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## ROS production in brown adipose tissue mitochondria: The question of UCP1-dependence <sup>☆</sup>



Irina G. Shabalina <sup>a</sup>, Marek Vrbacký <sup>b</sup>, Alena Pecinová <sup>b</sup>, Anastasia V. Kalinovich <sup>a</sup>, Zdeněk Drahotka <sup>b</sup>, Josef Houšťek <sup>b</sup>, Tomáš Mráček <sup>b</sup>, Barbara Cannon <sup>a,\*</sup>, Jan Nedergaard <sup>a</sup>

<sup>a</sup> Department of Molecular Biosciences, The Wenner-Gren Institute, The Arrhenius Laboratories F3, Stockholm University, SE-106 91 Stockholm, Sweden

<sup>b</sup> Department of Bioenergetics, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ 142 20 Prague, Czech Republic

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### ABSTRACT

Whether active UCP1 can reduce ROS production in brown-fat mitochondria is presently not settled. The issue is of principal significance, as it can be seen as a proof- or disproof-of-principle concerning the ability of any protein to diminish ROS production through membrane depolarization. We therefore undertook a comprehensive investigation of the significance of UCP1 for ROS production, by comparing the ROS production in brown-fat mitochondria isolated from wildtype mice (that display membrane depolarization) or from UCP1(−/−) mice (with a high membrane potential). We tested the significance of UCP1 for glycerol-3-phosphate-supported ROS production by three methods (fluorescent dihydroethidium and the ESR probe PHH for superoxide, and fluorescent Amplex Red for hydrogen peroxide), and followed ROS production also with succinate, acyl-CoA or pyruvate as substrate. We studied the effects of the reverse electron flow inhibitor rotenone, the UCP1 activity inhibitor GDP, and the uncoupler FCCP. We also examined the effect of a physiologically induced increase in UCP1 amount. We noted GDP effects that were not UCP1-related. We conclude that only ROS production supported by exogenously added succinate was affected by the presence of active UCP1; ROS production supported by any other tested substrate (including endogenously generated succinate) was unaffected. This conclusion indicates that UCP1 is not involved in control of ROS production in brown-fat mitochondria. Extrapolation of these data to other tissues would imply that membrane depolarization may not necessarily decrease physiologically relevant ROS production. This article is a part of a Special Issue entitled: 18th European Bioenergetics Conference (Biochim. Biophys. Acta, Volume 1837, Issue 7, July 2014).

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### 1. Introduction

Published observations concerning the effect of UCP1 on ROS production are not consistent, with data being interpreted as indicating that the absence of UCP1 leads to increased ROS production [1,2] – or that UCP1, even when active, has no effect on ROS production [3–5].

Whereas this issue is evidently of interest regarding brown adipose tissue function, its significance is broader than that. This is because UCP1 is the only verified uncoupling protein: it does lead

to decreased membrane potential when it is active [6–8]. Provided that ROS production is membrane-potential-sensitive, UCP1 should undoubtedly influence ROS production.

In this way, UCP1 effects on ROS production may be said to be a proof-of-principle test. It has been suggested [9], widely accepted [10] but also critically discussed [11–13] that the members of the so-called uncoupling protein family could be protective against oxidative damage, based on their suggested uncoupling (depolarizing) activity. However, as it may be doubted that any member of the uncoupling protein family (UCP2, UCP3, protoUCP1) other than mammalian UCP1 itself has uncoupling activity [12,14], it is not appropriate presently to examine this hypothesis in any tissue, except in the UCP1-expressing brown adipose tissue. If UCP1 does not function as a mitigator of ROS production in brown-fat mitochondria, the issue of developing possibilities to activate other uncoupling protein-family members to induce an uncoupling activity [15,16] becomes irrelevant as a means to diminish ROS production and oxidative damage.

Furthermore, as active UCP1 undoubtedly reduces the mitochondrial membrane potential [12,14], a diminished ROS production must unconditionally be observed when UCP1 is active if the so-called

**Abbreviations:** BSA, bovine serum albumin; DCPIP, 2,6-dichlorophenolindophenol; DHE, dihydroethidium; ESR, electron spin resonance; EDTA, ethylenediamine tetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoro-methoxy)-phenylhydrazone; GDP, guanosine 5'-diphosphate; mGPDH, mitochondrial glycerol 3-phosphate dehydrogenase; GCCR, glycerol-3-phosphate:cytochrome *c* reductase; PPH, 1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine; ROS, reactive oxygen species; SOD, superoxide dismutase; SCCR, succinate:cytochrome *c* reductase; UCP1, uncoupling protein 1

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\* Corresponding author at: Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, SE-106 91 Stockholm, Sweden. Tel.: +46 8 164120.

E-mail address: [barbara.cannon@su.se](mailto:barbara.cannon@su.se) (B. Cannon).

“mild-uncoupling” hypothesis for amelioration of oxidative damage is valid [17,18]. If this is not the case, the mild-uncoupling hypothesis would not provide a therapeutic pathway to protect against oxidative damage. The issue of the actual significance of oxidative damage for the ageing process [19] – and for mitochondrial diseases [20] – is outside the range of issues in the present paper.

As will be understood from the outline above, it is of considerable importance to establish the actual ability of UCP1 to affect ROS production. This is thus the aim of the present investigation. We conclude that, with the exception of a very specific and probably not physiologically related condition (exogenously added succinate respiration), we find no effect of UCP1 on ROS production in brown-fat mitochondria. Concerning the possible function of uncoupling proteins and membrane potential in ameliorating ROS production in any tissue, our observations would imply that such effects are unlikely, although dedicated experiments would be needed to establish this in general.

## 2. Materials and methods

### 2.1. Animals

UCP1-ablated mice (progeny of those described in [21]) were backcrossed to C57Bl/6 for 10 generations and after repeated intercrossing (every 10 generations) were maintained as UCP1(–/–) and UCP1(+/+)(wildtype) 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 the experiments on cold-acclimated animals, adult male mice were divided into age-matched (7–8 week old) groups, one per cage, and acclimated at 30 °C or at 4 °C during 4–6 weeks. The experiments were approved by the Animal Ethics Committee of the North Stockholm region.

### 2.2. Isolation of brown adipose tissue mitochondria

Mice were anaesthetised for 1 min with a mixture of 79% CO<sub>2</sub> and 21% O<sub>2</sub> and decapitated. The interscapular, periaortic, axillary, and cervical brown-adipose tissue depots were pooled from 3 to 5 mice and placed in ice-cold medium consisting of 250 mM sucrose, freed of white fat and used for isolation of brown-fat mitochondria. Preparations from wildtype and UCP1(–/–) mice (or warm- and cold-acclimated wildtype mice) were generally made and run in parallel.

Brown-fat mitochondria were isolated as described in [22]. The brown adipose tissue was finely minced with scissors and homogenized in a Potter homogeniser with a Teflon pestle. Throughout the isolation process, tissues were kept at 0–2 °C. Mitochondria were isolated by differential centrifugation as follows. The homogenates were centrifuged at 8 500 g for 10 min at 2 °C in a Beckman J2-21M centrifuge. The resulting supernatant, containing floating fat, was discarded. The pellet was resuspended in ice-cold medium containing 250 mM sucrose. The resuspended homogenate was centrifuged at 800 g for 10 min, and the resulting supernatant was centrifuged at 8 500 g for 10 min. The resulting mitochondrial pellet was resuspended in 100 mM KCl, 20 mM K<sup>+</sup>-Tes (pH 7.2), 1 mM EDTA, 0.6% (w/v) fatty-acid-free BSA and centrifuged again at 8 500 g for 10 min. The final mitochondrial pellets were resuspended by hand homogenisation in a small glass homogeniser in the same medium. The concentration of mitochondrial protein was measured using fluorescamine [23] with BSA as a standard.

### 2.3. Oxygen consumption

Oxygen consumption rates were monitored with a Clark-type oxygen electrode (Yellow Springs Instrument Co., USA) in a sealed chamber at 37 °C, as described [24]. Brown-fat mitochondria

(0.25 mg protein/ml) were incubated in a medium consisting of 125 mM sucrose, 20 mM K<sup>+</sup>-Tes (pH 7.2), 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 4 mM KPi, and 0.1% (w/v) fatty-acid-free BSA.

Respiratory activity of mitochondria was measured in the presence of 5 mM pyruvate plus 3 mM malate or of 50 μM palmitoyl-CoA plus 5 mM carnitine plus 3 mM malate or of 5 mM glycerol-3-phosphate or of 5 mM succinate. The respiration was initiated by the addition of substrate. Basal (i.e. UCP1-independent) respiration was determined as the residual respiration following addition of (routinely) 1 mM GDP (increased concentration up to 3 mM has no additional effect (not shown)). Maximal oxygen consumption rates were obtained by addition of FCCP to a final concentration of 0.7–2.1 μM.

### 2.4. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential measurements were performed with the dye safranin O [25] under the same conditions as those used for oxygen consumption. The changes in absorbance of safranin O were followed at 37 °C in an Olis® modernized Aminco DW-2 dual-wavelength spectrophotometer at 511–533 nm with a 3-nm slit. Olis GlobalWorks™ software was used for recording and quantification. Calibration curves were made for each mitochondrial preparation in K<sup>+</sup>-free medium and were obtained from traces in which the extramitochondrial K<sup>+</sup>, [K<sup>+</sup>]<sub>out</sub>, was altered by addition of KCl in a 0.1–500 mM final concentration range, in the presence of 3 μM valinomycin. The change in absorbance caused by each addition was plotted against [K<sup>+</sup>]<sub>out</sub> and the intramitochondrial K<sup>+</sup>, [K<sup>+</sup>]<sub>in</sub>, was estimated by extrapolation of the line to the zero uptake point, as described [25]. The absorbance readings were used to calculate the membrane potential (mV) by the Nernst equation according to:  $\Delta\psi = 61 \text{ mV} \cdot \log ([K^+]_{in}/[K^+]_{out})$ .

### 2.5. Mitochondrial superoxide production

Superoxide was measured using 2 methods: superoxide-induced conversion of dihydroethidium (DHE) into fluorescent 2-OH-ethidium [26], and superoxide-induced conversion of the spin-trapping reagent PPH (1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine) [27].

Conversion of DHE was monitored with a Sigma spectrofluorometer at 37 °C with an excitation wavelength of 495 nm and emission via a narrow band pass filter at 570 ± 5 nm [28]; the narrow band pass filter helps to increase the selectivity [26]. Detection of superoxide radical by this method has earlier been confirmed by inhibition of the fluorescent signal with exogenous superoxide dismutase and by observation of a lowered signal in hSOD2-overexpressing mice [28].

The cyclic hydroxyl-amine PPH (Noxygen Science Transfer & Diagnostics GmbH) was used for measurements of superoxide production by mitochondria [29]. PPH reacts with superoxide, producing stable PP-nitroxide that can be detected with electron spin resonance (ESR) spectroscopy [27]. Briefly, 10 mM PPH was dissolved in deoxygenated medium containing 50 μM deferoxamine. Mitochondria preparations and PPH stock solutions were kept on ice (50 μg protein mixed with 1 mM PPH and mitochondrial substrates in 100 μl incubation medium with the same composition as described for oxygen consumption). Accumulation of PP-nitroxide was measured with a Bruker ESP 300 X-band spectrometer and a super-high Q microwave cavity. The following ESR settings were used: microwave frequency 9.78 GHz, modulation amplitude 2 G, microwave power 10 dB, conversion time 1.3 s, and time constant 5.2 s.

### 2.6. Mitochondrial hydrogen peroxide production

Mitochondrial H<sub>2</sub>O<sub>2</sub> net production was determined fluorometrically with the Amplex Red reagent. Oxidation of Amplex Red coupled by horseradish peroxidase to reduction of H<sub>2</sub>O<sub>2</sub> produces the red fluorescent product resorufin. Mitochondria (0.05–0.12 mg mitochondrial

protein  $\text{ml}^{-1}$ ) were incubated under the same conditions as those used for oxygen consumption. All incubations also contained  $5 \mu\text{M}$  Amplex Red,  $12 \text{ units ml}^{-1}$  horseradish peroxidase and  $45 \text{ units ml}^{-1}$  superoxide dismutase. The reaction was routinely initiated by addition of mitochondria followed by successive addition of substrate (as described for oxygen consumption), followed by rotenone ( $0.7 \mu\text{M}$ ) or GDP ( $1 \text{ mM}$ ) or FCCP (actual concentrations indicated in figure legends). In some experiments, substrates and GDP or rotenone were added simultaneously. The increase in fluorescence emitted through a band pass filter of  $600 \pm 20 \text{ nm}$  from an excitation wavelength of  $545 \text{ nm}$  was followed in a  $3 \text{ ml}$  cuvette for 2–5 min with a Sigma spectrofluorometer. In some experiments, fluorescence was detected with an EnSpire® Multimode Plate Reader (PerkinElmer, USA) in 24-well plate. Optimisation was applied, the excitation wavelength was set to  $563 \text{ nm}$  and the fluorescence emission was detected at  $584 \text{ nm}$ . The rate of  $\text{H}_2\text{O}_2$  production was calculated as the change in fluorescence intensity during the linear increase phase, as earlier described [8,30]. Calibration curves were obtained by adding known amounts of freshly diluted  $\text{H}_2\text{O}_2$  (the concentration of stock solution was checked at  $240 \text{ nm}$  using a molar extinction coefficient of  $43.6$ ) to the assay medium. The standard curve was linear in a range up till  $500 \text{ nM H}_2\text{O}_2$ . The calibration was performed also in the presence of GDP, rotenone and all substrates used for this study; no additions had any effect on the calibration. We specifically paid attention to possible effects of glycerol-3-phosphate (DL- $\alpha$ -glycerolphosphate, disodium salt, 95–99% of  $\alpha$ -isomer), but in contrast to [31] we did not observe any assay interference with 5–10 mM of this compound.

### 2.7. Immunoblotting

Immunoblot analysis was performed principally as in [32]. Aliquots of freshly isolated mitochondrial suspensions were supplemented with a protease inhibitor cocktail (Complete Mini, Roche), placed in liquid nitrogen and then stored at  $-80^\circ\text{C}$ . Protein concentrations of the thawed mitochondrial samples were re-quantified using the Bradford assay. Mitochondrial proteins were analysed by Tricine SDS-PAGE on 10% gels [33] using the Mini-Protean III apparatus (BioRad). Proteins were transferred from gels to PVDF-membranes (Immobilon-P, Millipore) using semidry electrotransfer (BioRad). The membranes were blocked with 5% (w/v) non-fat dried milk in TBST ( $150 \text{ mM NaCl}$ ,  $10 \text{ mM Tris}$ ,  $0.1\%$  (v/v) Tween-20,  $\text{pH } 7.5$ ) for 1 h and incubated for 2 h with the specific primary antibodies diluted in TBST. A monoclonal antibody against subunit SDHA ( $70 \text{ kDa}$ ) of succinate dehydrogenase was obtained from Abcam; rabbit polyclonal antibodies to glycerol 3-phosphate dehydrogenase (mGPDH) were custom prepared [32]. The dilution of SDHA antibody was 1:2000. The dilution of mGPDH antibody was 1:5000. Membranes were then incubated for 1 h with corresponding secondary fluorescent antibodies – IRDye 680- or 800-conjugated goat anti-mouse IgG (Life Technologies) or goat anti-rabbit IgG (Rockland). Both secondary antibodies (mouse and rabbit) were diluted 1:3000. Detection of proteins was performed using an Odyssey fluorescence scanner (Li-Cor). The quantification of signals was carried out in Aida Image Analyzer programme version 3.21 (Raytest).

### 2.8. Enzyme activity assays

Activities of glycerol 3-phosphate dehydrogenase (mGPDH), glycerol-3-phosphate cytochrome c reductase (mGPDH + Complex III; systematic name, glycerol-3-phosphate:ferricytochrome c oxidoreductase; GCCR) and succinate cytochrome c reductase (Complex II + Complex III; systematic name, succinate:ferricytochrome c oxidoreductase; SCCR) were determined spectrophotometrically (Shimadzu UV-1601) in frozen-thawed mitochondria as described [34]. 2,6-dichlorophenolindophenol (DCPIP), electron acceptor for the dehydrogenase itself (mGPDH) was monitored at  $610 \text{ nm}$ , molar absorption coefficient  $\epsilon_{610} =$

$20.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ . Cytochrome c, electron acceptor for oxidoreductases (GCCR and SCCR) was monitored at  $550 \text{ nm}$ , molar absorption coefficient  $\epsilon_{550} = 19.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ . The assay medium contained  $50 \text{ mM KCl}$ ,  $10 \text{ mM Tris-HCl}$ ,  $1 \text{ mM EDTA}$ ,  $1 \text{ mg BSA ml}^{-1}$ ,  $1 \text{ mM KCN}$ ,  $\text{pH } 7.4$  and  $100 \mu\text{M DCPIP}$  or  $50 \mu\text{M cytochrome c}$  (from bovine heart) and approximately  $0.1 \text{ mg}$  mitochondrial protein. The reaction was started by addition of  $10 \text{ mM sn-glycerol 3-phosphate}$  or succinate, and changes in absorbance were monitored at  $30^\circ\text{C}$ . Enzyme activities were expressed as  $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  protein.

### 2.9. Chemicals

Fatty-acid-free bovine serum albumin (BSA), fraction V, was from Roche Diagnostics GmbH. Rotenone, FCCP (carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazine), GDP (guanosine 5'-diphosphate) (sodium salt), pyruvic acid (sodium salt), DL- $\alpha$ -glycerol-3-phosphate (disodium salt, 95–99% of  $\alpha$ -isomer), succinic acid (disodium salt), L(-) malic acid (disodium salt), palmitoyl coenzyme A (lithium salt), xanthine (2,6-dihydroxypurine), xanthine oxidase, safranin O, EDTA (ethylenediamine tetraacetic acid), bovine recombinant superoxide dismutase, horseradish peroxidase, alamethicin were all from Sigma-Aldrich Co. Fluorescamine (4-phenyl spiro-[furan-2(3H),1-phthalan]-3,3'-dione) was from Fluka Chemie GmbH. Amplex Red and dihydroethidium were from Life Technologies. GDP was dissolved in  $20 \text{ mM Tes}$  ( $\text{pH } 7.2$ ) and the pH of the solution readjusted to  $7.2$ . FCCP was dissolved in 95% ethanol and diluted in 50% ethanol; oligomycin was dissolved in 95% ethanol. Ethanol in a final concentration of  $0.1\%$  did not in itself have any effects on the parameters measured.

### 2.10. Statistics

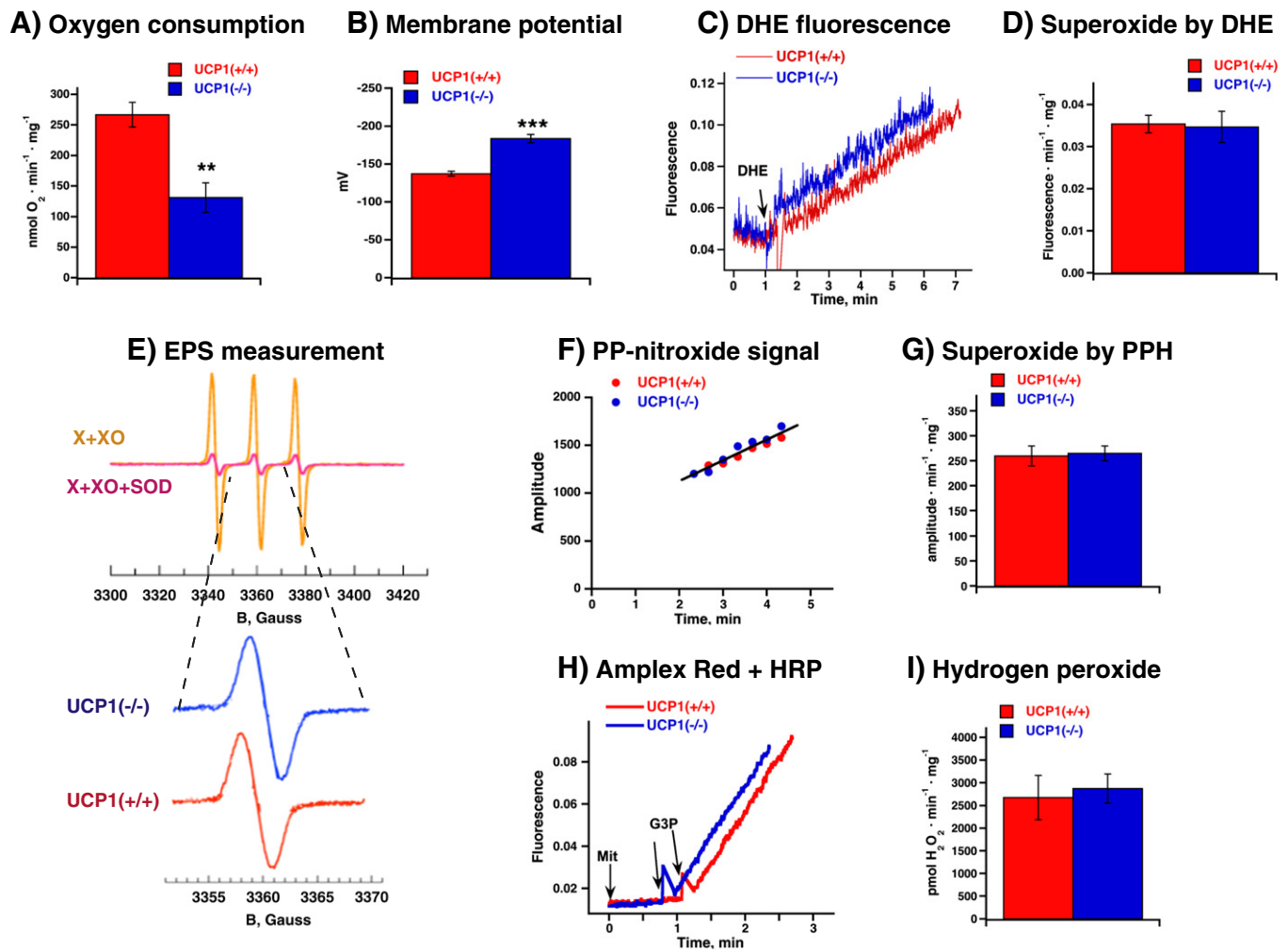
All data are expressed as means  $\pm$  standard errors. Statistical analysis was performed using KaleidaGraph 4.5.0 software, Student's *t*-test.

## 3. Results

The main aim of this investigation was to ascertain whether the presence or absence of UCP1 affected the generation of ROS in brown-fat mitochondria. Therefore, the experiments were basically comparisons between ROS production in brown-fat mitochondria isolated from wildtype or UCP1-ablated mice. The mice were housed at  $24^\circ\text{C}$  to provide a level of UCP1-mediated uncoupling that resulted in a fairly modest reduction in membrane potential, "mild uncoupling". The experiments were initially performed in the absence of GDP, i.e. UCP1 in the wildtype mitochondria is then innately active, which is expected to lead to a physiologically relevant, lower membrane potential and a high rate of oxygen consumption. The experiments were performed with different substrates (glycerol-3-phosphate, succinate, pyruvate and palmitoyl-CoA) and initially in the absence of rotenone, i.e. reverse electron transport was possible.

### 3.1. No effect of UCP1 on glycerol-3-phosphate-supported ROS production: verification by three different methods

Glycerol-3-phosphate is a favoured experimental substrate for brown-fat mitochondria, as compared not only to white- [35] and brite-fat mitochondria [36] but also to mitochondria from most other tissues [37]. The particular physiological role of glycerol-3-phosphate in brown adipose tissue metabolism has not been fully established, but mitochondrial glycerol-3-phosphate dehydrogenase activity is probably associated with the transfer of reducing equivalents from the cytosol to the mitochondria [38]. Due to the high activity of glycerol-3-phosphate dehydrogenase in brown-fat mitochondria, it is relevant to examine ROS production with glycerol-3-phosphate as substrate.



**Fig. 1.** No effect of UCP1 on ROS production in mitochondria energized by glycerol-3-phosphate. (A, B) Rates of oxygen consumption and level of membrane potential in brown-fat mitochondria from wildtype (UCP1(+/+)) and UCP1(-/-) mice. Respiration was supported by 5 mM glycerol-3-phosphate. These and all other experiments in this figure were performed in the absence of GDP and rotenone, i.e. allowing for full innate activity of UCP1 and for reverse electron flow. Data are means  $\pm$  SE from 10 to 12 independent experiments; \* indicates difference between genotypes (two symbols indicate  $P < 0.01$ , three symbols indicate  $P < 0.001$ ). (C) A representative trace of DHE fluorescence (superoxide release rates) measured in wildtype and UCP1(-/-) brown-fat mitochondria. Initial additions were 0.08 mg/ml brown-fat mitochondria and 5 mM glycerol-3-phosphate, DHE added as indicated. (D) Change in DHE fluorescence rate (measured principally as shown in C) in brown-fat mitochondria isolated from wildtype and UCP1(-/-) mice. Data are means  $\pm$  SE from 3 to 4 independent experiments. (E) Measurement of superoxide with ESR and the spin probe PPH. PPH reacts with superoxide (obtained here from xanthine plus xanthine oxidase), producing stable PP-nitroxide detected with ESR spectroscopy (orange signal, upper panel). The reaction was almost completely inhibited by 50 units/ml of superoxide dismutase (pink signal, upper panel). To measure biologically produced superoxide we followed the middle peak (as indicated) of the ESR signal, as studied in brown-fat mitochondria from UCP1(-/-) (blue signal, lower panel) and wildtype (red signal, lower panel) mitochondria. (F) Examples of changes of amplitude of the ESR signal (measured as in E (lower panel)) with time, followed in brown-fat mitochondria preparations. (G) Rate of superoxide generation measured by ESR (as shown in E and F) in wildtype and UCP1(-/-) mitochondria. Data are means  $\pm$  SE of 3–4 independent preparations. (H) A representative trace of Amplex Red fluorescence (H<sub>2</sub>O<sub>2</sub> levels) measured in wildtype and UCP1(-/-) brown-fat mitochondria. Additions were 0.03 mg/ml brown-fat mitochondria (Mit) and 5 mM glycerol-3-phosphate (G3P). (I) Hydrogen peroxide production rate (measured principally as shown in H and expressed as hydrogen peroxide amount, based on calibrations) in brown-fat mitochondria isolated from wildtype and UCP1(-/-) mice. Data are means of 10–12 independent preparations.

We initially verified the significance of UCP1 for glycerol-3-phosphate oxidation. As seen in Fig. 1A, wildtype mitochondria demonstrated a high rate of respiration which was about double that observed in UCP1(-/-) mitochondria; addition of GDP to wildtype mitochondria (Fig. 2A) reduced respiration to the level seen in UCP1(-/-) mitochondria (Fig. 2A and [24,39,40]). Concomitant with the difference in respiration seen between wildtype and UCP1(-/-) mitochondria, a clear difference in membrane potential was seen, with the membrane potential being markedly higher in UCP1(-/-) mitochondria (Fig. 1B).

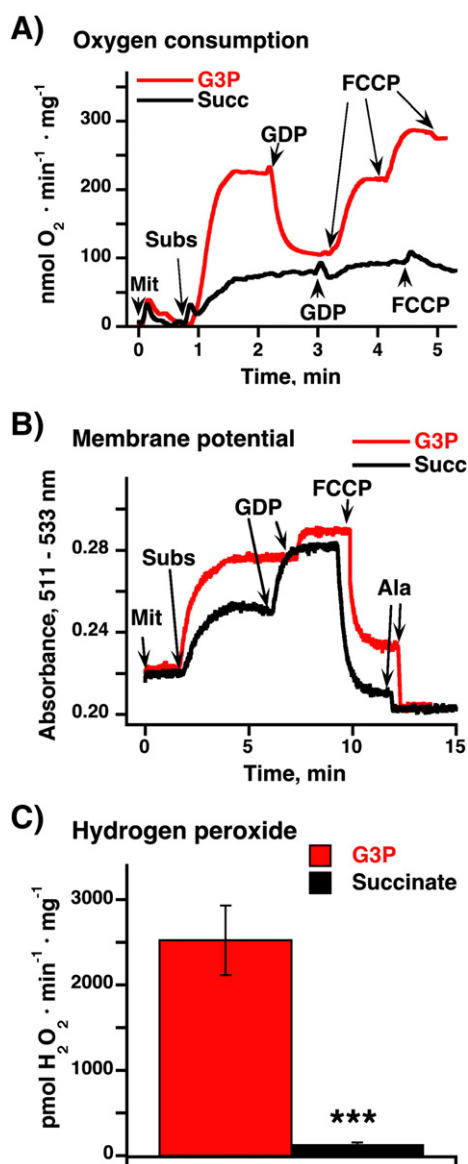
With regard to ROS production, we [3] and others [1] have earlier demonstrated that despite this clear difference in membrane potential, brown-fat mitochondria with or without UCP1 produce equal amounts of superoxide. These earlier experiments were conducted in the presence of rotenone, and reverse electron flow was thus inhibited. There is therefore a possibility that under conditions of unmanipulated electron flow through the respiratory chain, a UCP1 effect on ROS

production could become unveiled. Therefore here, brown-fat mitochondria were incubated without rotenone, to allow for reverse electron flow and therefore for superoxide generation possibly sensitive to membrane potential/proton motive force (Fig. 1C–I).

As it is recurrently discussed whether different methods for detecting ROS could yield different results, we used three different methods for ROS detection under otherwise similar circumstances.

### 3.1.1. Dihydroethidium

First, net superoxide release rates were assessed in isolated brown-fat mitochondria by following the fluorescence of OH-ethidium, which is generated when superoxide interacts with the dye dihydroethidium (DHE). Chemical and biological validation of this method has been performed earlier with brown-fat mitochondria [28]. In the present experiments, we found that wildtype brown-fat mitochondria incubated with glycerol-3-phosphate showed a linear increase in



**Fig. 2.** Bioenergetic parameters of wildtype brown-fat mitochondria, a comparison of glycerol-3-phosphate or succinate as substrate. (A, B) Representative recordings from oxygen consumption (A) and membrane potential (B) measurements of brown-fat mitochondria isolated from wildtype mice. Mitochondria were supported by either 5 mM glycerol-3-phosphate (red line) or 5 mM succinate (black line). Additions were 0.25 mg mitochondria (Mit), substrates (subs), 1 mM GDP and 0.7–2.1  $\mu$ M FCCP (added successively) for glycerol-3-phosphate in A, 0.7  $\mu$ M FCCP (single addition) for succinate in A and for both substrates in B, and 0.02 mg/ml alamethicin (Ala) (to allow for full de-energization). Note that no rotenone was present. To allow for direct comparisons, all parameters shown are from one experimental day, with parallel preparations of glycerol-3-phosphate and succinate, examined in parallel for respiration, membrane potential and hydrogen peroxide release. (C) Hydrogen peroxide production rate (measured principally as in Fig. 1H) in wildtype brown-fat mitochondria supported either by 5 mM glycerol-3-phosphate or by 5 mM succinate. Values are means  $\pm$  SE of 5 independent mitochondrial preparations analysed in parallel on two substrates. \*\*\*: statistically significant difference between glycerol-3-phosphate and succinate ( $P < 0.001$ ).

fluorescence (Fig. 1C); as exemplified in Fig. 1C, the result with brown-fat mitochondria from UCP1(–/–) mice was similar. A compilation of the results from a series of such experiments indicated an identical rate of superoxide formation in wildtype and UCP1(–/–) mitochondria (Fig. 1D). Thus, even in the absence of rotenone, we could not observe any effect of UCP1 on ROS production, when followed in this way.

### 3.1.2. Electron spin resonance spectroscopy

We also used the spin probe PPH (1-hydroxy-4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine) to follow superoxide production. This method for superoxide detection may be considered more specific than the dihydroethidium method (where other substances than superoxide may interfere [26]). Validation of this assay of superoxide radicals was performed with a superoxide ( $O_2^-$ )-generating system (xanthine + xanthine oxidase). The chemical generation of superoxide by the xanthine plus xanthine oxidase system resulted in a significant PP-nitroxide formation (Fig. 1E, upper panel). This was blocked by the addition of recombinant superoxide dismutase (Fig. 1E, upper panel), demonstrating the specificity of the signal. This method was then applied for comparison of superoxide production in brown-fat mitochondria from wildtype and UCP1(–/–) mice; superoxide was clearly detectable (Fig. 1E, lower panel). As seen in the example in Fig. 1F, the amplitude of the signal increased similarly with time in both types of mitochondria. Thus, also with this method, no effect of UCP1 on ROS generation was observable (Fig. 1G).

### 3.1.3. Amplex Red

ROS production may alternatively be followed as release of hydrogen peroxide, which can be monitored with the dye Amplex Red. Also here, ROS production was linear with time in both wildtype and UCP1(–/–) mitochondria (Fig. 1H), and no statistical difference in hydrogen peroxide production could be observed due to the presence or absence of UCP1 (Fig. 1I). It may be noted that there was no ROS production from endogenous substrates.

Thus, based on congruent results from three independent assays for ROS production, we conclude that the presence or absence of UCP1 does not influence ROS production supported by glycerol-3-phosphate. This is thus still the case even when reverse electron flow is possible, perhaps because most glycerol-3-phosphate-supported ROS production occurs within the enzyme itself (see below).

### 3.2. Succinate is a poor substrate for brown-fat mitochondria

In studies on the control of mitochondrial ROS production in any tissue, ROS production has primarily been examined with the complex II-coupled substrate succinate. Succinate reduces ubiquinone (Q) and generates a proton-motive force that is sufficiently high to drive electrons thermodynamically uphill through complex I to reduce  $NAD^+$  to NADH (i.e. reverse electron flow), resulting in superoxide production from a not fully clarified site, probably from semi-reduced coenzyme Q (semiquinone) at the coenzyme Q-binding site of complex I [41,42]. As both succinate and glycerol-3-phosphate dehydrogenases deliver electrons to coenzyme Q, we wanted to examine the influence of UCP1 on succinate-supported ROS production, in comparison with glycerol-3-phosphate-supported ROS production.

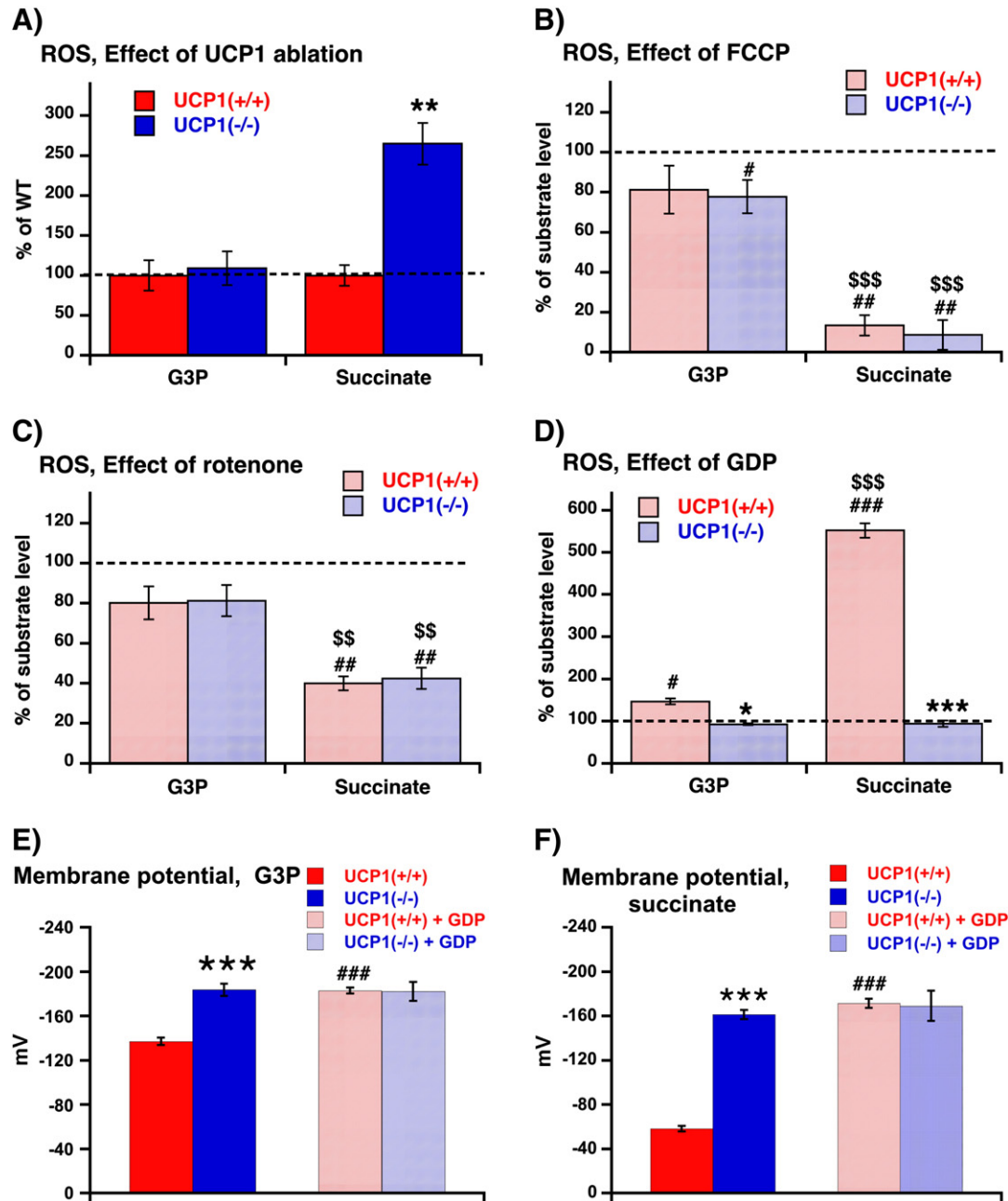
First, the functionality of isolated brown-fat mitochondria respiring on glycerol-3-phosphate versus succinate was examined (Fig. 2A and B) in classical bioenergetic experiments. As seen in Fig. 2A, wildtype brown-fat mitochondria responded to glycerol-3-phosphate addition with a large increase in respiration that was inhibitable by the UCP1 inhibitor GDP; respiration could be re-induced with FCCP. In contrast to glycerol-3-phosphate, succinate was a poor substrate for brown-fat mitochondria and unable to support a high rate of oxygen consumption (Fig. 2A), as pointed out earlier [5,7,36]. Based solely on succinate oxidation rates, no evidence for any role of UCP1 in brown-fat mitochondrial bioenergetics can (apparently) be observed (no effect of GDP or FCCP (Fig. 2A)). This misleading conclusion is presumably mainly due to a low capacity of the succinate transporter [43,44], although under these conditions, oxaloacetate may also exert inhibitory action on succinate dehydrogenase.

The results concerning the membrane potential were different. We assessed membrane potential by safranin O absorbance in mitochondria respiring on glycerol-3-phosphate or succinate (Fig. 2B). The addition of

glycerol-3-phosphate was in itself sufficient to markedly increase the membrane potential, with inhibition of UCP1 activity by GDP leading to a further increase. The addition of succinate led to a much smaller but still measurable increase in membrane potential, smaller probably because the ability of the brown-fat mitochondria to oxidise succinate was so low (Fig. 2A) that the respiratory chain proton pumping could not counteract the backflow of protons through UCP1. However, when UCP1 activity was inhibited by GDP, the pumping capacity was

sufficient to raise the membrane potential nearly to the fully coupled level. Thus, despite the low rate of succinate oxidation, the membrane potential of the brown-fat mitochondria could still be increased significantly when UCP1 was not active.

In these wildtype brown-fat mitochondria (where UCP1 activity is rather high), a much lower rate of ROS production was observed with succinate than with glycerol-3-phosphate (Fig. 2C). This is again probably due in part to the limitations caused by the low capacity of



**Fig. 3.** UCP1 has effect only on succinate-supported ROS production. (A) Effect of UCP1-ablation on rate of  $H_2O_2$  production supported by 5 mM glycerol-3-phosphate or 5 mM succinate in wildtype and UCP1(-/-) brown-fat mitochondria (measured principally as in Fig. 1H). To make this direct comparison we only used data from experiment where mitochondria from wildtype and UCP1(-/-) mice were isolated and analysed in parallel, examined with both glycerol-3-phosphate and succinate as substrate. The mean rate of  $H_2O_2$  production in wildtype mitochondria was expressed as 100% and the rate in UCP1(-/-) was expressed as percentage of this. The values are means  $\pm$  SE of 5 independent mitochondrial preparations for each genotype. (B, C, D) Effects of FCCP (B), rotenone (C) and GDP (D) on relative  $H_2O_2$  production rates in wildtype and UCP1(-/-) brown-fat mitochondria; a comparison between glycerol-3-phosphate and succinate as substrate. FCCP (0.7  $\mu$ M) or rotenone (0.7  $\mu$ M) or GDP (1 mM) was added following substrate addition or simultaneously with substrate. For each preparation day, the rate of hydrogen peroxide production on each substrate was expressed as 100% and the rate after addition in the same trace or in parallel traces was expressed as percentage of this. Values are means  $\pm$  SE of 3–5 independent mitochondrial preparations for each genotype. (E, F) Membrane potential levels in wildtype and UCP1(-/-) brown-fat mitochondria supported by 5 mM glycerol-3-phosphate (E) or 5 mM succinate (F) measured principally as in Fig. 2B. The values represent means  $\pm$  SE of 3–5 independent mitochondrial preparations for each genotype. For all these panels, \* indicates statistically significant differences between wildtype and UCP1(-/-) mitochondria, # statistically significant effect of additions (FCCP, rotenone or GDP) and \$ statistically significant differences between substrates used. One symbol indicates  $P < 0.05$ , two symbols indicate  $P < 0.01$ , and three symbols indicate  $P < 0.001$ .

the succinate transporter; e.g. in muscle mitochondria, the rates are substantially higher [8]. Since glycerol-3-phosphate is oxidised on the outside of the mitochondria, no transport limitations are relevant for this substrate. In addition direct ROS formation by the glycerol-3-phosphate dehydrogenase may also contribute to high ROS production rate [37].

### 3.3. UCP1-dependent ROS production in brown-fat mitochondria under classical conditions of ROS assessment

We proceeded to investigate the UCP1-dependence and further characteristics of succinate- versus glycerol-3-phosphate-supported ROS production (Fig. 3). For simple comparisons, and because the wildtype ROS production rates were so different with the two substrates, we have expressed the effects relative to the rates in wildtype mitochondria.

In contrast to what was the case for glycerol-3-phosphate-supported ROS production – that was unaffected by the presence or absence of UCP1 (Figs. 1D, G and 3A) – we found a marked effect of UCP1-ablation on succinate-supported ROS production (Fig. 3A). The higher membrane potential in UCP1-ablated mitochondria (Fig. 3F) was thus associated with nearly a tripling of ROS production.

In support of the succinate-driven higher ROS production in UCP1(–/–) mitochondria being due to the higher membrane potential, dissipation of the membrane potential with FCCP was associated with an almost complete elimination of succinate-supported ROS production (Fig. 3B). Notably, this FCCP effect was also evident in wildtype mitochondria that are generally considered to be innately “uncoupled”. However, as demonstrated in Figs. 2B and 3F, succinate was able to build up some membrane potential even in the wildtype mitochondria that were thus uncoupled without being totally de-energized – whereas FCCP addition leads to a full de-energization (Fig. 2B), probably explaining the inhibition of ROS production even in the wildtype mitochondria. These observations on succinate-supported ROS production in brown-fat mitochondria are fully in accordance with what would be expected based on classical data obtained with mitochondria from other tissues, demonstrating that succinate-supported ROS production is highly sensitive to changes in membrane potential [45,46], reviewed in [17,41,42].

There was also a small tendency to an inhibition of glycerol-3-phosphate-supported ROS production by de-energization with FCCP (Fig. 3B). This may imply that a small fraction of glycerol-3-phosphate-supported ROS production also occurs through membrane-potential-dependent reverse electron flow (see below); the reason that this is not visible as an effect of the presence or absence of UCP1 would be the same as that for succinate: that even in the presence of active UCP1, a fairly high membrane potential is reached (Figs. 2B and 3E).

To more directly examine the significance of reverse electron flow for ROS production from succinate or glycerol-3-phosphate, we examined the effect of rotenone (Fig. 3C). Rotenone inhibits complex I at subunit ND1 [47] and through this it eliminates reverse electron transport. We found, as expected from other tissues, that a substantial part of succinate-supported ROS production was inhibited by rotenone and thus was due to reverse electron flow. There was a markedly smaller effect of rotenone on glycerol-3-phosphate-supported ROS production (Fig. 3C) and the effect was thus comparable to the effect of FCCP (Fig. 3B). Taken together, these data thus imply that the major part of glycerol-3-phosphate-supported ROS production is not due to reverse electron transport, but also that a small fraction is indeed due to reverse electron transport, principally in agreement with observations in [31].

To further examine the significance of UCP1 activity on ROS production, we analysed the effect of the UCP1 inhibitor GDP. As expected, there was no effect of GDP on ROS production in UCP1(–/–) mitochondria with either substrate (Fig. 3D), in accordance with an absence of effect on membrane potential (Fig. 3EF). In accordance with expectations and with the data in Fig. 3A, inhibition of UCP1 activity by GDP

led to a large stimulation of succinate-dependent ROS production (Fig. 3D), explainable by an increase in membrane potential (Fig. 3F). Unexpectedly, GDP also increased glycerol-3-phosphate-supported ROS production (Fig. 3D), an observation that does not immediately concur with the absence of effect of UCP1 ablation (Figs. 1D, G, I and 3A). Indeed, the observation that the ROS production rate was higher than in UCP1(–/–) mitochondria (Fig. 3D) cannot be explained by an even higher membrane potential being observed in GDP-inhibited mitochondria than in UCP1-ablated mitochondria (Fig. 3E). Thus, the effect of GDP here may be of a different nature (see below).

It is clear from Figs. 1–3 that the magnitude of ROS production observed with succinate as compared to glycerol-3-phosphate is much lower: succinate generated about 10% of the ROS obtained from glycerol-3-phosphate. However, it is also clear that qualitatively remarkably different results were obtained when succinate or glycerol-3-phosphate was examined, yielding contrasting conclusions concerning the significance of membrane potential for ROS production. The conclusion remains that UCP1 does not affect glycerol-3-phosphate-supported ROS production but does notably affect succinate-supported ROS production, when succinate is presented as an externally added substance.

### 3.4. No effect of UCP1 on glycerol-3-phosphate dehydrogenase and succinate dehydrogenase amounts and activities

The possibility may be raised that some of the difference in ROS production observed between wildtype and UCP1(–/–) mitochondria (e.g. Fig. 3A) could be explained by an alteration in the levels of the enzymes responsible. In Fig. 4, we have examined the effect of UCP1 ablation on the protein levels and the enzyme activities of both glycerol-3-phosphate and succinate dehydrogenase systems. We saw no effect of UCP1 ablation on any of these parameters (Fig. 4).

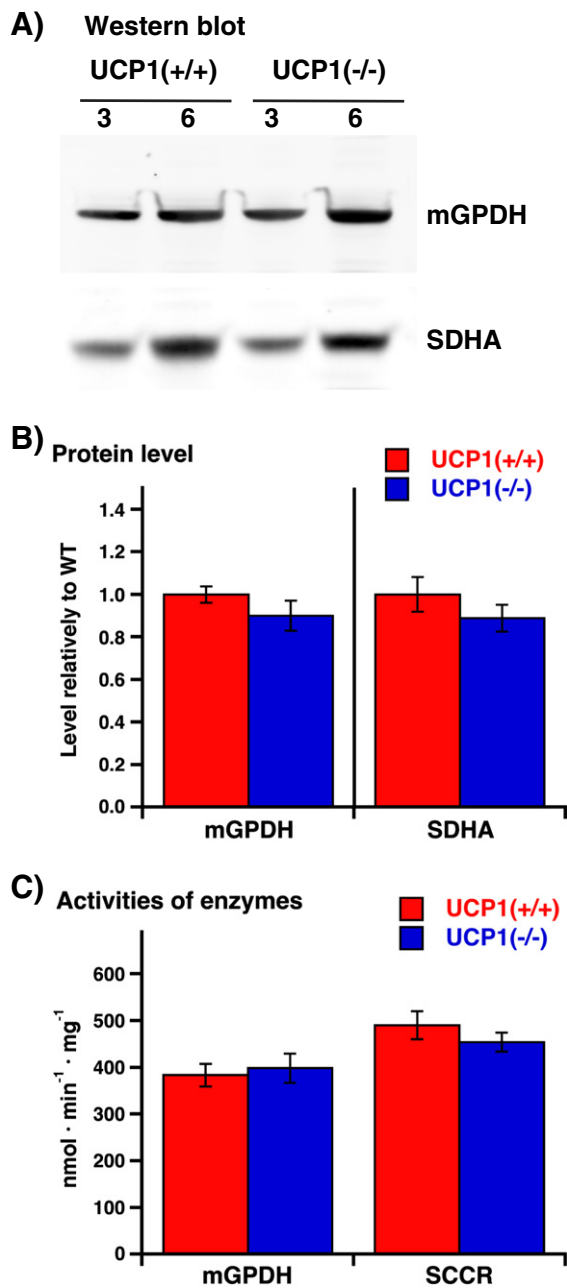
### 3.5. No effect of UCP1 on ROS production supported by physiologically relevant substrates (fatty acid/pyruvate)

Although succinate is a favourite substrate for ROS production studies, externally added succinate is not a physiologically relevant substrate for brown-fat mitochondria (hardly for any mitochondria). We therefore examined ROS production with more physiologically relevant substrates. Brown-fat mitochondria are normally energized mainly with fatty acids (supplying reducing equivalents from  $\beta$ -oxidation to complex I and to electron transferring flavoprotein-ubiquinone reductase) and with pyruvate (supplying reducing equivalents to complex I). For both substrates, there is good reason to believe that oxidation (in the presence of malate) proceeds through the citric acid cycle, and this means that physiologically relevant succinate oxidation will also take place and influence observed ROS production rates. Both fatty acids and pyruvate support UCP1-dependent changes in oxygen consumption and membrane potential in brown-fat mitochondria in a way reflecting naturally regulated uncoupling [7,24,36].

To examine the significance of UCP1 for ROS production with these physiologically relevant substrates, brown-fat mitochondria were allowed to respire on palmitoyl-CoA + carnitine + malate (Fig. 5A–D) or pyruvate + malate (Fig. 5E), and ROS production was measured.

As seen in Fig. 5AB, fatty acid-supported ROS production was observable in both wildtype and UCP1(–/–) mitochondria, although the rate was much lower than that for glycerol-3-phosphate-supported ROS production (Fig. 1I). Again, despite the membrane potential being lower in the wildtype than in the UCP1(–/–) mitochondria (Fig. 5D), ROS production rates were unaffected by the presence or absence of active UCP1 (Fig. 5AB).

We further added rotenone to the mitochondria. The addition of rotenone increased ROS production in both wildtype and UCP1(–/–) mitochondria (Fig. 5AB) to a similar degree. Thus, both endogenous fatty acid-supported ROS production and inhibitor-enhanced,



**Fig. 4.** No effect of UCP1 ablation on glycerol-3-phosphate dehydrogenase and succinate dehydrogenase levels and activities. (A) Western blot analyses of glycerol-3-phosphate dehydrogenase (mGPDH) and the subunit SDHA (70 kDa) of succinate dehydrogenase in wildtype and UCP1(-/-) brown-fat mitochondria. 3 or 6  $\mu$ g of protein was loaded as indicated. (B) Relative concentrations of mitochondrial proteins. Western blots as in A were quantified. The mean level of each protein in wildtype mitochondria was set to 100% and the levels of this protein in other mitochondria expressed relative to this. Values are means  $\pm$  SE of 5 independent mitochondrial preparations in each group, analysed in duplicate. (C) Activities of glycerol-3-phosphate dehydrogenase (mGPDH) and succinate cytochrome c reductase (SCCR) in wildtype and UCP1(-/-) brown-fat mitochondria. Means as in (B).

complex-I-derived ROS production were unaffected by the presence of UCP1 and the ensuing decreased membrane potential.

To further examine the significance of UCP1 activity for ROS production with fatty acid substrates, we analysed the effect of the UCP1 inhibitor, GDP. As seen in Fig. 5C, GDP increased the rate of ROS production in wildtype brown-fat mitochondria, as would indeed be expected if ROS production was influenced by membrane potential. However, the effect of GDP on membrane potential was not higher than the effect of UCP1 ablation (Fig. 5D) (that did not affect ROS production), so there was

no simple relationship between membrane potential and ROS production in this case. Even more remarkable, there was a clear stimulation of ROS production by GDP in UCP1(-/-) mitochondria (evidently without any effect on membrane potential (Fig. 5D)), making it clear that this GDP effect was unrelated to its inhibition of UCP1 activity. Rather, we would suggest that UCP1-independent GDP effects on ROS production may occasionally be observed. We do not know the nature of such effects; however, there is no reason to think that they are related to any uncoupling protein activity, as we have also seen such effects in liver mitochondria (not shown), i.e. in mitochondria that totally lack any of the so-called uncoupling proteins.

Also pyruvate is a good complex-I substrate for supporting thermogenesis in brown-fat mitochondria [7,24,36]. ROS production was low (Fig. 5E), the lowest observed with any of the substrates examined here. Pyruvate exhibits some capacity to scavenge ROS [48], and this could be a reason that ROS appeared lower than with fatty acid substrate. We did not observe any effect of UCP1 on pyruvate-supported ROS production, and similar to what was the case for fatty acid-supported ROS production, rotenone increased ROS production, again to the same extent in wildtype and UCP1(-/-) mitochondria (Fig. 5E).

Thus, with physiologically relevant substrates, we find no evidence that the presence of active UCP1 affects ROS production. As the total oxidation process in the mitochondria would include the oxidation of succinate in the citric acid cycle, the data also imply that when succinate is generated in the mitochondria as part of the physiologically relevant oxidation process, there is no measurable effect of the presence or absence of UCP1 on endogenous-succinate-supported ROS production.

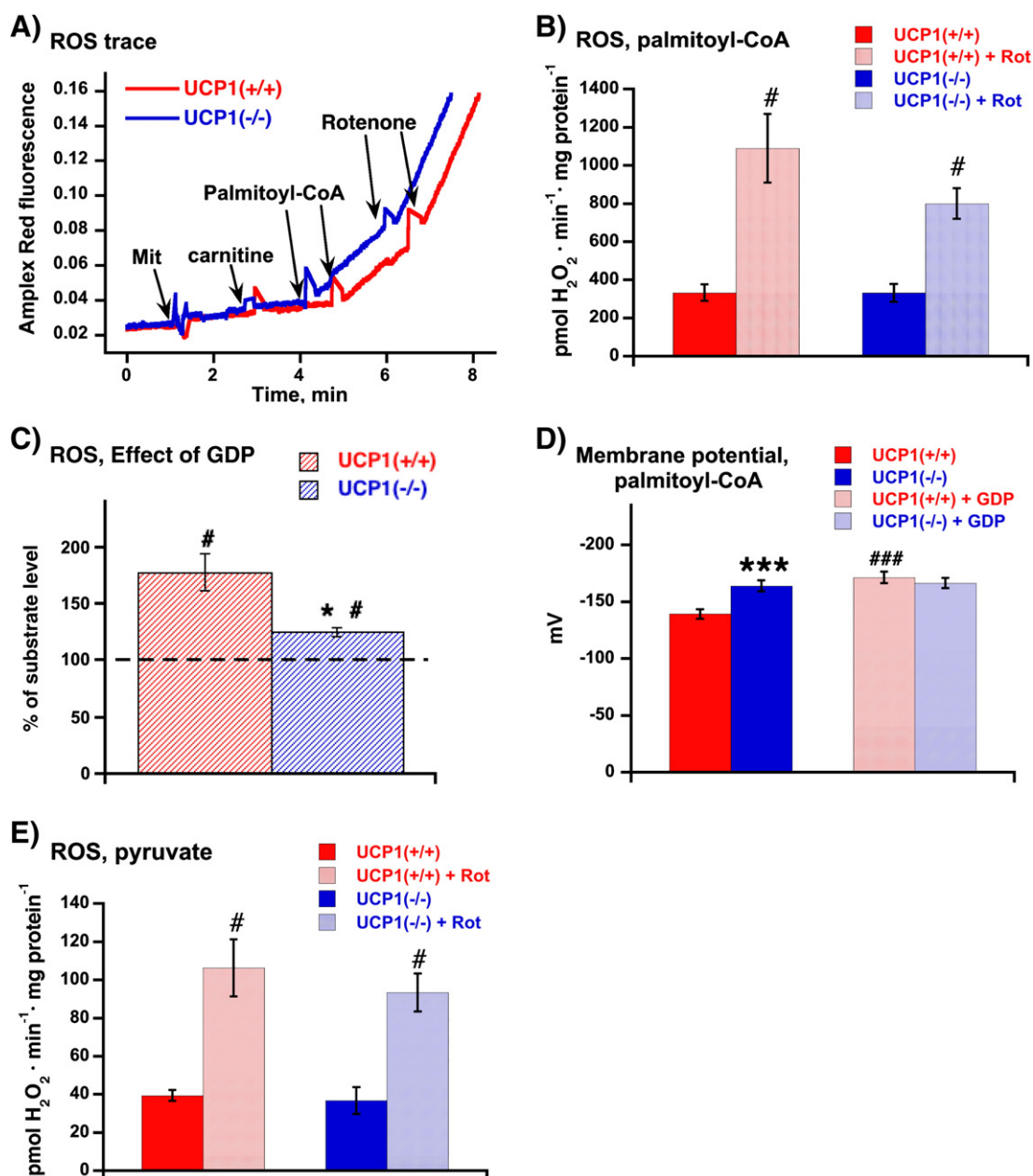
### 3.6. Cold acclimation induces increased uncoupling but concurrently high ROS production

To directly study the relation between UCP1 amount and ROS production rate, we examined ROS production in brown-fat mitochondria isolated from warm-acclimated and cold-acclimated mice (Fig. 6). As compared to mitochondria from warm-acclimated (30 °C) mice, mitochondria from cold-acclimated (4 °C) mice possess about 3-fold higher amounts of UCP1 [7,36]. This difference is reflected in the mitochondrial membrane potential being much higher in the warm-acclimated than in the cold-acclimated mitochondria, both when palmitoyl-CoA and when glycerol-3-phosphate were used as substrates (Fig. 6A and D). Despite these large differences in UCP1 amount and membrane potential, ROS production rates supported by palmitoyl-CoA were identical in mitochondria from warm-acclimated and cold-acclimated mitochondria (Fig. 6B), also after the addition of rotenone (cold acclimation does not alter the concentration of complex I in brown-fat mitochondria [36]). There was a stimulatory effect of GDP on ROS production in both types of mitochondria (Fig. 6C), an effect that could have been ascribed to inhibition of UCP1 activity by GDP and an ensuing higher membrane potential. However, the effect of GDP was higher in the warm-acclimated than in the cold-acclimated mitochondria (Fig. 6C), which is the opposite of what would be expected if the effect was due to inhibition of UCP1. We therefore also here suggest that GDP effects observed in these and other types of mitochondria are not (necessarily) due to UCP1 inhibition.

Concerning glycerol-3-phosphate-supported ROS production, acclimation to cold was associated with a higher and not lower rate, as would have been expected if UCP1 activity was protective against ROS production (Fig. 6E). Also in these mitochondrial preparations, inhibition of ROS production by rotenone implied that some part of the ROS originated from reverse electron flow (as implied above (Fig. 3)). The effect of GDP addition was similar in the two types of preparations (+21  $\pm$  5% in mitochondria from warm- and +29  $\pm$  9% in mitochondria from cold-acclimated mice; not shown), again indicating that the GDP effect is probably not related to UCP1 activity.

As one possible cause of the increased ROS production in cold-acclimated mitochondria, we examined whether the activity of the



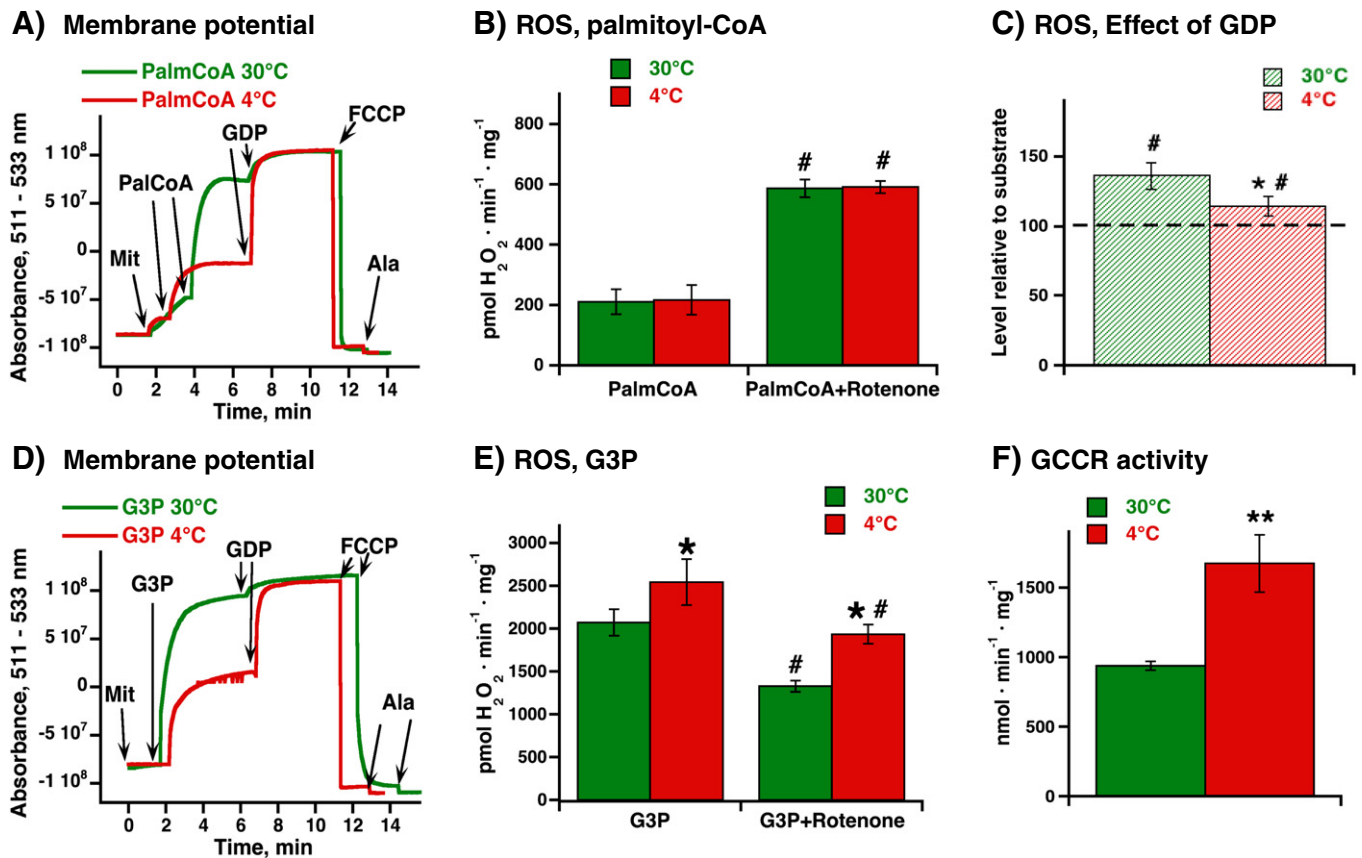


**Fig. 5.** No effect of UCP1 on ROS production from complex-I-linked substrates. (A) A representative trace of Amplex Red fluorescence measured in wildtype and UCP1(−/−) brown-fat mitochondria. Successive additions were 0.08 mg/ml brown-fat mitochondria (Mit), 5 mM carnitine, 50 μM palmitoyl-CoA and 0.7 μM rotenone. Note that these experiments are initially run in the absence of GDP and rotenone; thus, neither UCP1 activity nor forward electron flow is inhibited. (B) ROS production (measured principally as shown in A and expressed as hydrogen peroxide amount, based on calibrations) in brown-fat mitochondria isolated from wildtype and UCP1(−/−) mice. In this panel and the following ones, values are means ± SE of 4 independent mitochondrial preparations of each genotype; statistics are as in Fig. 3. (C) Comparison of effects of 1 mM GDP on relative H<sub>2</sub>O<sub>2</sub> production rates in wildtype and UCP1(−/−) brown-fat mitochondria supported by 50 μM palmitoyl-CoA plus 5 mM carnitine. GDP was added following substrate addition, principally as shown for rotenone in (A). The rate of hydrogen peroxide production supported by substrate was expressed as 100% and the rate after GDP addition from the same trace was expressed as percentage of this. (D) Membrane potential level in wildtype and UCP1(−/−) brown-fat mitochondria supported by 50 μM palmitoyl CoA plus 5 mM carnitine, measured principally as in Fig. 2B. (E) ROS production rate (measured principally as shown in A but after addition of malate instead of carnitine and pyruvate instead of palmitoyl-CoA) in brown-fat mitochondria isolated from wildtype and UCP1(−/−) mice.

glycerol-3-phosphate dehydrogenase system was altered as an effect of cold acclimation. We observed nearly a doubling of the activity of this system (Fig. 6F), in agreement with our earlier observations [49]. We therefore find it probable that the increased ROS production in the cold with glycerol-3-phosphate as substrate reflects the higher amount of the dehydrogenase system in itself and has nothing to do with UCP1 activity (where, as said, the opposite relation would have been expected).

#### 4. Discussion

The present study addressed the issue whether UCP1 regulates ROS production. The answer was qualitatively dependent on the experimental approach used. Under standard conditions for examining ROS production, i.e. with reverse electron flow from exogenously added succinate, UCP1 activity led to diminished ROS production. However, with physiologically relevant substrates, representing lipid and carbohydrate oxidation, ROS



**Fig. 6.** Effect of increased UCP1 levels on ROS production. In each panel, comparisons were made between brown-fat mitochondria isolated from wildtype mice acclimated to either thermoneutrality (30 °C) or to cold (4 °C). (A, D) Representative recordings from membrane potential measurements of brown-fat mitochondria supported by palmitoyl-CoA plus carnitine (A) or by glycerol-3-phosphate (D). Additions were 0.25 mg mitochondria (Mit), 50 μM palmitoyl CoA (PalmCoA) (5 mM carnitine was already present in the medium) or 5 mM glycerol-3-phosphate (G3P), 2 mM GDP, 1.4 μM FCCP and 0.02 mg/ml alamethicin (Ala). (B, E) ROS production rates (measured principally as shown in Figs. 1H and 5A). Values here and in (C) and (F) are means ± SE from 3 to 5 independent mitochondrial preparations for each environmental temperature. (C) Comparison of effects of 1 mM GDP on relative ROS production rates in brown-fat mitochondria supported by 50 μM palmitoyl-CoA plus 5 mM carnitine. GDP was added following substrate addition, principally as in Fig. 5A and C. (F) Activities of glycerol 3-phosphate cytochrome c reductase (GCCR) in brown-fat mitochondria. \*: statistically significant difference between mitochondria isolated from mice acclimated at 30 °C versus 4 °C temperatures; #: statistically significant effect of additions (rotenone or GDP). One symbol indicates  $P < 0.05$ , two symbols indicate  $P < 0.01$ .

production was independent of UCP1 amount and activity. These results may not only be of significance for understanding the ROS issues of brown adipose tissue. If extrapolation to other tissues is allowed, our results may have an important bearing on the physiological relevance of the “mild uncoupling” hypothesis for protection against oxidative damage, and particularly on the hypothesis that oxidative damage may be prevented by activation of members of the uncoupling protein family. The implication from the present experiments would be that activation of uncoupling proteins and decreases of mitochondrial membrane potential may not be feasible avenues for decreasing ROS production and oxidative damage. However, dedicated experiments would be needed to prove the generality of our observations.

#### 4.1. The singular substrates and ROS production

As implied above, the relationship between UCP1 activity, membrane potential and ROS production is different depending on the experimental system studied, particularly which substrate is used to support mitochondrial respiration and thus ROS production. These substrates will therefore be discussed separately.

##### 4.1.1. Glycerol-3-phosphate

We found no effect of the presence or activity of UCP1 on glycerol-3-phosphate-supported ROS production. These results expand those

reported by us [3] and others [1] demonstrating that despite a clear difference in membrane potential levels, brown-fat mitochondria with or without UCP1 produce equal amounts of superoxide, as determined by the DHE method. The earlier experiments were conducted in the presence of rotenone, and reverse electron flow was thus inhibited. The absence of effect of uncoupling on ROS was therefore as expected, as discussed above. However, the continued absence of UCP1 effect even in the absence of rotenone would indicate that reverse electron flow is not important for glycerol-3-phosphate-supported ROS production. This in turn is in agreement with our observations of only a small inhibitory effect of either rotenone or FCCP on glycerol-3-phosphate-supported ROS production. This low but existing ROS production from reverse electron flow is in agreement with what has been suggested [2,31]. The reason that FCCP has an inhibitory effect but UCP1 has not may be understood as reflecting the only limited decrease in membrane potential observed from UCP1 activity (Fig. 1B), as compared to the total de-energisation introduced by FCCP (Fig. 2B) in these mitochondria from 24 °C-acclimated mice with limiting UCP1 capacity.

Thus, we adhere to the idea that the main part of glycerol-3-phosphate-supported ROS production in brown-fat mitochondria comes directly from the glycerol-3-phosphate-dehydrogenase complex itself or its interaction with coenzyme Q [50,51], rather than from activity elsewhere in the respiratory chain. It may thus accurately be said that a membrane potential effect cannot be expected

for glycerol-3-phosphate-supported ROS production. However, in that case the possibility of diminishing ROS production and oxidative damage through membrane depolarization becomes untenable.

The ROS production we observed with glycerol-3-phosphate was much higher than that seen with any other substrate. This primarily reflects the very high level of glycerol-3-phosphate dehydrogenase in brown-fat mitochondria [35,38,52]. An issue is whether such high rates would ever be relevant in-vivo. This is doubtful, as the rates reported here are observed with 5 mM concentrations of glycerol-3-phosphate. Measurements of tissue levels of glycerol-3-phosphate imply much lower levels of cytosolic glycerol-3-phosphate ( $\approx 0.2$  mM) [53,54]. Thus, in reality the brown-fat mitochondria may not become exposed to the very high ROS levels observed with the high glycerol-3-phosphate concentrations experimentally used.

#### 4.1.2. Succinate

Concerning succinate-supported ROS production, we obtained exactly the type of results that would be predicted from many similar studies in other cell types. When reverse electron flow was allowed, membrane potential-sensitive ROS production could be demonstrated, which was thus also sensitive to the activity of UCP1. This is also what has been observed by others working with brown-fat mitochondria [2]. However, this ROS production is supported by what are probably high concentrations of exogenously added succinate; there are indications that endogenous succinate concentrations (from the citric acid cycle) may be much lower [13,55,56]. Thus, had succinate been a “typical” substrate, there would have been good evidence for UCP1 and uncoupling as protectors against enhanced ROS production and oxidative damage. However, since succinate represents the exception rather than the rule, the results obtained with succinate cannot be extrapolated to be relevant for ROS production in general.

#### 4.1.3. Acyl-CoA/acyl-carnitine

We found no effect of the presence or activity of UCP1 on ROS production supported by the palmitoyl-CoA. In contrast, Oelkrug et al. [2] suggested that UCP1 mitigates mitochondrial superoxide production during fatty acid oxidation. This was based on experiments different from those used here and of a somewhat more complex type. First Oelkrug et al. [2] ran what is traditionally referred to as a “carnitine cycle” in brown-fat mitochondria from wildtype and UCP1(–/–) mice. During this ATP-induced oxidation of endogenous fatty acids, more ROS was produced in the UCP1(–/–) than in wildtype mice. While this would be in accordance with UCP1 activity diminishing ROS production, another possibility would be that the difference could be due to different amounts of endogenous fatty acids being present in the two types of mitochondria. We have observed that UCP1(–/–) brown-fat mitochondria contain more fatty acids than do wildtype mitochondria (not shown), and the difference in ROS production may adequately be explained based on this difference in amount of substrate. Oelkrug et al. [2] further found that addition of palmitate to the mitochondria enhanced ROS production more in UCP1(–/–) mitochondria than in wildtype mitochondria. However, palmitate addition to the UCP1(–/–) mitochondria seemed to decrease the respiratory rate, and electrons may accumulate in Complex-I due to limitations in electron flow (cf. our Figs. 5B and 6B). In our conditions, we see no effect of the presence or amount of UCP1 on ROS production (Figs. 5B and 6B).

If it is accepted that only reverse electron flow is sensitive to membrane potential [41,42], and fatty acid oxidation does not support reverse electron flow [57], our results of lack-of-effect of UCP1 on palmitoyl-CoA-supported ROS production would indeed be the expected outcome. Our findings of UCP1-independence of palmitoyl-CoA-supported ROS are also principally in agreement with those of Schönfeld et al. [5] that suggested that acylcarnitine-supported ROS generation in brown-fat mitochondria is mainly associated with acyl-CoA dehydrogenase and electron transferring flavoprotein-ubiquinone reductase, rather than with the central complexes of the

respiratory chain. Still, it is in the form of external substrates that electrons are fed into the respiratory chain, and if alterations in membrane potential should alter physiologically relevant ROS production rates, these effects must be observable when the relevant substrates are made available to the mitochondria.

Thus, the indications are that acyl-CoA-supported ROS production is independent of UCP1 activity in brown-fat mitochondria. There are, however, further implications of these observations. Fatty acids are undoubtedly a major substrate source for brown-fat mitochondria in-situ, and the implication from the present investigations would therefore be that there is no reason to think that UCP1 activity in-situ should affect the risk for oxidative damage in brown-fat mitochondria. Indeed, we have observed that, as compared with wildtype mice, there is 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) [3]. The absence of oxidative damage in UCP1(–/–) mitochondria was not due to enhanced activity of antioxidant enzymes [3]. There was also no difference in phospholipid fatty acyl composition in brown-fat mitochondria between wildtype and UCP1-ablated mice [58] in contrast that would be expected if lipid peroxidation was activated due to absence of UCP1.

Thus, the present and the earlier observations converge to indicate that UCP1 would appear not to be physiologically involved in defence against oxidative stress.

Additionally, as mentioned above, mitochondrial oxidation of complex-I-linked substrates is the only source for succinate in-situ. Succinate being the only substrate with a demonstrated membrane potential effect on ROS production, any ROS originating from succinate oxidation should be visible in the ROS production observed during acyl-CoA oxidation. No part of the ROS production was, however, affected by the membrane potential when acyl-CoA was the substrate. Thus, despite the accumulation of biochemically convincing evidence for decreased ROS production due to decreased membrane potential when succinate is solely used as substrate, these observations can clearly not be extrapolated to physiological circumstances.

#### 4.1.4. Pyruvate

We found no effect of UCP1 presence or activity on ROS production supported by pyruvate. Our results are in agreement with [2,4], and principally this is as expected, based on the conclusions by Brand and by Murphy that forward electron flow supported by pyruvate is insensitive to membrane potential [41,42]. However, when Dlaskova et al. [1] used Amplex Red (as we did here), they observed UCP1 dependence. These data are difficult to reconcile with ours and others [2,4]. One reason for this discrepancy may be related to the experimental conditions. As pointed out earlier [7], UCP1(–/–) mitochondria are very sensitive to medium tonicity and the low tonicity medium used by Dlaskova et al. [1] may have influenced the results.

The reasoning above concerning acyl-CoA-supported ROS production is equally relevant for pyruvate-supported ROS production, with the addition that the very low rates of ROS production observed with this substrate (about 100-fold lower than with glycerol-3-phosphate) would indicate that pyruvate-supported ROS production may not be a quantitatively important contributor to ROS in the cell. Thus, our data again imply that uncoupling activity has no effect on ROS production with physiological substrates.

#### 4.2. Possible misleading effects of GDP

In several types of experiments, we observed that the addition of GDP to the mitochondria increased ROS production. The obvious explanation would be that this was caused by GDP inhibition of UCP1, leading to an increase in the membrane potential and an ensuing increase in ROS production. Indeed, this is probably what happens when succinate-supported ROS production is followed, as in Fig. 3D. However,

several other experiments indicate that this explanation is not relevant for most GDP stimulation observed here. We see effects of GDP even in UCP1(–/–) mitochondria, and these effects are not accompanied by (further) increases in membrane potential, eliminating the possibility that GDP could inhibit any other “uncoupling”(-like) protein. We have also observed such ROS production-stimulating effect of GDP in liver mitochondria, i.e. mitochondria that do not possess any of the named “uncoupling” proteins (not shown).

GDP could interact with other membrane proteins, such as the adenine nucleotide transporter [59,60], but again, the effects we saw were not paralleled with any membrane potential effects which could have been anticipated if the adenine nucleotide transporters are responsible for a significant fraction of the “basal proton leak” of the mitochondria [61,62]. Suski et al. found [63] effects of GDP on ROS production in brain mitochondria – but they were inhibitory and are thus not relevant for the discussion here. Others have seen very varying effects of GDP on ROS production in thymus mitochondria [64], and there is indeed good reason to think that GDP can interact in different ways with many processes.

What we conclude from all observations of GDP and ROS production is that although GDP effects may be observable and may even in some cases be truly due to UCP1 inhibition, the observation that GDP has an effect can in no way be taken in general as a demonstration that UCP1 (or any so-called uncoupling protein) is involved in the process, at least not in a way related to mitochondrial membrane potential control.

#### 4.3. The relationship between membrane potential and ROS production in brown-fat mitochondria

In this paper, we have a series of conditions where the membrane potential of the mitochondria is decreased (Figs. 1B, 3E, 5D) but where parallel measurements of ROS production do not indicate any effect of this depolarization (Figs. 1D, G, I, 3A, 5B). Taken together, these results imply that the mitochondrial membrane potential is of minor significance for controlling mitochondrial ROS production. The further implication of this would be that protection against oxidative damage cannot be obtained by reduction in membrane potential, at least not in brown-fat mitochondria. This may mainly be because most ROS is released from enzymes (dehydrogenases) slightly distant from the core respiratory chain. However, this does not alter the conclusion that alterations in membrane potential do not markedly affect ROS production.

#### 4.4. The significance of UCP1 for ROS production in brown adipose tissue

The data presented here – and most of the data presented elsewhere – arrive at the conclusion that UCP1 is not a regulator of ROS production in brown-fat mitochondria; we have not been able to replicate the few data claiming the opposite. Indeed, it has been pointed out by Mailloux et al. [4] that the increased level of UCP1 in the cold is actually associated with a higher ROS production (cf. also our Fig. 6). We have examined the literature to establish the relationship between increased UCP1 amounts and the activation of other protective means (which would be expected to be less needed if UCP1 was active). Although cold acclimation is a situation with increased amounts of UCP1 [65], the antioxidant system of brown adipose tissue is significantly upregulated in the cold [4,66,67]. We have also observed significant upregulation of SOD2 and thioredoxin reductase 2 in brown-fat mitochondria isolated from cold-acclimated mice (G. Mathy, I. Shabalina, J. Nedergaard, F. Sluse, unpublished observations). Thus, even if UCP1 would have any effect against oxidative damage, it is clearly not so that the tissue would rely solely on this for protection.

#### 4.5. The general significance of uncoupling proteins for protection against oxidative damage

In a broader context, this investigation of the significance of UCP1 for ROS production also constitutes a general investigation of the possibility that other uncoupling proteins (or other proteins that could have uncoupling characteristics) could function as ameliorators of oxidative damage. For this thesis, observations of positive effects of UCP1 could have been seen as a proof-of-principle. As the data presented here arrive at the opposite conclusion, i.e. that UCP1 through membrane depolarization has little ability to protect against oxidative damage, suggestions that other UCPs could be protective by mediating uncoupling appear less relevant. Several suggestions have been made for the existence of agents that could activate an uncoupling activity in other UCPs [9,15]; the validity of such agents has been questioned [3,8,28,68,69]. However, based on extrapolation of the data presented here, this issue would be of less interest – in that even if activated, the UCPs could not be expected to affect ROS production and oxidative damage through an uncoupling activity.

Of course, the present observations do not exclude the possibility that UCP1 or other UCPs may be able to protect against oxidative damage through other means. Indeed, several such suggestions have been made [69–72]. What is demonstrated here is merely that innate uncoupling (as mediated through UCP1) at physiological conditions does not seem to have the potential to decrease ROS production and oxidative damage, at least not in brown-fat mitochondria.

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