability of fatty acids to re-activate UCP1 in the presence of purine nucleotides is the basis for the concept that fatty acids are the physiological activators of UCP1 (reviewed in Ref. 4). We found that irrespective of concentration added, the lipid metabolite HNE was unable to overcome inhibition caused by GDP, the nucleotide classically used experimentally to mimic the situation in the cell (Fig. 1).

Thus, the present experiments indicate that HNE cannot overcome the chronic *in situ* inhibition of UCP1. Physiologically, this means that UCP1 cannot be part of a self-regulating system where an increased level of HNE would activate UCP1, thus decreasing the membrane potential and through this the generation of ROS, leading to a lower rate of generation of HNE. Basically, HNE lacks the necessary property of being a UCP1 (re)activator.

**Oxidized Aldehydes Activate UCP1-independent and UCP1-dependent Proton Leak**—Mitochondria detoxify HNE through several pathways: glutathione conjugation (33, 46), formation of 1,4-dihydroxy-trans-2-nonene (33), and oxidation to 4-hydroxy-2-nonenonic acid by the NAD⁺-dependent aldehyde dehydrogenases ALDH2 and ALDH5A (47, 48). The mitochondrial aldehyde dehydrogenases are highly expressed and active in many tissues (liver, heart, kidney, muscle, brain, lung, spleen, and stomach) (48). Thus, in mitochondrial preparations from these tissues, the aldehyde dehydrogenase present could oxidize HNE into a fatty acid. However, in brown adipose tissue, the expression of aldehyde dehydrogenase is remarkably low (49).

We did not observe signs of spontaneous fatty acid formation from HNE in brown fat mitochondria (Figs. 1 and 2), in agreement with the low expression of aldehyde dehydrogenase. The situation in our experiments with addition of exogenous aldehyde dehydrogenase (Fig. 2) may thus be said to mimic the situation in many other tissues. In those tissues, the endogenous aldehyde dehydrogenase may convert a significant amount of the added HNE to 4-hydroxy-2-nonenonic acid; e.g., in isolated intact kidney mitochondria about 80% of HNE is degraded within 3 min, and the main product is hydroxynonenonic acid (50). As seen in Fig. 1A, such fatty acids can uncouple mitochondria in an UCP1-independent way. There is therefore a possibility that apparent aldehyde-induced increases in proton conductance in mitochondria from different tissues (24) could in part be because of the effect of formation of hydroxynonenonic acid.

**4-Hydroxy-2-nonenonic Is Not a Regulatory Cofactor for UCP1**—When directly added to brown fat mitochondria, HNE did not augment the activity of uninhibited UCP1 (Fig. 3B). In experiments investigating proton leak in the standard manner by successive addition of respiratory inhibitor, results corresponding to an increase in proton leak caused by HNE were observed, but the effect was essentially the same whether UCP1 was present or not (Fig. 4C). Indeed, when the proton leak ascribable to UCP1 was calculated, the result was that UCP1 activity was nearly halved in the presence of HNE (Fig. 4D). This evidence for non-involvement of UCP1 in response to HNE of brown fat mitochondria questions earlier indications that UCP1 is activated by HNE (24).

It has also been discussed whether HNE could be the activating cofactor explaining the difference between the functional statuses of UCP1 in reconstituted systems (where UCP1 is inactive) and in brown fat mitochondria (where UCP1 is innately active); HNE could be present in the brown fat mitochondria but absent in the reconstituted systems (10). The inability of added HNE to augment the activity of uninhibited UCP1 (Figs. 4 and 5) indicates that the amount of HNE present in isolated brown fat mitochondria cannot be rate-limiting for the activity of UCP1. Thus, the present experiments cannot preclude the possibility that HNE (or similar compounds) present in brown fat mitochondria is necessary for the activity of native UCP1, but if so, HNE is clearly present endogenously in sufficient amounts to allow full UCP1 activity, and HNE can therefore not have a controlling role. The alternative possibility would be that HNE does not have a role as a cofactor for UCP1 activity.

**The Nature of the UCP1-independent, HNE-induced Proton Leak**—Although we clearly observed no ability of HNE to activate a proton leak ascribable to UCP1 in brown fat mitochondria (Fig. 4D), we observed effects of HNE addition that were independent of UCP1 but had characteristics (Fig. 4C) similar to those earlier demonstrated in brown fat mitochondria (24). The nature of these proton leaks may be discussed. It is noteworthy that HNE can cause direct permeabilization of mitochondrial membranes, revealed as high amplitude swelling (51), i.e., HNE-induced mitochondrial swelling represents a so-called permeability transition. There thus seems to be a possibility that such a permeabilization of the mitochondrial membrane may also cause the UCP1-independent increase in proton conductance in mitochondria (24) after HNE treatment.

**Relation to HNE Activation of Other UCPS**—We have only examined the ability of HNE to activate one of the members of the uncoupling protein family, UCP1. HNE activation has been suggested as a general mechanism for uncoupling protein activation (24); it has, e.g., been suggested that this was the original mode of action of all members of the uncoupling protein family and that the ability of UCP1 to be thermogenic was a later evolutionary event (3, 11). The inability shown here of HNE to activate UCP1 would then be considered a consequence of this development, i.e., an evolutionary loss of function. Concerning the other UCPS, results published indicate that the HNE effect in muscle mitochondria from UCP3-ablated mice is smaller than in wild-type mice (24), which would support a contribution from UCP3. Direct data concerning UCP2 have not been published. The possibility that a UCP1-mediated HNE effect in our wild-type mitochondria has been taken over by an increased amount of UCP3 in the UCP1-ablated mice is unlikely, as UCP3 gene expression is decreased in UCP1-ablated mice (34). Concerning UCP2, it would seem that although mRNA levels are high in the brown adipose tissue of UCP1-ablated mice (34), the protein is not translated in these mice (52). It is therefore very unlikely that UCP2/UCP3 has taken over as the mediator of HNE effects on proton leak in brown fat mitochondria of UCP1-ablated mice. Based on our data we can, thus, at least conclude that activation by HNE is not a general feature of all uncoupling proteins.

**Covalent Modification of UCP1 Is Not Involved in Regulation of Its Activity**—HNE is a highly reactive compound and several functional groups on mitochondrial proteins are modified by interaction with HNE, particularly sulphydryl groups to form thioether adducts (53), and histidine and lysine residues of proteins to form stable Michael adducts (37–39). Covalent modification of UCPS by HNE has been proposed as
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a molecular mechanism of activation of UCPs (3, 24). However, the existence of UCP1 adducts has not so far been demonstrated. UCP1 constitutes a large fraction of the mitochondrial membrane proteins, and if HNE/UCP1 adducts were formed to any significant degree in vivo, as part of the activation process, they should reasonably be identifiable. However, we could not identify any protein/HNE adducts that would correspond to UCP1 (Fig. 5). This implies that covalent modification of UCP1 is not directly involved in regulation of UCP1 activity.

Absence of Increased Oxidative Damage in UCP1-ablated Mice Implies That UCP1 Does Not Have an Antioxidative Effect in Vivo—The above conclusions have been based on in vitro data and were restricted to examination of HNE effects, and these conditions may not reflect physiological conditions. However, we were also unable to observe any increase in oxidative damage (measured as total amount of HNE adducts) in brown fat mitochondria from UCP1-ablated mice in different physiological conditions (Fig. 5). The absence of effect of UCP1 ablation could have been due to a compensatory activation of alternative antioxidant pathways in the brown adipose tissue of UCP1-ablated mice, but we found no evidence for this (Fig. 6). In extension of this, it may be concluded that not only HNE but also other compounds associated with oxidative stress, such as superoxide (11) and compounds downstream of superoxide (carbon-centered radicals (15), hydroperoxy fatty acids (16, 17), and lipid peroxi-
dation product in addition to HNE (24), which have been suggested to activate UCP1 in a self-regulatory feed-back process, either do not induce such a process or do not depend on UCP1 for this process. Thus, physiologically, UCP1 does not seem to be an indispensable antioxidant.

Absence of Increased Oxidative Damage in Cold-acclimated UCP1-ablated Mice May Have Implications for Theories for Oxidative Damage—At a general level, the observation that no difference was found in the mitochondria of UCP1-ablated mice, but we found no evidence for this (Fig. 6). In extension of this, it may be concluded that not only HNE but also other compounds associated with oxidative stress, such as superoxide (11) and compounds downstream of superoxide (carbon-centered radicals (15), hydroperoxy fatty acids (16, 17), and lipid peroxidation product in addition to HNE (24), which have been suggested to activate UCP1 in a self-regulatory feed-back process, either do not induce such a process or do not depend on UCP1 for this process. Thus, physiologically, UCP1 does not seem to be an indispensable antioxidant.

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45. Bernal-Mizrachi, C., Gates, A. C., Weng, S., Imamura, T., Knutsen, R. H., DeSantis, P., Coleman, T., Townsend, R. R., Muglia, L. J., and Semenkovich, C. F. (2005) Nature 435, 502–506
46. Meyer, M. J., Mosely, D. E., Amarnath, V., and Picklo, M. J., Sr. (2004) Chem. Res. Toxicol. 17, 1272–1279
47. Murphy, T. C., Amarnath, V., and Picklo, M. J., Sr. (2003) J. Neurochem. 84, 1313–1321
48. Ohta, S., Ohsawa, I., Kamino, K., Ando, F., and Shimokata, H. (2004) Ann. N. Y. Acad. Sci. 1011, 36–44
49. Unami, A., Shinohara, Y., Kajimoto, K., and Baba, Y. (2004) Biochem. Pharmacol. 67, 555–564
50. Ullrich, O., Grune, T., Henke, W., Esterbauer, H., and Siems, W. G. (1994) FEBS Lett. 352, 84–86
51. Vieira, H. L., Belzacq, A. S., Haouzi, D., Bernassola, F., Cohen, I., Jacotot, E., Ferri, K. F., El Hamel, C., Bartle, L. M., Melino, G., Brenner, C., Goldmacher, V., and Kroemer, G. (2001) Oncogene 20, 4305–4316
52. Pecqueur, C., Alves-Guerra, M. C., Gelly, C., Levi-Meyrueis, C., Couplan, E., Collins, S., Ricquier, D., Bouillaud, F., and Miroux, B. (2001) J. Biol. Chem. 276, 8705–8712
53. Petersen, D. R., and Doorn, J. A. (2004) Free Radic. Biol. Med. 37, 937–945
54. Foster, D. O., and Frydman, M. L. (1978) Can. J. Physiol. Pharmacol. 56, 110–122
55. Matthias, A., Ohlson, K. E. B., Fredriksson, J. M., Jacobsson, A., Nedergaard, J., and Cannon, B. (2000) J. Biol. Chem. 275, 25073–25081