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Original Contribution

Antioxidant properties of UCP1 are evolutionarily conserved in mammals and buffer mitochondrial reactive oxygen species

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ABSTRACT

Mitochondrial uncoupling reduces reactive oxygen species (ROS) production and appears to be important for cellular signaling/protection, making it a focus for the treatment of metabolic and age-related diseases. Whereas the physiological role of uncoupling protein 1 (UCP1) of brown adipose tissue is established for thermogenesis, the function of UCP1 in the reduction of ROS in cold-exposed animals is currently under debate. Here, we investigated the role of UCP1 in mitochondrial ROS handling in the Lesser hedgehog tenrec (*Echinops telfairi*), a unique protoendothermic Malagasy mammal with recently identified brown adipose tissue (BAT). We show that the reduction of ROS by UCP1 activity also occurs in BAT mitochondria of the tenrec, suggesting that the antioxidative role of UCP1 is an ancient mammalian trait. Our analysis shows that the quantity of UCP1 displays strong control over mitochondrial hydrogen peroxide release, whereas other factors, such as mild cold, nonshivering thermogenesis, oxidative capacity, and mitochondrial respiration, do not correlate. Furthermore, hydrogen peroxide release from recoupled BAT mitochondria was positively associated with mitochondrial membrane potential. These findings led to a model of UCP1 controlling mitochondrial ROS release and, presumably, being controlled by high membrane potential, as proposed in the canonical model of "mild uncoupling". Our study further promotes a conserved role for UCP1 in the prevention of oxidative stress, which was presumably established during evolution before UCP1 was physiologically integrated into nonshivering thermogenesis.

Introduction

Mitochondrial uncoupling tunes three physiologically relevant bioenergetic outputs in mitochondria: mitochondrial reactive oxygen species production, ATP, and heat [1]. The proton gradient over the mitochondrial inner membrane, named the mitochondrial proton-motive force, is central to these three outputs. Whereas mitochondrial superoxide production occurs during electron transport at the respiratory chain complexes for the production of the proton-motive force, consumption of the proton-motive force is mediated by ATP synthesis and proton leak.

The magnitude of mitochondrial reactive oxygen species (ROS) production depends on the redox state of the respiratory chain complexes. When complexes are reduced at high proton-motive force, this increases the probability of electrons escaping from the respiratory chain and forming superoxide [1,2]. Conversely, a reduction of the proton-motive force reduces mitochondrial ROS output. This can be achieved either by less substrate oxidation (electron influx), although intermediate reduction states may also form radicals [3], or by increased consumption of the proton-motive force. Uncoupling/proton leak activity of mitochondrial uncoupling proteins (UCPs) and the adenine nucleotide translocase (ANT) has been implicated in cellular ROS handling [2].

Under conditions of physiological homeostasis, the proton-motive force, as a central intermediate, is perfectly integrated to balance cellular energy metabolism. The dynamic alteration of a single oxidative phosphorylation parameter will affect proton-motive force and all other modules of oxidative phosphorylation, with

Abbreviations: HPRR, hydrogen peroxide release rate; ANT, adenine nucleotide translocase; BAT, brown adipose tissue; CAT, carboxyatractylate; COX, cytochrome *c* oxidase; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; GDP, guanosine diphosphate; G3P, glycerol 3-phosphate; PWR, peak rewarming rate; ROT, rotenone; MMP, mitochondrial membrane potential; UCP1, uncoupling protein 1; ROS, reactive oxygen species

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physiological consequences on cellular energy homeostasis and ROS production. For example, during glucose-stimulated insulin secretion, glucose increases the substrate input to mitochondria, increasing ATP levels to trigger insulin secretion [4]. Mitochondrial uncoupling controls the efficiency of ATP production, altering the magnitude of insulin secretion [5]. Recently, the modulation of insulin secretion by UCP2 has also been attributed to alterations in ROS rather than to ATP levels [6]. The bioenergetic design of the β cells demonstrates that one mitochondrial parameter, such as uncoupling, may have an impact on multiple aspects of cellular metabolism and function.

In mammalian brown adipose tissue (BAT), the role of UCP1 has been solely attributed to heat production to defend body temperature [7]. Others and we have shown that UCP1 activity may also reduce mitochondrial ROS production, as shown in isolated thymus and BAT mitochondria of wild-type and UCP1-knockout mice [8–10]. The impact of UCP1 on ROS is greatest in response to prolonged cold acclimation [10]. Although the role of UCP1 in heat production has been established for the past 35 years [11], given pronounced mitochondrial biogenesis in BAT during cold acclimatization, the physiological importance of ROS prevention can only be hypothesized. Increased content of mitochondrial complexes potentially increases the probability of electrons escaping from the respiratory chain, thus increasing deleterious levels of ROS. Recently, increased systemic oxidative stress during cold exposure has been found in UCP1-knockout mice [12] but whether this contributes to the reduced life span of these mice in the cold [13] remains elusive.

So far, the potential role of UCP1 in ROS prevention has been investigated only in mice, although there is a diversity of species that possess UCP1. Our laboratory discovered an ancient evolutionary history of UCP1, demonstrating the presence of UCP1 orthologs in ectothermic vertebrates [14]. The function of these orthologs is unknown to date but a thermogenic function as found in modern eutherian mammals seems to be unlikely. However, the thermogenic role and molecular characterization of UCP1 were shown in a protoendothermic afrotherian species. Afrotheria is a mammalian clade that diverged during early evolution of modern eutherian mammals [15,16]. Whether UCP1 in afrotherian species also prevents ROS production in BAT mitochondria is unknown but may elucidate whether these properties were already present at the evolutionary origin of eutherian nonshivering thermogenesis of BAT.

Here we studied hydrogen peroxide release from BAT mitochondria of the Lesser hedgehog tenrec (*Echinops telfairi*), a protoendothermic mammal that sporadically exhibits homeothermic endothermy at a body temperature of 32 °C. The animals were either acclimated to mild cold (20 °C) or kept at room temperature (27 °C), which resulted in group differences in β3-adrenergic-mediated thermogenesis during rewarming phases, cytochrome *c* oxidase (COX) activity, and UCP1 content, as shown previously [16]. The differences in UCP1 content between acclimation groups, however, were not as extreme as in previous cold-acclimation experiments in mice, leading us to apply regression analysis to explore the interrelation between UCP1 and ROS.

Material and methods

Animals

The maintenance of the laboratory bred Lesser hedgehog tenrecs (*E. telfairi*) was described previously [16]. One group of six animals remained at 27 °C (warm-acclimated group, WA) and the other group of six animals was stepwise acclimated to 20 °C (cold-acclimated group, CA). The average body mass was 129.9 ± 10.5 g for the WA group and 145.4 ± 8.1 g for the CA group ($P=0.267$, Student's *t* test). Body temperature was recorded using temperature-sensitive transmitters (Mini Mitter Co., Inc., USA) to ensure that all animals were endothermic at the time of organ harvest (>29 °C [16]). All experiments were approved by the German Animal Welfare Authorities.

Peak rewarming rates

Peak rewarming rate (PWR, °C/min) was calculated from the body temperature time course during arousal, assuming a four-parameter sigmoid curve [17]. For these calculations, the Regression Wizard function of SigmaPlot was used (SigmaPlot 10 for Windows, Systat Software, Inc., USA). BAT-mediated thermogenesis was inhibited by subcutaneously injecting animals with a β3-adrenergic receptor antagonist, SR 59230A (1 mg/kg, 3-(2-ethylphenoxy)-1-[[(1*S*)-1,2,3,4-tetrahydronaphth-1-yl]amino]-(2*S*)-2-propanol oxalate salt, Sigma), at the onset of torpor arousal [18]. ΔPWR represents the difference between peak rewarming rates without and with injection of the antagonist, reflecting the BAT-associated part of thermogenesis.

Isolation of mitochondria

Tenrecs were sacrificed and all brown adipose tissue depots were removed, weighed, and rapidly placed in ice-cold buffer for mitochondrial isolation as described previously [19]. Mitochondrial protein concentrations were determined with a biuret assay using fatty acid-free bovine serum albumin as a standard.

COX activity, UCP1 detection, mitochondrial respiration, and membrane potential

UCP1 content, COX activity, mitochondrial respiration, and membrane potentials were measured previously [16]. In short, UCP1 protein levels of BAT homogenates and isolated mitochondria were detected by Western blot analysis using a rabbit anti-UCP1 polyclonal antibody (1:10,000, 3046, Chemicon). COX activity was determined polarographically using a Clark-type oxygen electrode (Rank Brothers, UK) in BAT homogenates and mitochondria at 25 °C. COX activity was calculated per gram of BAT wet weight or per milligram of mitochondrial protein (determined with the BCA method).

Mitochondrial oxygen consumption was measured using a Clark-type oxygen electrode (Rank Brothers Ltd.) kept at 32 °C and calibrated with air-saturated medium (50 mM KCl, 5 mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES), 2 mM $MgCl_2 \cdot 6 \times H_2O$, 4 mM KH_2PO_4, 1 mM EGTA, 0.4% bovine serum albumin (w/v), pH 7.2 at room temperature) that was assumed to contain 432 nmol of O ml^{-1} at 32 °C [20]. Concentrations of inhibitors in the reaction chamber were 4.8 μM rotenone (inhibitor of complex I), 1 μg/ml oligomycin (inhibitor of the ATP synthase), and, where indicated, 2.5 μM carboxyatractylate (CAT; inhibitor of the ANT). Mitochondria were energized by the addition of succinate (4 mM, substrate for complex II) or glycerol 3-phosphate (G3P; 15 mM, substrate of the glycerol-3-phosphate dehydrogenase). Subsequently GDP (5 mM) was administered to inhibit UCP1 and to obtain coupled mitochondria and finally FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone) to induce maximum substrate oxidation.

Simultaneous with succinate respiration, mitochondrial membrane potential was assessed using a triphenylmethylphosphonium (TPMP+)-sensitive electrode in the presence of inhibitors as described previously [16]. Nigericin was added to convert ΔpH to membrane potential, thereby assessing proton-motive force.

Measurement of mitochondrial hydrogen peroxide release

Measurements of hydrogen peroxide production of isolated mitochondria were performed similar to the procedures of Oelkrug et al. [10]. Brown adipose tissue mitochondria (20 μg) were incubated in 200 μl of pre-equilibrated assay buffer (50 mM KCl, 5 mM TES, 2 mM $MgCl_2 \cdot 6 \times H_2O$, 4 mM KH_2PO_4, 1 mM EGTA, 0.4% bovine serum albumin (w/v), pH 7.2 at room temperature). This assay contained the fluorescent probe Amplex red (50 μM, Invitrogen), 30 units/ml superoxide dismutase, 6 units/ml horseradish peroxidase, and 1 μg/ml oligomycin. Furthermore, superoxide production was also measured in the presence of rotenone (ROT; 2 μM, inhibiting complex I-derived reactive oxygen species production), GDP (5 mM, to inhibit UCP1), and CAT (2.5 μM) to distinguish from adenine nucleotide transporter (ANT)-dependent effects. H_2O_2 formation was initiated by the addition of succinate (5 mM) or glycerol 3-phosphate (15 mM). Fluorescence was detected at 32 °C in a microplate reader (FLUOstar Optima, BMG Labtech) in 96-well microplates (Greiner 96-well, clear, F-bottom, black), at an excitation wavelength of 560–10 nm and an emission wavelength of 590 nm. Fluorescence was calibrated using known amounts of H_2O_2 at each experimental day.

Statistical analysis

Statistical analysis was performed using SPSS version 20.0 (IBM) for correlation and regression or SigmaStat 3.5 (Jandel Scientific) for paired *t* test and Student's *t* tests, on raw data. Distributions of all variables were tested for normality using the Kolmogorov–Smirnov test. In figures and text, the data are expressed as means ± standard error of the mean.

For any bivariate analysis, r^2 is the squared Spearman correlation coefficient (r), giving the proportion of the variance (fluctuation) of one variable that is predictable from the other variable. To explore potential independent variables for their impact on the target variable (here, ROS release rates) we used GLM (general linear modeling). For this, the parameter "acclimation state" was dummy coded. After independent variable variance was accounted for, the residual variances (expressed as standardized residuals) were explored for potential significant associations with other measured parameters of the BAT mitochondria. The 0.05 level of probability was set to indicate statistical significance.

Results

Cold acclimation improves the oxidative quality of tenrec BAT

We studied BAT-mediated thermogenesis in the protoendothermic tenrec by analyzing PWR from torpor, as rates are sensitive to β3-adrenergic stimulation [16]. Mild cold ambient temperature of 20 °C (vs 27 °C) recruited BAT-mediated thermogenesis and increased mitochondrial biogenesis, BAT COX activity, and UCP1 content [16]. Here we confirm that total BAT weight was unchanged (mean BAT weight: 27 °C, 0.94 ± 0.14 g, and 20 °C, 1.24 ± 0.10 g, $P=0.124$, Student's *t* test), suggesting endogenous remodeling of brown adipocytes and their mitochondria in the cold.

Mitochondrial ROS production is buffered by tenrec UCP1 activity

The dynamic changes in oxidative capacity and UCP1 levels in response to cold prompted us to investigate mitochondrial ROS production in isolated tenrec BAT mitochondria. The hydrogen peroxide release rates were monitored to assess mitochondrial superoxide production. The mitochondria were energized with either succinate (5 mM) or G3P (15 mM) at an assay temperature of 32 °C, which resembled the endothermic tenrec body temperature. The measurements were also performed in the presence of CAT, a specific inhibitor of the ANT, to exclude potential uncoupling effects of the ANT. Under resting conditions (State 4), hydrogen peroxide release rates in tenrec BAT mitochondria were low on both substrates. That said, State 4 respiration rate in the presence of G3P was higher compared to State 4 rates in the presence of succinate (Fig. 1A and C; $P=0.007$, paired *t* test). There was no difference in hydrogen peroxide release with either substrate in response to acclimation temperature (20 °C vs 27 °C). Administration of GDP resulted in inhibition of tenrec UCP1 and recoupling of BAT mitochondria [16]. This increased hydrogen peroxide release (Fig. 1A and C) revealed significant group differences between mitochondria from cold- and warm-acclimated animals respiring on succinate ($P=0.04$, Student's *t* test) but not on G3P ($P=0.134$, Student's *t* test). The production of superoxide was sensitive to the complex I inhibitor rotenone. With succinate, the major proportion of superoxide was presumably derived from the I_Q site of complex I [3], whereas with G3P, a significant proportion of superoxide was derived from the G3P dehydrogenase [21]. Measurements in the absence of CAT showed little effect on hydrogen peroxide release (Supplementary Fig. 1). The measurements shown in Fig. 1A and C allowed us to calculate the proportion of hydrogen peroxide that is quenched by tenrec UCP1 activity (GDP/ANT inhibited hydrogen release rates minus State 4 hydrogen peroxide release rates), which was labeled as UCP1-mediated ROS prevention (Fig. 1B and D).

Mitochondrial ROS production correlates with UCP1 content

When the proportion of hydrogen peroxide release that was prevented by UCP1 was plotted against UCP1 content (determined by Western blot analysis), we found a significant positive correlation for mitochondria respiring on succinate ($P<0.001$, $r=0.928$; Fig. 2A), confirming the positive relationship between UCP1 content and ROS release rates/prevention under conditions of complex II-linked substrates. The association for mitochondria respiring on G3P with UCP1 content was less strong ($P=0.019$, $r=0.688$; Fig. 2B), presumably because of the confounding effect of ROS release rates mediated by the G3P dehydrogenase, independent of proton-motive force [22].

Based on previous findings in mice and rats, we also expected UCP1 content to correlate with BAT-associated nonshivering thermogenesis and respiratory capacities. Cold-induced improvement of nonshivering thermogenesis was estimated as the ΔPWR before and after injection of β3-adrenergic inhibitors [16]. Interestingly, the association with ΔPWR and UCP1 content was much weaker compared to the association of ROS with UCP1 (Table 1). There was also no correlation between UCP1 content and COX activity or FCCP-induced respiration.

These analyses suggested that the interdependence of UCP1 content with ROS was stronger than with oxidative capacity and mitochondrial respiration.

Variation in maximal mitochondrial ROS output is explained by UCP1 content and mitochondrial membrane potential

Because ROS release rates significantly increased with UCP1 concentrations (Fig. 2), and cold acclimation increased UCP1 content per milligram of protein, presumably improving rewarming rates [16], we used regression analysis aiming to dissect the specific impact of these parameters on ROS release rates from tenrec BAT mitochondria.

Using GLM we removed the significant effect of UCP1 on ROS levels (with succinate as substrate). The remaining, unexplained variation, expressed as standardized residuals, was uncorrelated

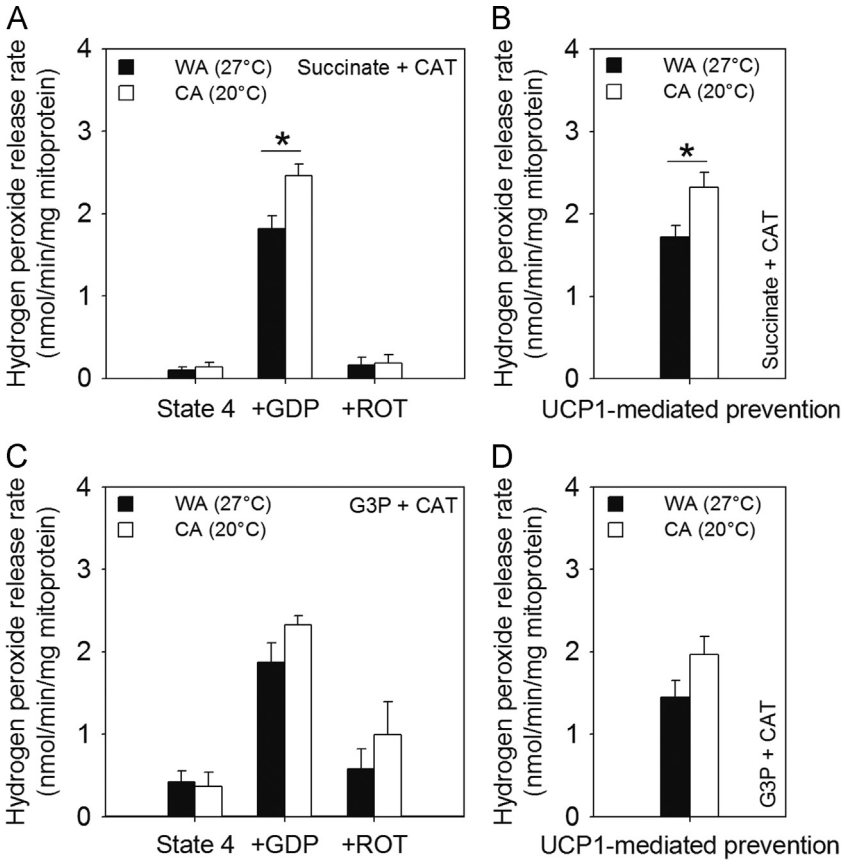

Fig. 1. Hydrogen peroxide release rates of tenrec BAT mitochondria in the absence and presence of UCP1 inhibitor, respiring either (A, B) on succinate (5 mM) or (C, D) on glycerol 3-phosphate (15 mM). GDP (5 mM) was used as a potent inhibitor of UCP1, ROT (2 μM) of complex I, and carboxyatractylate (2.5 μM) of the ANT. After inhibition of UCP1 (+GDP) increases in hydrogen peroxide release rates were detected, with significant differences between acclimatization groups when respiring on succinate. The differences can be solely attributed to UCP1 activity (D; GDP rate minus State 4 rate, $n=5$ or 6 per group (means ± SEM), $^*P<0.05$, Student's t test).

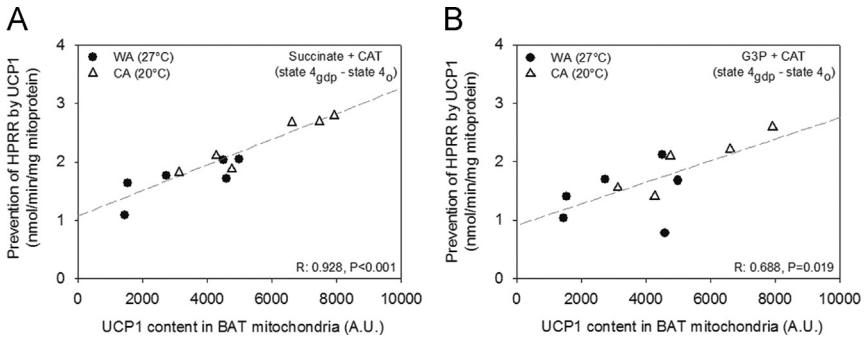

Fig. 2. Correlation between hydrogen peroxide release rate (HPRR) and UCP1 content (using 5 mM succinate and 15 mM glycerol 3-phosphate as substrate) in tenrec BAT mitochondria. (A) The addition of GDP inhibited UCP1, recoupled mitochondria, and revealed a strong correlation between hydrogen peroxide release and UCP1 content of mitochondria respiring on succinate. (B) The correlation between hydrogen peroxide release rate and UCP1 content was less pronounced for mitochondria respiring on glycerol 3-phosphate (symbols represent single animals; $n=5$ or 6 per group).

Table 1
Association of UCP1 content (per milligram of protein) and relevant mitochondrial output parameters *ex vivo*.

	n	*r*	*P*
ΔPWR (°C/min)	10	0.698	**0.025**
ROS succinate+CAT (nmol/min/mg mitoprotein)	12	0.928	**<0.001**
ROS G3P+CAT (nmol/min/mg mitoprotein)	11	0.688	**0.019**
COX (nmol O/min/mg mitoprotein)	12	0.406	0.191
FCCP succinate+CAT (nmol O/min/mg mitoprotein)	12	0.493	0.103
FCCP G3P+CAT (nmol O/min/mg mitoprotein)	12	0.067	0.863

After accounting for multiple comparisons (Bonferroni method) the positive association between UCP1 and ROS (G3P+CAT) and UCP1 with ΔPWR did not remain significant.

with rewarming rate or any other of the respiratory parameters studied (Supplementary Table 1). The residual variances from this regression model were homogeneous (Levene test, $P=0.182$) and did not differ between warm- and cold-acclimated tenrecs (ANOVA, $P=0.462$). Thus, as a single predictor, UCP1 content accounted for 85% ($R^2_{adj}=0.847$) of the variation in ROS release rates from BAT mitochondria respiring on succinate, irrespective of acclimation state (Table 2, Model 1).

In search of an additional predictor for ROS release rates, we also explored the mitochondrial membrane potential (MMP) as a surrogate for proton-motive force. It is established that high MMP causes increased electron leakage, thus increasing ROS production [1,23–26]. Similar to findings in rodent BAT mitochondria, the

Table 2
Parameter estimates from regression analysis of ROS release rates with (Model 1) UCP1 content as a single parameter and (Model 2) UCP1 content and membrane potential as predictors in tenrec brown adipose tissue mitochondria.

	Slope	SE of slope	β	t	P
Model 1: ANOVA, $df=1$, $F=61.95$, $P<0.001$, adj $R^2=0.847$					
Constant	1.033	0.137		7.556	<0.001
UCP1 content/mg protein (A.U.)	0.000218	0.000028	0.928	7.871	<0.001
Model 2: ANOVA, $df=2$, $F=47.20$, $P<0.001$, adj $R^2=0.902$					
Constant	−1.522	0.934		−1.63	0.142
UCP1 content/mg protein (A.U.)	0.000148	0.000027	0.693	5.529	0.001
Mitochondrial membrane potential (mV)	0.016	0.005	0.363	2.896	0.020

A.U., arbitrary units.

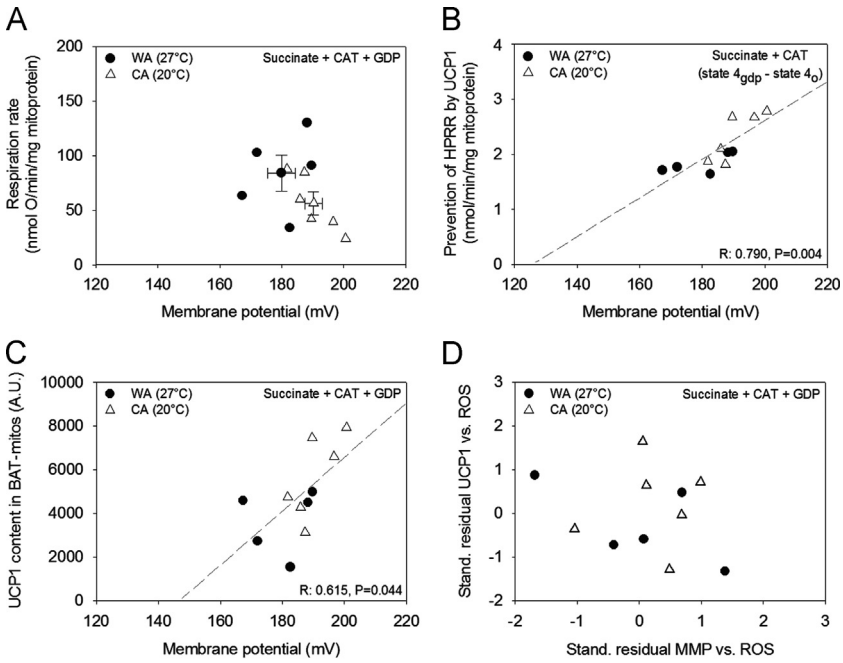

Fig. 3. Association between mitochondrial membrane potential and coupled proton leak respiration and between hydrogen peroxide release rate (HPRR) and UCP1 content. (A) Average coupled proton leak respiration was higher in warm-acclimated animals (WA, $n=5$, means ± SEM) compared to cold-acclimated animals (CA, $n=5$, means ± SEM) and decreased with higher membrane potential. (B) Higher membrane potentials were accompanied by elevated hydrogen peroxide release rates, demonstrating a strong positive correlation. (C) Similarly, UCP1 content and membrane potential were associated. (D) Standardized residuals derived from regressing UCP1 content and mitochondrial membrane potential with ROS release rates showed no correlation. Symbols represent single animals, $n=5$ per group (except averages in (A)).

determination of "basal" MMPs in tenrec BAT mitochondria was below confident assessment levels for two reasons: (a) use of the TPMP+-sensitive electrode and (b) use of a moderate concentration of bovine serum albumin, as excessive concentrations may damage the mitochondria [27]. Therefore, we recoupled mitochondria maximally with GDP, thus fully excluding UCP1 activity from the mitochondrial system [27,28]. The obtained MMPs were plotted against State 4 respiration (Fig. 3A). Although there was no association between respiration and MMP ($r=-0.157$, $P=0.625$), the average values suggested that, in the absence of UCP1 activity, mitochondria were more strongly coupled in cold-acclimated animals. This is further supported by lower leak respiration at higher MMPs (Fig. 3A). The association of MMP with ROS was strong and significant ($r=0.790$, $n=11$, $P=0.004$; Fig. 3B). Accordingly, the UCP1 content correlated with MMP ($r=0.615$, $P=0.044$; Fig. 3C), but this association did not remain significant after accounting for multiple comparisons (Bonferroni method).

Inclusion of UCP1 levels and MMP into a single regression model confirmed the two parameters as significant predictors of ROS release rates and improved the amount of explained variation in ROS release rates of mitochondria respiring on succinate ($R^3_{adj}=90\%$; Table 2, Model 2). The lack of association between residuals derived from regressing each parameter with ROS release rates ($r=-0.251$, $P=0.457$, Fig. 3D) further supported the conclusion that MMP and UCP1 jointly modulated ROS release rates.

Discussion

It is broadly accepted that UCP1 is important for the thermogenic function of BAT, and the lack of classical nonshivering thermogenesis in UCP1-ablated mice confirms the pivotal role of UCP1. Furthermore, cold-induced expression of UCP1 suggests a direct relationship to heat output, although the "engine" of heat production, mitochondrial oxidative capacity, also requires coordinated recruitment for enhanced thermogenesis, as reviewed in [29]. The relationship between proton leak and heat is straightforward; proton-motive force is dissipated as heat. From the mitochondrial bioenergetic point of view, the modular system of mitochondrial oxidative phosphorylation predicts that increases in proton leak also affect other mitochondrial outputs, such as ATP and ROS production, simultaneously. Whereas mitochondrial ATP

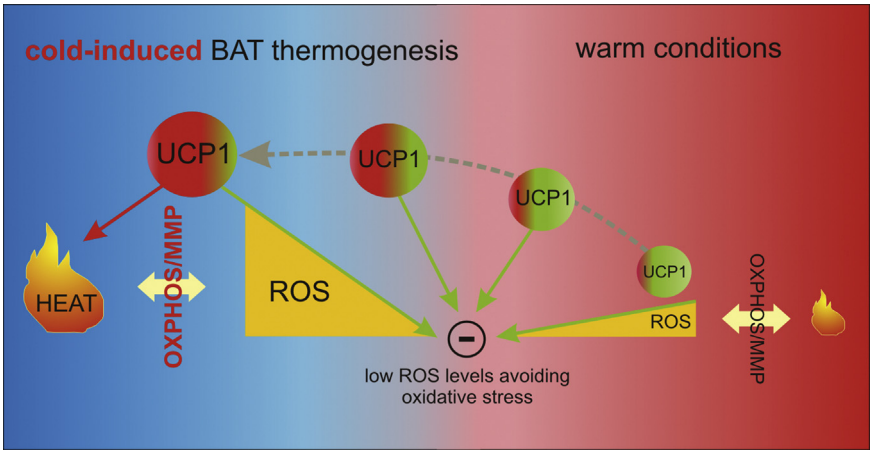

Fig. 4. Graphical model of the physiological significance of UCP1 for oxidative stress. Cold acclimation recruits the thermogenic capacity of brown fat (OXPHOS), and the integration of UCP1 in thermogenesis by dissipation of MMP is well established. Cold-induced recruitment of OXPHOS will also lead to increased ROS production, but the MMP-sensitive proportion may be reduced by UCP1 activity. Under warm conditions, the thermogenic importance of UCP1 becomes less important, but our model suggests that UCP1, modulated by mitochondrial membrane potential, buffers superoxide production, possibly allowing ROS homeostasis during fluctuating substrate influx.

production may play only a minor role in BAT [30], the role of mitochondrial ROS may be of higher importance. The dynamic increases in mitochondrial oxidative capacity in brown adipocytes should also affect the probability of mitochondrial ROS release, thus requiring either cellular ROS buffering by antioxidants or prevention of ROS release from the respiratory chain.

Mitochondrial uncouplers reduce ROS release from mitochondria by decreasing proton-motive force and the reduction state of respiratory chain complexes [31]. An antioxidant role for UCP1 activity was suggested previously using UCP1-knockout mice [8–10,12]. In contrast to previous mouse studies, in which pronounced cold acclimatization to 5 °C ambient temperature led to severalfold increases in UCP1 levels and oxidative capacity [10], the comparably mild cold acclimatization of the tenrecs in this study resulted in rather small increases in mitochondrial UCP1 levels and COX activity [16].

When investigating mitochondrial ROS production, we found that hydrogen peroxide release rates were buffered toward low levels, and the buffering was mediated by UCP1 activity. Furthermore, taking advantage of the expanded range in UCP1 content from warm- and mild-cold-acclimated tenrecs, we show that the amounts of hydrogen peroxide release that were reduced by UCP1 activity strongly correlate with the quantity of UCP1 per milligram mitochondrial protein. A single regression model confirmed UCP1 levels and MMP as significant predictors of ROS release rates and supported the conclusion that MMP and UCP1 jointly modulated ROS release rates.

Our analyses, therefore, generally promote the concept that UCP1 levels per se and, to a lesser extent, MMP are the most relevant parameters associated with ROS release in tenrec BAT mitochondria: inhibition of uncoupling activity increases ROS release rates in proportion to UCP1 content, and high MMPs further increase ROS rates. Owing to the restricted number of tenrecs and the colinearity between MMP and UCP1, the extent to which the two parameters independently affect ROS rates cannot be specified. However, this finding may suggest that UCP1 activity is important, in particular at high MMPs, when there is excessive mitochondrial ROS release (Fig. 4). Changes in MMP may occur because of fluctuating substrate input, as brown adipose tissue controls glucose and lipid metabolism [32,33], presumably even at thermoneutrality [34].

Whereas the antioxidative role of UCP1 in BAT had been put forward previously in UCP1-knockout mouse models by others and our laboratory [8–10,12], this study of a phylogenetically basal, protoendothermic eutherian mammal suggests that the antioxidative role of UCP1 was already present at the divergence of afrotherian and laurasiatherian species about ~65 mya [15]. UCP1 in BAT mitochondria may enable lesser hedgehog tenrecs to maintain high tissue respiration rates in the absence of deleterious superoxide production rates.

Conclusion

The association between UCP1 and ROS prevention seems stronger than the association between UCP1 and thermogenesis under mild cold conditions. Antioxidative properties of UCP1 represent an additional or possibly “rudimentary” function that should be considered in studies of nonthermogenic organs (e.g., thymus), in cells in which thermogenesis is currently under debate (e.g., brite/beige adipocytes), and in UCP1 orthologs from ectotherms, in which the function of UCP1 is unknown and unlikely to be thermogenic. The antioxidative role of UCP1 opens the window to new clues on the evolutionary origin of UCP1 and new venues to target UCP1 in pathological phenotypes of oxidative stress.

Author contributions

R.O. performed all experiments; R.O. drafted the manuscript; M.J. wrote and edited the manuscript; C.W.M. and M.J. conceptualized and designed the study.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.09.004.

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