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Hydroxynonenal, a lipid peroxidation end product, stimulates uncoupling protein activity in *Acanthamoeba castellanii* mitochondria; the sensitivity of the inducible activity to purine nucleotides depends on the membranous ubiquinone redox state

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Abstract We studied the influence of exogenously generated superoxide and exogenous 4-hydroxy-2-nonenal (HNE), a lipid peroxidation end product, on the activity of the *Acanthamoeba castellanii* uncoupling protein (AcUCP). The superoxide-generating xanthine/xanthine oxidase system was insufficient to induce mitochondrial uncoupling. In contrast, exogenously added HNE induced GTP-sensitive AcUCP-mediated mitochondrial uncoupling. In non-phosphorylating mitochondria, AcUCP activation by HNE was demonstrated by increased oxygen consumption accompanied by a decreased membrane potential and ubiquinone (Q) reduction level. The HNE-induced GTP-sensitive proton conductance was similar to that observed with linoleic acid. In phosphorylating mitochondria, the HNE-induced AcUCP-mediated uncoupling decreased the yield of oxidative phosphorylation. We demonstrated that the efficiency of GTP to inhibit HNE-induced AcUCP-mediated uncoupling was regulated by the endogenous Q redox state. A high Q reduction level activated AcUCP by relieving the inhibition caused by GTP while a low Q reduction level favoured the inhibition. We propose that the regulation of UCP activity involves a rapid response through the endogenous Q redox state that modulates the inhibition of UCP by purine nucleotides, followed by a late response through lipid peroxidation products resulting from an increase in the formation of reactive oxygen species that modulate the UCP activation.

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Keywords *Acanthamoeba castellanii* · Mitochondria · Hydroxynonenal · Proton conductance · Uncoupling protein · Ubiquinone

Abbreviations

AcUCP	Uncoupling protein of *Acanthamoeba castellanii* mitochondria
BSA	Bovine serum albumin
FFA	Free fatty acid
HNE	4-hydroxy-2-nonenal
LA	Linoleic acid
PN	Purine nucleotide
ROS	Reactive oxygen species
Q	Ubiquinone coenzyme Q
QH_2	Reduced Q (ubiquinol)
Qtot	Total pool of endogenous Q in the inner mitochondrial membrane
QH_2/Qtot	Reduction level of Q (Q redox state)
TPP^+	Tetraphenylphosphonium
UCP	Uncoupling protein
$\Delta\Psi$	Membrane electrical potential of mitochondria

Introduction

Uncoupling proteins (UCPs) are members of the mitochondrial anion carrier protein (MACP) family. UCPs are present in the mitochondrial inner membrane and mediate an inducible, purine nucleotide (PN)-inhibited proton conductance that dissipates the proton electrochemical gradient generated during the oxidation of respiratory substrates. Therefore, UCPs modulate the coupling of mitochondrial respiration and ATP synthesis. Since 1999,

the presence of UCPs has been demonstrated in some non-photosynthesizing unicellular eukaryotes, including amoeboid and parasite protists, as well as in non-fermentative yeast and filamentous fungi (for an overview, see (Jarmuszkiewicz et al. 2010)).

The UCP of the amoeba *Acanthamoeba castellanii* (AcUCP) is the best functionally characterized of the UCPs of unicellular eukaryotes. In the mitochondria of this amoeboid protozoon, the action of AcUCP has been shown to mediate the free fatty acid (FFA)-activated, PN-inhibited H^+ leak that can divert energy from oxidative phosphorylation during phosphorylating respiration (Jarmuszkiewicz et al. 1999, 2005). It has also been shown that the treatment of amoeba cell cultures with cold temperatures increases the activity and protein levels of AcUCP, indicating that UCP could be a cold-response protein in unicellular eukaryotes (Jarmuszkiewicz et al. 2004a). Moreover, we have shown that UCPs of the mitochondria of unicellular organisms, such as of the amoeba *A. castellanii*, may play a role in decreasing reactive oxygen species (ROS) production, resulting in the constant level observed throughout the growth cycle of this organism (Czarna and Jarmuszkiewicz 2005; Czarna et al. 2007). Recently, PNs have been observed to inhibit FFA-activated AcUCP in phosphorylating and non-phosphorylating mitochondria of *A. castellanii* in a manner dependent upon the redox state of membranous ubiquinone (coenzyme Q, Q) (Jarmuszkiewicz et al. 2005; Swida et al. 2008). Therefore, we have proposed that the Q redox state could be a universal metabolic sensor that modulates the PN inhibition of FFA-activated UCPs (Jarmuszkiewicz et al. 2010) as is the case in the mitochondria of skeletal muscle (UCP3 and UCP2), potato tuber (plant UCP), and rat brown adipose tissue (UCP1) (Jarmuszkiewicz et al. 2004b; Navet et al. 2005; Swida-Barteczka et al. 2009). For *A. castellanii* mitochondria, it has been shown that ubiquinol (QH_2), but not oxidized Q, functions as a negative regulator of UCP inhibition by PNs (Woyda-Ploszczyca and Jarmuszkiewicz 2011).

In unicellular organisms, as well as in non-thermogenic animal and plant tissues, UCPs can limit the production of ROS by the mitochondrial respiratory chain (Jarmuszkiewicz et al. 2010; Mailloux and Harper 2011; Vercesi et al. 2006; Nicholls 2006; Cannon et al. 2006; Echtay 2007). Mitochondria are the main source of ROS, which are important cell signalling molecules that are the natural by-products of aerobic respiration but can severely impair cellular functions and induce cell death when they accumulate to a toxic level (Mailloux and Harper 2011; Murphy 2009). High concentrations of ROS can induce the oxidation of biomolecules. The polyunsaturated fatty acids of phospholipids in cell membranes are especially susceptible to oxidation. The non-enzymatic and self-propagating process of lipid peroxidation produces various compounds, including carbon-centred fatty acyl radicals, peroxyl radicals, fatty acid hydroperoxides and finally multiple breakdown products (Murphy et al. 2003). 4-Hydroxy-2-nonenal (HNE), a reactive unsaturated aldehyde, is the most abundant end product of lipid peroxidation resulting from the production of mitochondrial superoxide. At a high concentration, HNE can be toxic because it covalently modifies and inactivates biopolymers (Grimsrud et al. 2008). In animal mitochondria, the products of lipid peroxidation that arise from ROS, such as HNE and the structurally related compounds *trans*-retinoic acid, *trans*-retinal and other 2-alkenals, specifically induce the uncoupling of mitochondria through the uncoupling proteins UCP2 and UCP3 (Rial et al. 1999; Echtay et al. 2003). Similarly, the induction of mitochondrial uncoupling by HNE and *trans*-retinal has been observed in plant mitochondria (Smith et al. 2004). Moreover, it has been shown that exogenously generated superoxide activates UCPs in both animal and plant mitochondria in the presence of FFAs (Considine et al. 2003; Echtay et al. 2002a). The activation of UCP1 from thermogenic mammalian brown adipose tissue by HNE remains controversial (Cannon et al. 2006; Shabalina et al. 2006; Esteves et al. 2006). However, animal UCP2 and UCP3 and plant UCPs are generally thought to be activated by lipid peroxidation products (such as HNE) to induce a proton leak, thus providing a negative feedback regulation of mitochondrial ROS production. Specifically, ROS production is a prerequisite of lipid peroxidation and HNE production, which in turn induce mild uncoupling mediated by UCPs resulting in ROS attenuation (Murphy et al. 2003; Echtay et al. 2003). Thus far, it is unknown whether UCP activity can be induced by ROS or lipid peroxidation products in the mitochondria of unicellular eukaryotes.

The aim of this work was to study the effect of exogenously generated superoxide and exogenous HNE on the activity of protozoan UCPs, such as amoeba AcUCP. Our results indicate that the stimulation of UCP activity by the alkenal product of lipid peroxidation and the negative feedback loop for mitochondrial ROS production could occur in the mitochondria of unicellular organisms. Moreover, we performed the first study of the influence of the endogenous Q redox state on the PN inhibition of HNE-induced UCP activity.

Materials and methods

Chemicals

HNE was obtained from MP Biomedicals, and the other chemicals were purchased from Sigma-Aldrich. HNE and linoleic acid (LA) were dissolved in methanol. For each experiment, the HNE solution was prepared fresh and stored at−20 ° C during measurements to minimize degradation.

All LA stocks were stored at−80 ° C under argon and prepared in brown glass vials.

Cell culture and isolation of mitochondria

The soil amoeba *A. castellanii*, strain Neff, was cultured as previously described (Czarna et al. 2007). Trophozoites of the amoeba were collected between 46 and 48 h following inoculation at the late exponential phase at a density of approximately 4–5×10^6 cells/ml. The mitochondria were isolated and purified on a self-generating Percoll gradient (29 %) as previously described (Jarmuszkiewicz et al. 1997). The presence of 0.3 % bovine serum albumin (BSA) in isolation media allowed the endogenous FFA to be chelated from the mitochondrial suspension. The mitochondrial protein concentration was determined by the Biuret method.

Mitochondrial oxygen consumption and mitochondrial membrane potential measurements

The oxygen uptake was measured polarographically with a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) in 2.8 ml of incubation medium (25 °C), with 1.5–1.7 mg of mitochondrial protein. The mitochondria were incubated in a standard incubation medium containing: 120 mM KCl, 20 mM Tris/HCl (pH 7.2), 3 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.5 mM EGTA, and 0.025 % BSA. Succinate (7 mM) was used as an oxidizable substrate in the presence of rotenone (4 μM) to block the electron input from complex I. As a control, phosphorylating respiration was measured for each mitochondrial preparation to check the coupling parameters. Only high quality mitochondrial preparations, i.e., those with an ADP/O value of approximately 1.3 (with succinate as a respiratory substrate) and a respiratory control ratio of approximately 2.5–3.2, were used in all experiments. The values of O_2 uptake are given in nmoles of O per min per mg of protein.

The electrical potential (ΔΨ) of the mitochondrial membrane was measured simultaneously with the oxygen uptake using a tetraphenylphosphonium (TPP^+)-specific electrode as previously described (Woyda-Ploszczyca and Jarmuszkiewicz 2011). The TPP^+ electrode was calibrated with 4 sequential additions (0.4, 0.4, 0.8, and 1.6 μM) of TPP^+. After each run, 0.5 μM carbonylcyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was added to release TPP^+ for the baseline correction. To calculate the ΔΨ value, the matrix volume of the amoeba mitochondria was assumed to be 2.0 μl/mg protein. The calculation assumes that the TPP^+ distribution between the mitochondria and medium followed the Nernst equation. The values of ΔΨ were corrected for TPP^+ binding using the apparent external and internal partition coefficients of TPP^+. The correction shifted the calculated ΔΨ values to lower values (a shift of approximately 30 mV), but it did not alter the relative changes in the resulting ΔΨ. The values of ΔΨ are given in mV.

Proton leak measurements

The response of proton conductance to its driving force can be expressed as the relationship between the oxygen consumption rate and ΔΨ (flux-force relationship) when varying the potential by titration with respiratory chain inhibitors. The proton leak rates can be calculated from the respiration rates by multiplying the respiration rates by a H^+/O ratio of 6. The measurements were performed in the absence of exogenous ADP, i.e., in the resting state (non-phosphorylating respiration; State 4). To inhibit the activity of an ATP/ADP antiporter and ATP synthase, 1.8 μM carboxyatractyloside and 0.7 μg/ml oligomycin were used respectively. Benzohydroxamate (1.5 mM) was used to inhibit alternative oxidase activity.

The respiratory rate, ΔΨ and the Q redox state were varied by modulating the Q-reducing or QH_2-oxidizing pathways. To decrease the rate of the Q-reducing pathway, succinate dehydrogenase was titrated with malonate (up to 5 mM). To decrease the rate of the QH_2-oxidising pathway, either complex III was inhibited with antimycin A (up to 0,6 μg/mg protein) or complex IV was inhibited with cyanide (up to 100 μM). To avoid possible errors due to non-steady-state conditions, such as during the sequential additions of inhibitors, and to assess the Q redox state for a given steady state, the data from separate measurements with different (given) inhibitor concentrations were combined to generate common curves. To assess the statistical significance of the induced shifts in the proton leak curves, we generally compared the respiration rates at the common ΔΨ values for pairs of curves from 5 to 7 independent experiments using Student's *t* test for unpaired data.

Exogenous superoxide was generated using xanthine (up to 200 μM) and xanthine oxidase (0.01 units per 50 μM xanthine). Xanthine was prepared in the standard incubation medium. Xanthine and xanthine oxidase were added prior to the TPP^+ calibration and incubated with mitochondria for 5, 10, 15 or 20 min (Fig. 1a). The release of superoxide by the xanthine/xanthine oxidase system in the presence of the mitochondrial suspension was examined by monitoring the reduction of nitroblue tetrazolium (NBT) (Baehner et al. 1976). NBT (40 μM) was added into a cuvette with the stirred standard incubation medium (2.8 ml), and its reduction upon xanthine/xanthine oxidase addition was measured spectrophotometrically at 560 nm.

To induce AcUCP activity, LA (9 μM) or HNE (at different concentrations) was used. HNE was always added to the mitochondria before the TPP^+ calibration and the

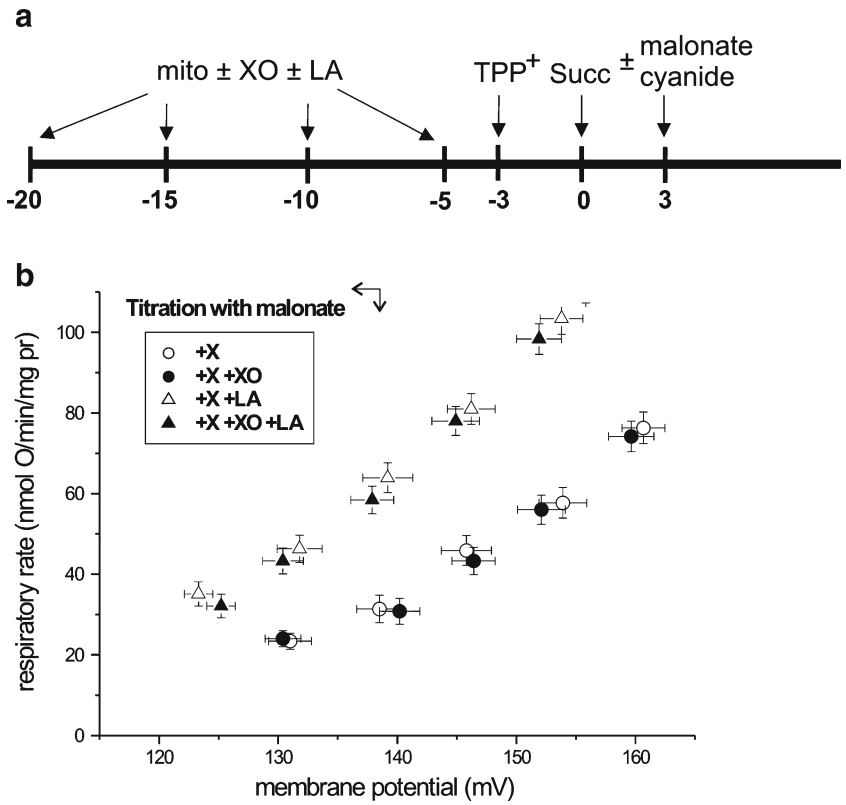

Fig. 1 The effect of exogenously generated superoxide on proton leak kinetics in non-phosphorylating amoeba mitochondria. **a** Experimental scheme and timing of additions. An incubation medium containing 4 μM rotenone, 1.8 μM carboxyatractyloside, 0.7 μg/ml oligomycin, 1.5 mM benzohydroxamate, and 0.025 % BSA was supplemented with 50, 100, 150 or 200 μM xanthine. The negative numbers represent the min prior to energization and reflect the different times of mitochondrial (mito) incubation with or without the xanthine/xanthine oxidase system in the presence or absence of 9 μM linoleic acid (LA). The mitochondria (1.5 mg protein) were added at $t=-20$, −15, −10 or −5 and incubated in the open chamber in 2.8 ml of the continuously stirred incubation medium. The amount of xanthine oxidase (XO) units changed proportionally to the xanthine concentration (0.01 units per 50 μM xanthine). When the chambers were closed, the TPP^+-specific electrodes were calibrated 3 min prior to mitochondrial energization ($t=-3$). At $t=0$, an oxidizable substrate (Succ, succinate) was added. The respiration was titrated with malonate or cyanide 3 min after energization ($t=3$). Measurements in the presence of xanthine alone or in the presence of the xanthine/xanthine oxidase system were performed simultaneously. **b** The relationship between the respiratory rate and membrane potential. The mitochondria were incubated for 15 min according to scheme (**a**) with 100 μM xanthine and 0.02 units of xanthine oxidase. The succinate oxidation was sequentially decreased by the addition of increasing concentrations of malonate (0.7, 1.4, 2.8, and 4.2 mM) in the absence or presence of 9 μM LA. The values are the means ± S.E. of five independent mitochondrial preparations, for which each determination was performed in duplicate

mitochondrial energization with succinate. The incubation time with HNE was varied to obtain the strongest stimulatory effect (Fig. 2). LA was added after mitochondrial energization except when performing experiments using the xanthine/xanthine oxidase system (Fig. 1). The LA-induced and HNE-induced AcUCP activities were inhibited by 2 mM GTP, which is the strongest AcUCP inhibitor among the PNs (Woyda-Ploszczyca and Jarmuszkiewicz 2011).

Phosphorylating respiration measurements

The ADP/O ratio was determined by an ADP (450 nmoles) pulse method with succinate (plus rotenone) as the oxidizable substrate. The total amount of oxygen consumed during phosphorylating (State 3) respiration was used to calculate the ratio. The simultaneous measurements of $\Delta\Psi$ enabled the fine control of the duration of phosphorylating respiration. To decrease the rate of the Q-reducing pathway during phosphorylating respiration, succinate oxidation was inhibited by n-butylmalonate (5–10 mM), a competitive inhibitor of succinate uptake.

Measurements of the ubiquinone reduction level

The endogenous Q in *A. castellanii* mitochondria is Q_9 (Jarmuszkiewicz et al. 1998). The redox state of Q in steady-state respiration was determined by an extraction technique followed by HPLC detection as previously described (Swida et al. 2008). Commercial Q9 (Sigma) was used for peak calibration. The Q reduction levels are expressed as the percentage of total Q (QH_2/Qtot).

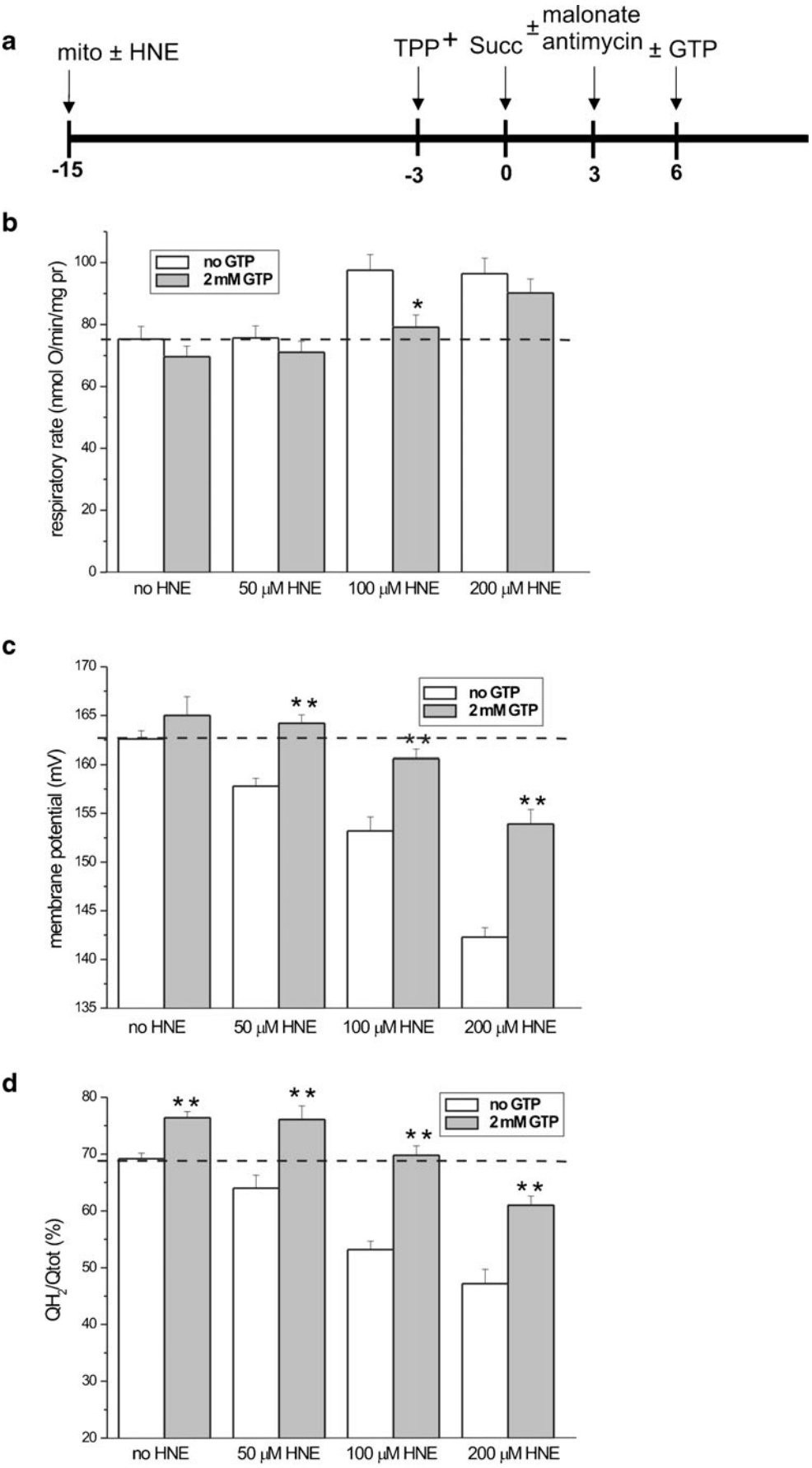

Fig. 2 The effect of exogenous 4-hydroxy-2-nonenal (HNE) on the bioenergetics of non-phosphorylating amoeba mitochondria. **a** Experimental scheme and timing of additions to the incubation chamber. The incubation medium (as in Fig. 1a) was supplemented with 0, 50, 100, or 200 μM HNE. Under the control conditions, the mitochondria were treated with an equivalent volume of methanol (the HNE solvent). The negative numbers represent the min before energization and reflect the time of mitochondrial (mito) incubation with or without HNE. The mitochondria (1.7 mg protein) were added at $t=-15$ and incubated in the open chamber in 2.8 ml of the continuously stirred incubation medium. When the chambers were closed, the TPP^+-specific electrodes were calibrated 3 min prior to mitochondrial energization ($t=-3$). After 15 min of incubation ($t=0$), the oxidizable substrate (Succ, succinate) was added. The respiration was titrated with malonate or antimycin A 3 min after energization ($t=3$). Measurements in the presence or absence of a given concentration of HNE were performed simultaneously. **b**, **c**, **d**, the influence of three concentrations of HNE (50, 100, and 200 μM) on the respiratory rate, membrane potential and Q redox state, respectively, in the absence of respiratory chain inhibitors. The measurements were performed in the absence or presence of 2 mM GTP. The values are the means ± S.E. of five independent mitochondrial preparations, for which each determination was performed in duplicate. *, ** Where indicated, the p values are significantly different from the respective values in the absence of GTP ($p<0.05$ or $p<0.01$, respectively; Student's unpaired t test). The dashed vertical lines show the control values in the absence of HNE and GTP

Results

No influence of the xanthine/xanthine oxidase system is observed on isolated *A. castellanii* mitochondria

Xanthine oxidase plays an important role in the catabolism of purines and under certain conditions, it can produce superoxide anion (Vincent and Taylor 2006). We treated the isolated mitochondria of *A. castellanii* with the xanthine/xanthine oxidase system, which generates superoxide, to determine its ability to stimulate AcUCP. The same approach was previously employed with mammalian (Echtay et al. 2002a, b), plant (Considine et al. 2003) and avian (Talbot et al. 2003) mitochondria. Under our experimental conditions (Fig. 1a), the standard incubation medium was supplemented with xanthine, and superoxide generation was initiated by xanthine oxidase addition. Varing concentrations of xanthine (from 50 to 200 μM) and appropriate amounts of xanthine oxidase (0.01 units per 50 μM xanthine) were used. The presence of xanthine or xanthine oxidase alone in the incubation medium had no effect on the basal proton conductance of amoeba mitochondria (data not shown), similar to the effect observed in mammalian and plant mitochondria (Considine et al. 2003; Echtay et al. 2002b). The proton leak measurements were always performed in pairs, i.e., with the complete xanthine/xanthine oxidase system and with xanthine alone. Different times of mitochondrial incubation (from 5 to 20 min) were tested. Because enzymatic systems that produce superoxide can lead to oxygen consumption (Li et al. 1998), the incubation of the amoeba mitochondria with xanthine/xanthine oxidase was performed in an open chamber in which the medium was constantly stirred to balance the air saturation. For our experimental conditions, we confirmed the xanthine concentration-dependent exogenous superoxide generation using the nitroblue tetrazolium (NBT) reduction test (data not shown).

Figure 1b shows the representative proton leak kinetics of non-phosphorylating *A. castellanii* mitochondria in the presence of the superoxide generating system after 15 min of incubation with 100 μM xanthine in the presence or absence of 0.02 units of xanthine oxidase. There was no significant change in the respiration rate or $\Delta\Psi$ in the presence of the complete or incomplete xanthine/xanthine oxidase system. The titration of succinate oxidation with malonate did not reveal any uncoupling effect in the presence of exogenous superoxide. Because the superoxide effect was not observed in the absence of LA in plant mitochondria (Considine et al. 2003), we investigated the effect of superoxide on the LA-induced proton conductance. However, the presence of the xanthine/xanthine oxidase system did not enhance the LA-induced proton conductance. Moreover, no exogenous superoxide-induced proton conductance was observed under the following conditions (data not shown): (i) when a different respiratory substrate was used (1.4 mM NADH), (ii) during titration with cyanide, (iii) when BSA was omitted from the incubation medium (i.e., in the presence of endogenous FFAs), (iv) with different concentrations of xanthine (50, 100, 150, and 200 μM), and (v) with different times of incubation (5, 10, 15, and 20 min). Taking these results into account, we can conclude that a 5 to 20 min exposure of amoeba mitochondria to superoxide that was exogenously produced by the xanthine/xanthine oxidase system (up to 200 μM xanthine) is insufficient to stimulate uncoupling in these mitochondria.

4-Hydroxy-2-nonenal (HNE) activates the uncoupling protein of *A. castellanii* mitochondria during non-phosphorylating respiration

Because exogenously generated ROS did not increase the proton leak catalyzed by AcUCP, we used exogenous HNE, which has been reported to be a potent UCP activator in mammalian and plant mitochondria (Echtay et al. 2003; Smith et al. 2004). It was necessary to incubate the amoeba mitochondria with HNE for 15 min before energization (Fig. 2a) because the direct addition of HNE to the respiring mitochondria did not influence AcUCP activity (data not shown). No effect or a weak effect of HNE was observed if the mitochondria were not incubated long enough with HNE or if the HNE concentration was not high enough. We determined the optimal HNE concentration required to obtain a simultaneous increase in oxygen consumption, a decrease in $\Delta\Psi$ and a decrease in the Q reduction level for non-phosphorylating mitochondria respiring in the absence of respiratory chain inhibitors, (Fig. 2b, c, d). When these changes are sensitive to PNs, they can be considered to indicate UCP activation. To exclude AcUCP activation by endogenous FFAs, all measurements were performed in the presence of defatted BSA. To exclude uncoupling mediated by an adenine nucleotide carrier, all measurements were performed in the presence of carboxyatractyloside. Figure 2 shows the influence of three different concentrations of HNE (50, 100 and 200 μM) in the presence or absence of 2 mM GTP on the respiratory rate (Fig. 2b), $\Delta\Psi$ (Fig. 2c) and the Q redox state (Fig. 2d) in *A. castellanii* mitochondria during non-phosphorylating respiration. The optimal HNE-induced GTP-inhibited uncoupling effect was observed with 100 μM HNE. At 100 μM HNE, the respiration rate increased significantly, by approximately 30 %. This increase was accompanied by an approximately 10 mV decrease in $\Delta\Psi$ and a 14 % decrease in the Q reduction level. The effects were almost fully sensitive to GTP. At 50 μM HNE, we observed no effect on the respiratory rate and a relatively low decrease in $\Delta\Psi$ (approximately 5 mV) and in the Q redox state (approximately 5 %). At 200 μM HNE, there was no further increase in the oxygen consumption compared

to 100 μM HNE, and the inhibitory effect of GTP was impaired compared to the control values of respiration, ΔΨ and Q reduction level.

The above results indicate the operation of HNE-induced, GTP-inhibited, AcUCP-mediated uncoupling in *A. castellanii* mitochondria. To our knowledge, these are the first experimental data describing the effect of HNE-induced GTP-inhibited uncoupling activity on the redox state of endogenous mitochondrial Q.

GTP inhibition of the HNE-induced AcUCP-mediated proton leak is under the control of the endogenous Q reduction level

Recently, we have demonstrated that membranous ubiquinol (the reduced form of Q) negatively regulates the PN inhibition of FFA-induced AcUCP activity (Woyda-Ploszczyca and Jarmuszkiewicz 2011). Therefore, in the present study, we investigated the influence of the Q redox state on the GTP inhibition of HNE-induced AcUCP activity. Figure 3 shows an example experiment in which the respiratory rate, ΔΨ, and the Q redox state were measured in *A. castellanii* non-phosphorylating mitochondria that were incubated with 100 μM HNE concomitant with the inhibition of succinate oxidation (Fig. 3b) by malonate or antimycin A to ~30 %, leading to the same level of ΔΨ (120–122 mV). The corresponding Q redox state was decreased from 55 % to 25 % by malonate or increased from 55 % to 84 % by antimycin A. After the subsequent addition of GTP, the inhibition of the HNE-induced uncoupling manifested in the inhibition of the respiratory rate as well as the restoration of ΔΨ and the Q redox state. However in the presence of respiratory chain inhibitors, the inhibitory effect of GTP was much stronger when the nucleotide was added to mitochondria that had been inhibited by malonate (leading to the Q redox state of 41 %) compared to those inhibited by antimycin A (leading to the Q redox state of 85 %). This stronger inhibition by GTP can be seen in the larger effect of the nucleotide on ΔΨ and the Q redox state in the presence of malonate. In the absence of respiratory chain inhibitors (Fig. 3a), i.e., at an intermediate Q redox value (55 %), the inhibitory effect of GTP was also intermediate. These results indicate that the efficiency of GTP to inhibit the HNE-induced uncoupling in non-phosphorylating *A. castellanii* mitochondria depends on the endogenous Q redox state. The inhibition by GTP can be diminished when Q is sufficiently reduced.

The same conclusion can be drawn from the study of proton leak kinetics. Figure 4 displays the H^+ conductance curves (the flux-force relationships) (Fig. 4a and c) and the relationships between the respiratory rate and the Q reduction level (Fig. 4b and d) of *A. castellanii* mitochondria treated with 100 μM HNE in the presence or absence of 2 mM GTP during titration with inhibitors of the Q-reducing (malonate) or QH_2-oxidizing (antimycin A) pathways. The same H^+ leak curves in the absence of GTP were observed during titration with malonate or antimycin A. These results clearly indicates that the redox state of endogenous Q does not affect the HNE-induced proton conductance in *A. castellanii* mitochondria.

Figure 4a and b show the enhancement of the GTP inhibitory effect on the HNE-induced uncoupling upon the gradual reduction of the rate of the Q-reducing pathway. The progressive decrease of succinate oxidation, as revealed by the gradual decrease of ΔΨ (Fig. 4a) and the Q redox state

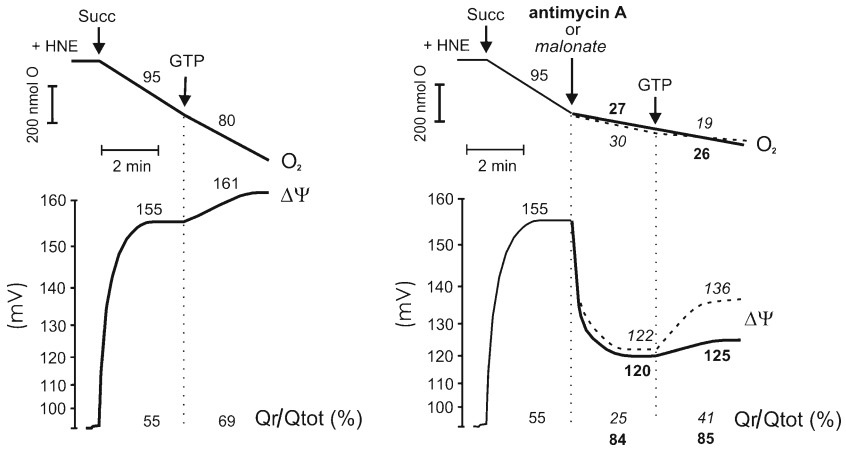

Fig. 3 The effect of GTP on the changes in respiration, membrane potential and the Q redox state caused by an HNE-induced proton leak (AcUCP activity) when the Q reduction level is varied under non-phosphorylating conditions. The sensitivity to GTP was tested (**a**) without inhibitors and (**b**) with malonate (*broken lines and numbers in italics*) or antimycin A (*solid lines and bold numbers*). The assay conditions were the same as in Fig. 2a. The mitochondria were incubated with 100 μM HNE for 15 min prior to energization. Additions: 7 mM succinate (Succ), 2.8 mM malonate, 0,6 μg/mg protein antimycin A, 2 mM GTP. The numbers on the traces refer to the O_2 consumption rates in nmol O/min/mg protein or to the ΔΨ values in mV. The Q redox state values (in %) correspond to the given experimental steady-state conditions. Examples of ten measurements (using mitochondria from five different preparations) are shown

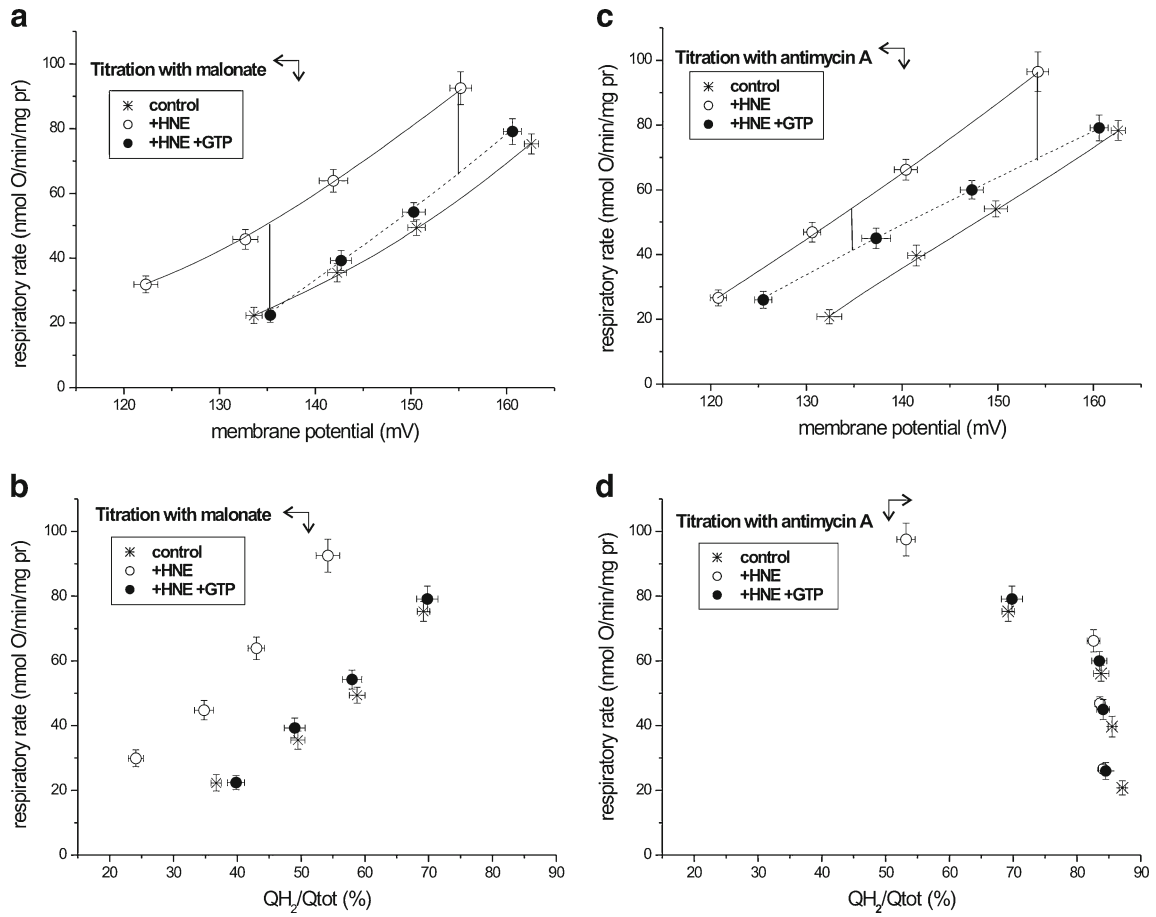

Fig. 4 The regulation of the GTP sensitivity of the HNE-induced proton leak through the endogenous Q redox state under non-phosphorylating conditions. The inhibitory effect of GTP on the HNE-induced proton leak was altered when the Q-reducing pathway (**a**, **b**) or the QH_2-oxidizing pathway (**c**, **d**) was decreased during non-phosphorylating respiration. The relationships between the respiratory rate and $\Delta\Psi$ (proton leak kinetics) (**a**, **c**) and the respiratory rate and the Q redox state (**b**, **d**) are shown. The oxidation of succinate (**a**, **b**) was gradually decreased by increasing the concentration of malonate (0.7, 1.4, and 2.8 mM). The QH_2-oxidizing pathway (**c**, **d**) was titrated with antimycin A (0.1, 0,3 and 0,6 μg/mg protein). The assay conditions were the same as in Fig. 2a. The measurements were performed in the absence or presence of 100 μM HNE, which was added 15 min prior to mitochondrial energization. GTP (2 mM) was added after energization. The control values were obtained with mitochondria not treated with HNE in the absence of GTP. The vertical lines indicate the proton leak size at two different $\Delta\Psi$ values (154 and 135 mV). The values are the means ± S.E. of five independent experiments (mitochondrial isolations), each performed in duplicate

(Fig. 4b), led to the increase in the inhibitory effect of GTP on the HNE-induced H^+ leak. The full inhibitory effect was achieved when the points obtained in the presence of HNE and GTP progressively came forward to match the control points obtained in the absence of HNE and GTP, i.e., at a $\Delta\Psi$ of approximately 135 mV, which corresponded to approximately 40 % of the Q reduction level. Figure 4c and d depict the attenuation of the GTP inhibitory effect on the HNE-induced H^+ leak upon the gradual decrease of the rate of the QH_2-oxidizing pathway. When the Q reduction level was gradually increased, the inhibition by GTP was progressively reduced, and the points obtained with HNE and GTP progressively came forward to the levels of the points obtained without GTP. The lowest inhibitory effect was observed at approximately 84 % of the Q reduction level, which corresponded to a $\Delta\Psi$ of approximately 125 mV. Because the Q redox state gradually increased within a very narrow range (from 70 % to 87 %) during titration with antimycin A, the alleviation of the GTP inhibitory effect was less visible within the steep relationship between the respiratory rate and the Q reduction level. Moreover, a comparison of the HNE-induced GTP-sensitive H^+ leak at the lowest common $\Delta\Psi$ (135 mV) for mitochondria titrated with malonate or antimycin A (180±7 and 86±5 nmol H^+/min/mg protein, respectively) suggested a twofold higher sensitivity to GTP when the Q redox state was significantly decreased (vertical lines in Fig. 4a and c and Table 1).

These results indicate that the efficiency of GTP to inhibit the HNE-induced AcUCP-mediated uncoupling in non-phosphorylating mitochondria is under the control of the endogenous Q redox state. Increased concentrations of oxidized Q facilitate the inhibition of AcUCP by GTP, while

Table 1 Comparison of the HNE-induced GTP-sensitive and LA-induced GTP-sensitive effects on non-phosphorylating *A. castellanii* mitochondria

	V4 (nmol O/min/mg pr)	ΔΨ4 (mV)	QH_2/Qtot (%)	Malonate-titrated H^+ leak		AA- or KCN-titrated H^+ leak	
				154 mV	135 mV	154 mV	135 mV
HNE-induced GTP-sensitive	16.0±1.0	8.6±0.6	16.5±1.1	152±9	180±7*	154±9	86±5**
LA-induced GTP-sensitive	13.8±0.8	6.9±0.5	14.2±0.9	160±8	197±10*	144±9	89±6*

Measurements with 100 μM HNE were performed as described in Fig. 2. During measurements with LA (9 μM), the fatty acid was added after mitochondrial energization during steady-state non-phosphorylating respiration. 2 mM GTP was used. The HNE- or LA-induced GTP-sensitive respiratory rates (V4), ΔΨ4 and the Q reduction level in the absence or presence of respiratory chain inhibitors are shown. The proton leak values (in nmol H^+/min/mg protein) at two different ΔΨ values (154 and 135 mV) are shown. The HNE-induced H^+ leaks titrated with malonate or antimycin A (AA) are calculated from proton leak kinetics (Fig. 4). The LA-induced H^+ leaks titrated with malonate or cyanide are calculated from the proton leak kinetics presented in (Woyda-Ploszczyca and Jarmuszkiewicz 2011). The values are the means ± S.E. of five independent mitochondrial preparations, for which each determination was performed at least in duplicate. *, ** Where indicated, the p values are significantly different from the respective values at 154 mV ($p<0.05$ or $p<0.01$, respectively; Student's unpaired t test)

increased concentrations of QH_2 (ubiquinol) diminish the affinity of AcUCP for GTP.

Comparison of AcUCP activation by HNE and LA

Linoleic acid is the best known stimulator of AcUCP (Swida et al. 2007) and the sensitivity of the LA-induced AcUCP-catalyzed proton leak to PNs is an elegant example of the posttranslational regulation of UCPs, including those of the mitochondria of unicellular organisms (Swida et al. 2008; Woyda-Ploszczyca and Jarmuszkiewicz 2011). Therefore, we compared the effect of both HNE (100 μM) and LA (9 μM) on AcUCP activity. Table 1 shows that the HNE- or LA-induced GTP-sensitive respiration, ΔΨ and Q reduction levels measured during non-phosphorylating respiration in the absence of respiratory chain inhibitors were similar. Moreover, a comparison of the proton leak kinetics in the absence or presence of 100 μM HNE (Fig. 4) or 9 μM LA (Woyda-Ploszczyca and Jarmuszkiewicz 2011) for the same ΔΨ values (at 154 or 135 mV) during the titration of the Q-reducing pathway with malonate or the titration of the Q-oxidizing pathway with antimycin A or cyanide indicated that the inducible GTP-sensitive AcUCP-mediated proton conductance did not change when HNE or LA were used as activators (Table 1). In addition, the inducible GTP-sensitive proton leak was significantly increased in the presence of both of the AcUCP activators during titration with malonate (i.e., when the Q reduction level was decreased) and significantly diminished during titration with antimycin A or cyanide (i.e., when the Q reduction level was increased). These results confirm that the sensitivity of AcUCP activity to PNs depends on the Q reduction level regardless of which activator, HNE or LA, is used to induce uncoupling.

The only noticeable difference between the effects of HNE and LA is the concentration required to obtain a similar uncoupling effect. Indeed, uncoupling requires over tenfold more HNE than LA, indicating a lower AcUCP affinity for HNE than LA. Probable reflection of the lower AcUCP affinity for HNE is the 15 min mitochondrial incubation required during in vitro experiments with HNE. However, our results strongly support the role of HNE as a potent activator of UCPs in unicellular organisms.

HNE activates AcUCP during phosphorylating respiration

UCPs (including AcUCP), as energy-dissipating systems, lead to a decrease in the oxidative phosphorylation efficiency, i.e., the ATP synthesis yield per oxygen consumed (ADP/O ratio) (Jarmuszkiewicz et al. 2010). If HNE is a potent activator of AcUCP, its presence in the incubation medium during phosphorylating respiration should decrease the ADP/O ratio and the respiratory control ratio, the best known parameters used to estimate the efficiency of oxidative phosphorylation. Table 2 shows the values of the phosphorylating respiratory rates, ΔΨ and the coupling parameters measured during succinate oxidation in phosphorylating amoeba mitochondria in the absence or presence of HNE (100 μM). At the concentration used, HNE did not disturb the activity of the respiratory chain, as the phosporylating respiration and ΔΨ remained unaffected. The ADP/O ratio and the respiratory control ratio were significantly lowered by HNE, indicating the induction of the proton leak. However, the coupling parameters did not return to the control values (i.e., values in the absence of HNE) when the measurements were performed in the presence of GTP. To observe the sensitivity of the HNE-induced

Table 2 The effect of HNE-induced GTP-sensitive uncoupling on the respiratory rate, membrane potential and coupling parameters in phosphorylating *A. castellanii* mitochondria

	no inhibitor				+ n-butylmalonate			
	V3	ΔΨ3	ADP/O	RCR	V3	ΔΨ3	ADP/O	RCR
− HNE + GTP	203±8	131±7	1.28±0.06	2.6±0.1	97±4	129±7	1.26±0.04	1.78±0.1
+ HNE, − GTP	205±10	130±5	1.11±0.04	1.7±0.1	104±3	130±7	1.02±0.03	1.18±0.1
+ HNE, + GTP	199±12	133±8	1.12±0.03	1.8±0.1	101±6	132±8	1.16±0.03	1.42±0.1

The mitochondria were incubated in the absence or presence of 100 μM HNE for 15 min prior to energisation as described in Fig. 2A. The measurements were performed with 7 mM succinate as a respiratory substrate (plus rotenone) with 480 nmol ADP as a pulse in the absence of oligomycin and carboxyatractyloside. 0.6 mM GTP was employed. To decrease the rate of the Q-reducing pathway and thus decrease the Q reduction level, succinate oxidation was inhibited by 50 % by n-butylmalonate (5–10 mM). The phosphorylating respiration (V3) and ΔΨ (ΔΨ3) are expressed in nmol O/min/mg protein and mV, respectively. RCR, respiratory control ratio. The values are the means ± S.E. of 3 independent experiments (mitochondrial isolations), each performed in duplicate

proton leak to GTP, the phosporylating respiration was decreased by 50 % with n-butylmalonate (at an unchanged phosphorylating ΔΨ of approximately 130 mV). In HNE-treated mitochondria in the absence of GTP, the decrease in succinate oxidation was accompanied by a decrease in the ADP/O ratio and the respiratory control ratio. The comparison of the values obtained in the absence or presence of GTP indicated that, in HNE-treated mitochondria, a partial restoration of both coupling parameters by GTP was observed when the Q-reducing pathway, and thus the Q reduction level, was lessened. These results are consistent with previous studies (Jarmuszkiewicz et al. 2005) that show that the inhibition of the LA-induced uncoupling by GTP in *A. castellanii* mitochondria is observed only when membranous Q is sufficiently oxidized, not during uninhibited phosphorylating respiration.

We demonstrated for the first time that HNE is a potent UCP activator in both phosphorylating and non-phosphorylating mitochondria, which highlights its important role at a physiological level. The results indicate that the HNE-induced GTP-inhibited proton leak mediated by AcUCP diverts energy from ATP synthesis during phosphorylating respiration.

Discussion

It has been shown that in the presence of FFAs, exogenously generated superoxide activates UCPs in some animal and plant mitochondria (Considine et al. 2003; Echtay et al. 2002a; Talbot et al. 2003). However, in our study, the exposure (up to 20 min) of amoeba *A. castellanii* mitochondria to exogenous superoxide from the xanthine/xanthine oxidase system (with up to 200 μM xanthine) was insufficient to stimulate uncoupling in these mitochondria, independent of the presence of LA or BSA in the incubation medium. A lack of activation of UCPs by exogenously (Couplan et al. 2002; Criscuolo et al. 2005) or endogenously (Silva et al. 2005; Nabben et al. 2011) generated superoxide has also been observed in some animal mitochondria. It is possible that in amoeba mitochondria under our experimental conditions, the endogenous antioxidant defense depressed the effect of the exogenous superoxide generating system to prevent lipid peroxidation. Therefore, for further studies, we altered our experimental procedure to apply an exogenous lipid peroxidation product, HNE.

Using the isolated mitochondria of *A. castellanii*, we demonstrated for the first time that the UCP of unicellular eukaryotes is activated by the exogenous reactive aldehyde HNE. Under our experimental conditions, an adequate incubation time before energization (15 min) and after energization (3 min) in the absence of PNs was crucial to the induction of the proton leak by HNE. If the incubation was performed in the presence of HNE and GTP or if HNE was added after mitochondria energization, the traces of the control and HNE-treated mitochondria were comparable (data not shown). To exclude AcUCP activation by endogenous FFAs, all measurements were performed in the presence of BSA. The maximal uncoupling effect of HNE was observed with 100 μM HNE at 0.6 mg of amoeba mitochondrial protein per ml of incubation medium, which corresponds to the 35–50 μM HNE concentration at approximately 0.35 mg of mitochondrial protein per ml of assay medium usually used in studies with mammalian mitochondria (Murphy et al. 2003; Echtay et al. 2003; Parker et al. 2008a; Aguirre and Cadenas 2010). In addition, a 100 μM HNE concentration can be considered to be physiologically relevant (Uchida 2003). Moreover, to study the effect of HNE in vitro, the applied HNE concentration has to be elevated because exogenous HNE is rapidly metabolized in the presence of mitochondria (Ullrich et al. 1994).

The mechanism of HNE action on UCPs is unknown. Allosteric interactions (Murphy et al. 2003) or covalent modifications (Azzu and Brand 2010) have been proposed.

Because the effect of HNE is highly sensitive to PNs, as confirmed in this work for the UCP of a unicellular organism, HNE may bind reversibly to UCP in a PN-dependent manner. However, the covalent modification of UCP cannot be excluded because HNE is a highly reactive aldehyde that modifies the functional groups of proteins (Grimsrud et al. 2008). HNE forms stable adducts with proteins so this kind of interaction with UCP would not be reversible, but specific HNE adducts of UCPs have not been detected thus far. Interestingly, in the case of hydroperoxy fatty acids, which are also lipid peroxidation products, a classical cycling mechanism of UCP action has been proposed (Jaburek et al. 2004).

In mammalian mitochondria, the HNE-induced uncoupling is sensitive to GDP and carboxyatractyloside (a potent inhibitor of the adenine nucleotide carrier), indicating that the uncoupling is a cumulative effect of UCP and adenine nucleotide carrier stimulation, similar to the FFA-induced uncoupling (Echtay et al. 2003; Parker et al. 2008a; Aguirre and Cadenas 2010; Azzu et al. 2008). Taking this phenomenon into account, all of our measurements with non-phosphorylating amoeba mitochondria were performed in the presence of carboxyatractyloside. Therefore, we attributed the HNE-induced uncoupling only to AcUCP. Moreover, there is currently no evidence that FFA-induced UCP-independent uncoupling could occur in the mitochondria of unicellular eukaryotes (Jarmuszkiewicz et al. 2010; Uyemura et al. 2000, 2004). Because the proton conductance of the adenine nucleotide carrier is partially inhibited by GDP (Khailova et al. 2006; Parker et al. 2008b), we used GTP in our study, which also exhibits the highest inhibitory affinity for AcUCP (Woyda-Ploszczyca and Jarmuszkiewicz 2011).

In non-phosphorylating *A. castellanii* mitochondria, the HNE-induced GTP-sensitive AcUCP activity was manifested as an increase in the oxygen uptake accompanied by a decrease in $\Delta\Psi$ and the Q redox state. Moreover, the effect of HNE was similar to that induced by LA. The inducible GTP-sensitive AcUCP-mediated proton conductance did not differ when HNE or LA were used as activators. Our results indicated that HNE induced the AcUCP-mediated proton conductance both in non-phophorylating and phosphorylating mitochondria, decreasing the yield of oxidative phosphorylation in the latter. Therefore, we can conclude that activation by alkenal lipid peroxidation products also affects the UCPs of unicellular organisms, such as that of protozoan *A. castellanii*, similar to plant (Smith et al. 2004) and mammalian UCPs (Echtay et al. 2003). In mammalian and plant mitochondria, the stimulation of UCPs by physiologically relevant concentrations of HNE (Echtay et al. 2003) and FFAs (Kowaltowski et al. 1998) decreases mitochondrial ROS generation. It has also been shown that the FFA-induced AcUCP activation decreases ROS formation in *A. castellanii* mitochondria (Czarna and Jarmuszkiewicz

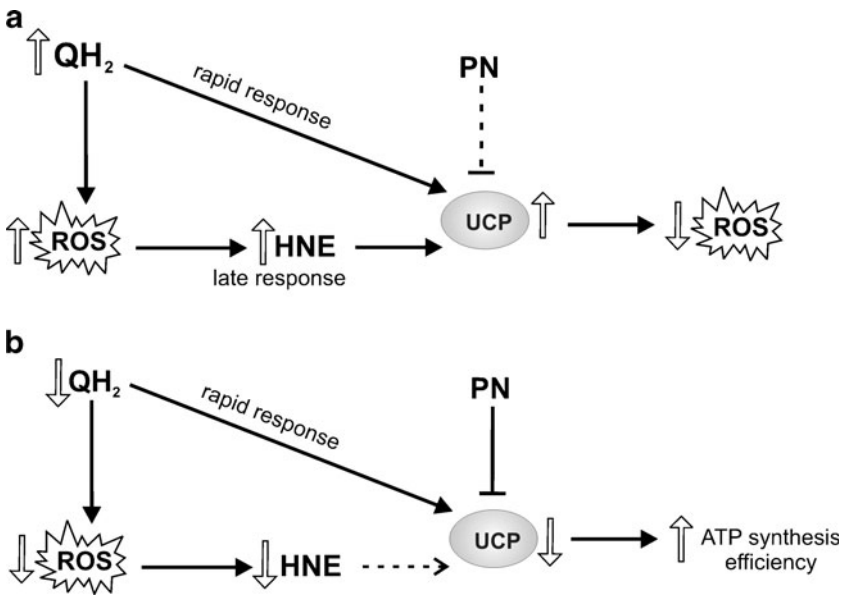

Fig. 5 A model for the regulation of AcUCP involving the membranous Q redox state (*a rapid response*) and the ROS-induced HNE-mediated pathway (*a late response*). **a** A high endogenous Q reduction level (a high QH_2 concentration) activates UCP by relieving the inhibition caused by PNs. This rapid response is followed by a late response through the ROS-induced HNE-mediated pathway. Along with a high Q reduction level, the increased level of ROS leads to increased membrane phospholipid peroxidation, resulting in the formation of HNE, which induces a proton leak through UCP (*a late response pathway*). As a consequence of UCP activation, mitochondrial ROS production is limited. **b** At low endogenous QH_2 concentrations, the proton conductance through UCP is inhibited by PN in the rapid response. The activation of UCP through the ROS-induced HNE-mediated pathway, thus the late response, does not occur, favouring a high ATP synthesis efficiency. The empty arrows represent the activation or high level (*up*) and inhibition or low level (*down*)

2005). Therefore, the current model proposed for animal and plant mitochondria (Murphy et al. 2003; Echtay et al. 2003; Considine et al. 2003) for the activation of UCPs by ROS through the initiation of lipid peroxidation (the HNE pathway), leading to the feedback down-regulation of mitochondrial ROS production, can be applied to the UCPs of unicellular eukaryotes. Thus, a common regulation of UCPs through lipid peroxidation products that arise from ROS could exist in all eukaryotes that possess UCPs.

The results presented in this work clearly indicate that the membranous Q redox state regulates the inhibition of HNE-induced AcUCP by GTP. So far, it has been shown that the Q redox state modulates the PN inhibition of FFA-activated UCPs in the isolated mitochondria of skeletal muscle (UCP3 and UCP2), potato tuber (plant UCP), and *A. castellanii* (AcUCP) respiring under phosphorylating conditions (Jarmuszkiewicz et al. 2004b, 2005; Navet et al. 2005). Moreover, a Q redox state-dependent inhibition of FFA-induced UCPs by PNs has also been observed in the isolated non-phosphorylating mitochondria of *A. castellanii* (AcUCP) and rat brown adipose tissue (UCP1) (Swida et al. 2008; Swida-Barteczka et al. 2009). In the present study, we demonstrated that conditions of high ubiquinone (oxidized Q) concentrations facilitated the inhibition of HNE-induced AcUCP-mediated uncoupling by PNs. In contrast, the inhibition was largely weakened when the Q redox state (ubiquinol concentration) was increased. In mammalian mitochondria, it has been shown that the endogenous and HNE-induced activation of proton conductance (including UCP-mediated uncoupling) increases with incubation time and requires a high $\Delta\Psi$ (Parker et al. 2008a, b) and, thus, a high Q reduction level. We propose that the activation by endogenous lipid peroxidation products could, therefore, be a late UCP response because it requires a prolonged period of increased endogenous superoxide formation. Our studies indicate that a rapid response through the endogenous Q redox state (particularly through the endogenous QH_2) could directly regulate UCP activity and does not require (although it does not exclude) activation by lipid peroxidation products. Thus, in our model for the activation of UCPs, an increased concentration of QH_2 (at a higher Q reduction level) activates UCP by relieving the inhibition from PNs (Fig. 5a). This rapid response is followed by a late response through the ROS-induced HNE-mediated pathway. Namely, the increased level of ROS formation, which results from the high membranous Q reduction level, leads to increased phosholipid peroxidation. The increased HNE level induces the proton conductance through UCP, which, in turn, attenuates the mitochondrial ROS production. This antioxidant role of UCPs could be especially important during oxidative stress conditions, when the high redox state of the electron carriers of the mitochondrial respiratory chain promotes superoxide formation. Thus, HNE, as both a lipid peroxidation product and a UCP activator, constitutes a late response pathway to prevent further lipid peroxidation and downstream detrimental effects. However, at a lower QH_2 concentration (i.e., at a low endogenous Q reduction level), PN may bind to UCP and proton conductance through UCP is inhibited during the rapid response (Fig. 5b). At the same time, the activation of UCP by ROS and the resulting lipid peroxidation products (the late response) would not occur. This UCP inactivation and the resulting efficient ATP synthesis would be favourable when ATP demand is high and the reducing substrate supply is low. Thus, the QH_2-dependent rapid response and the HNE-dependent late response lead to the same physiological effects (Fig. 5a and b). Therefore, QH_2, the mitochondrial respiratory chain component that is involved in ROS formation, and HNE, a marker of oxidative stress, are potential physiological signals for recruitment of UCPs, which are considered to be a first line of defense against mitochondrial ROS production. However, at elevated levels, both QH_2 and HNE determine the severe impairment of cellular functions and the induction of cell death.

Our work provides strong evidence for the universality of HNE-induced UCP-mediated mitochondrial uncoupling among eukaryotes. This study also supports the idea that the first and ancestral function of UCPs could be the attenuation of mitochondrial ROS production through a negative feedback loop involving lipid peroxidation products as a late response to the elevated ROS level. The rapid response through the endogenous Q redox state could directly regulate the sensitivity of the HNE-induced (and FFA-induced) UCP activity to PNs.

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