Mitochondrial Proton Conductance in Skeletal Muscle of a Cold-Exposed Marsupial, *Antechinus flavipes*, Is Unlikely to Be Involved in Adaptive Nonshivering Thermogenesis but Displays Increased Sensitivity toward Carbon-Centered Radicals

Martin Jastroch1,*, Kerry W. Withers2, Sigrid Stoehr1, Martin Klingenspor3

1Department of Animal Physiology, Faculty of Biology, Philipps-Universität Marburg, Karl-von-Frisch-Str. 8, 35032 Marburg, Germany; 2Centre for Systems Biology, University of Southern Queensland, Toowoomba, Queensland, Australia; 3Technische Universität München, Molecular Nutritional Medicine, Else Krön er-Fresenius Center, Freising-Weihenstephan, Germany

Accepted 1/13/2009; Electronically Published 7/17/2009

ABSTRACT

The organs and molecular mechanisms contributing to adaptive thermogenesis in marsupials are not known because some species apparently lack brown adipose tissue (BAT). The increased oxidative capacity and presence of uncoupling protein 3 (UCP3) in skeletal muscle led to speculations on whether uncoupled respiration sustains endothermy in the cold, as found for BAT. Here, we investigated the role of mitochondrial proton conductance in the small Australian marsupial *Antechinus flavipes* during cold exposure. Although there was a tendency toward higher oxidative capacity in skeletal muscle, indicating metabolic adjustments to the cold, we observed no change in basal proton conductance of isolated myotubular and liver mitochondria. In eutherians, 4-hydroxynonenal (HNE) is an activator of mitochondrial uncoupling mediated by UCP3 and ANT (adenine nucleotide translocase). In the marsupial *A. flavipes*, proton conductance in myotubular mitochondria could be induced by HNE selectively in the cold-acclimated group. Induced uncoupling activity could be attributed to the ANT as judged by inhibition with carboxyatractylate, while GDP, a putative inhibitor of rodent UCP3, had no detectable effects on marsupial UCP3. In contrast to previous expectations, basal proton conductance in the myotubular mitochondria of marsupials does not contribute to adaptive thermogenesis, as found for eutherian BAT. Increased sensitivity of proton conductance to HNE by the ANT suggests a greater requirement for mild uncoupling activity that may convey protection from lipid peroxidation and mitigate reactive oxygen species production during cold stress.

Introduction

In eutherian mammals, it has been well established that brown adipose tissue (BAT) contributes significantly to adaptive nonshivering thermogenesis (NST; Cannon and Nedergaard 2004). Uncoupling protein 1 (UCP1), exclusively expressed in BAT and recently found in thymus (Carroll et al. 2004; Adams et al. 2008), uncouples respiration from ATP production and dissipates proton motive force as heat (Nicholls and Locke 1984). In response to cold, UCP1 expression and mitochondrial biogenesis in BAT increase about two- to threefold, thereby providing higher NST capacity (Klingenspor et al. 1996).

Marsupials also thermoregulate well in the cold, although the presence of BAT has not been unequivocally demonstrated in all species (Hayward and Lisson 1992; Kabat et al. 2003; Jastroch et al. 2008). It has been suggested that shivering is the main source of heat production in marsupials, but cold-acclimated (CA) kowari (*Dasyuroides byrnei*) showed a decrease in shivering tremor, indicating some form of NST that replaced shivering thermogenesis (May 2003). Other studies claimed the presence of NST similar to eutherians. For example, noradrenaline injections led to an increase of metabolic rate in marsupials, but cold-acclimated (CA) kowari (*Dasyuroides byrnei*) showed a decrease in shivering tremor, indicating some form of NST that replaced shivering thermogenesis (May 2003). Other studies claimed the presence of NST similar to eutherians. For example, noradrenaline injections led to an increase of metabolic rate in marsupials, but cold-acclimated (CA) kowari (*Dasyuroides byrnei*) showed a decrease in shivering tremor, indicating some form of NST that replaced shivering thermogenesis (May 2003). Other studies claimed the presence of NST similar to eutherians. For example, noradrenaline injections led to an increase of metabolic rate in marsupials, but cold-acclimated (CA) kowari (*Dasyuroides byrnei*) showed a decrease in shivering tremor, indicating some form of NST that replaced shivering thermogenesis (May 2003).

Studies in the South American marsupial *Monodelphis domestica* suggested a role for skeletal muscle in NST because CA individuals showed higher costs of locomotory transport,
thereby indicating an increase in muscle energetics (Schaeffer et al. 2005). The concordant upregulation of the muscle-specific UCP3 implied a molecular explanation for thermogenic processes seen in M. domestica. In Ca macropods, NST mediated by UCP3 was suggested on the basis of gene expression analysis showing the presence of UCP3 but was not supported by functional protein data (Kabat et al. 2003; Schaeffer et al. 2005). Taken together, these observations suggested that in absence of UCP1 and BAT, skeletal muscle including UCP3 may be responsible for adaptive heat production in marsupials.

In eutherians, there is minor evidence for UCP3-mediated heat production. Although UCP3 knockout mice have a diminished thermogenic response to the drug 3,4-methylenedioxyamphetamine (MDMA) in skeletal muscle (Mills et al. 2003), the physiological significance under nonpharmacological conditions is questioned. There is doubt as to whether low protein concentration as compared with UCP1 in BAT can produce sufficient amounts of heat (Nedergaard and Cannon 2003). Furthermore, thermogenic uncoupled respiration should be reduced in response to fasting, but in contrast, UCP3 gene expression is increased (Boss et al. 1998; Jastroch et al. 2004b). That rodent UCP3 exhibits an uncoupling function was confirmed by activation with superoxide and 4-hydroxynonenal (HNE) in isolated mitochondria, but activation by these molecules is implicated in protection from lipid peroxidation by mild uncoupling (Echtay et al. 2002, 2003). Furthermore, it has been shown that HNE activates uncoupling activity not only of UCPs but also of the adenine nucleotide translocase (ANT). Recently, UCP3 has been identified in muscle of Antechinus flavipes (Jastroch et al. 2004b), but the function of UCP3 in marsupials has not been investigated so far. Adult A. flavipes do not express UCP1 (Jastroch et al. 2008) and represent a suitable model to investigate the role of UCP3 in skeletal muscle during cold exposure.

Our approach was to acclimate the small marsupial A. flavipes to the cold and test for increases in oxidative capacity as found for M. domestica (Schaeffer et al. 2003). In a previous study on A. flavipes, we showed that UCP3 protein levels increase during cold exposure despite a maintenance of UCP3 mRNA levels (Jastroch et al. 2004a). In this study, we aimed to substantiate previous speculations on uncoupling activity by measuring mitochondrial proton conductance in myotubular mitochondria.

**Material and Methods**

**Animal Experiments**

All experimental protocols were approved by the Animal Ethics Committee of the University of Southern Queensland, Queensland Environmental Protection Agency (permit WISP02633304), and Environment Australia (export no. WT2005-12380). Seven Antechinus flavipes were captured with Elliott traps from several subtropical habitats in southeast Queensland (Australia) between January and March 2005 and housed individually for at least 1 wk at 24°C (12L:12D, lights on at 0700 hours) with free access to water and food (mealworms and cat food mix including calcium carbonate). To investigate the effect of cold exposure, four individuals were transferred to a climate chamber at 10°C for 17–22 d (CA), whereas three individuals remained at 24°C (warm acclimated [WA]). The body weight of the individuals ranged from 24.9 to 38.5 g. The two acclimation groups were balanced for body weight.

**Tissue Dissection**

Antechinus flavipes were killed (carbon dioxide), and the bulk of skeletal muscle and liver was used to isolate mitochondria. Small samples of skeletal muscle for molecular analysis were immediately snap frozen in liquid nitrogen and shipped to Marburg, Germany.

**Cytochrome c Oxidase (COX) Activity**

Cytochrome c oxidase (COX) activity was measured polarographically in a temperature-controlled chamber. Forty to 60 mg of frozen skeletal muscle tissue was homogenized in 500 μL ice-cold tissue buffer (33.9 mM KH₂PO₄, 66.1 mM K₂HPO₄, 2 mM EDTA, 10 mM glutathione, pH 7.5) with a UltraTurrax tissue homogenizer. Protein extracts from skeletal muscle were quantified using the Bradford method. The homogenate was diluted 1:2 with a detergent, 1.5% polyoxyethylenehexyl ether W1 (Sigma), to release the protein from membrane fractions. Oxygen consumption was measured using a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK) maintained at 25°C and calibrated with air-saturated measuring buffer (79 mM K₂HPO₄, 20.1 mM KH₂PO₄, 5 mM EDTA, 3 mM horse cytochrome C [Sigma], 0.25 M ascorbate; pH 7.4), which was assumed to contain 479 nmol O₂ mL⁻¹ (Reynafarje et al. 1985). Ten, 20, or 30 μL of diluted homogenate were suspended in 1 mL medium.

**Isolation of Skeletal Muscle and Liver Mitochondria**

Skeletal muscle tissue was immediately placed in ice-cold isolation CP1 medium containing 100 mM KCl, 50 mM Tris-HCl, and 2 mM EGTA, pH 7.4, at 4°C. Muscle tissue was cleaned from adipose tissue patches, minced with scissors, and repeatedly rinsed with CP-1 medium, stirred for 3 min in CP-2 medium (CP-1 plus 1 mM ATP, 5 mM MgCl₂, 0.5% [w/v] bovine serum albumin [BSA], 2.1 U mL⁻¹ protease [Subtilisin A]), pH 7.4, at 4°C) and homogenized in CP-2 medium using a polytron tissue homogenizer (Kinetics). The homogenate was stirred in CP-2 for 3 min, then mitochondria were isolated using differential centrifugation and resuspended in CP-1 medium. The liver was placed in ice-cold STE medium (250 mM sucrose, 5 mM Tris, 2 mM EGTA, pH 7.4, at 4°C), minced with scissors, and disrupted with a Dounce homogenizer with a medium-fitted pestle. Protein concentration was determined using the biuret method with fatty acid free BSA as standard (Sigma A3803).
Mitochondrial Proton Conductance in Skeletal Muscle of Marsupials

Measurement of Mitochondrial Oxygen Consumption

Oxygen consumption was measured using a Clark-type oxygen electrode (Rank Brothers) maintained at 35°C and calibrated with air-saturated medium (120 mM KCl, 5 mM KH₂PO₄, 3 mM HEPES, 1 mM EGTA, and 0.3% [w/v] defatted BSA, pH 7.2), which was assumed to contain 416 nmol O mL⁻¹ (Reynafarje et al. 1985). Mitochondria were suspended at 0.35 mg mL⁻¹ to 0.5 mg mL⁻¹ (for skeletal muscle) and 1.0 mg mL⁻¹ (for liver) in 2.5 mL medium and incubated with 6 mM succinate, 8 μM rotenone, 4 μM oligomycin, and 150 nM nigericin. Respiratory control ratio (RCR) was measured in the first experiments to determine the accuracy of the mitochondrial preparation and the integrity of the mitochondria (RCR > 3) using 600 μM ADP for the establishment of state 3 respiration.

Measurement of Proton Conductance

The respiration rate of mitochondria, in the presence of oligomycin to inhibit ATP synthesis (state 4), is proportional to the rate at which protons leak across the mitochondrial inner membrane. The kinetic response of the proton conductance to its driving force (proton motive force) can therefore be measured as the relationship between respiration rate and membrane potential when the potential is varied by titration of the potential-dependent probe TPMP⁺ (Brand 1995). The TPMP⁺ electrode was calibrated with sequential additions of up to 1.25 μM TPMP⁺. Six mM succinate was added to start the reaction. GDP, CAT, and HNE were added 6 min before energization with succinate, GDP and CAT immediately before HNE. TPMP⁺ correction factor was assumed to be 0.4 for liver and 0.35 for skeletal muscle, as described previously (Rolfe et al. 1994).

RNA Isolation, Reverse Transcriptase–Polymerase Chain Reaction, and Northern Blot Analysis

Total RNA was isolated with TRIzol (GIBCO-BRL) according to the manufacturer’s protocol. As an additional step, the RNA pellet was redissolved in a solution containing 6.3 M guanidinium thiocyanate, 40 mM sodium citrate pH 7, 0.8% sarcosyl, and 8 mM 2-mercaptoethanol, precipitated with 1 vol isopropanol, washed in 75% ethanol, and finally dissolved in DEPC-treated water. Total RNA was photometrically quantified at 260 nm and stored at −70°C. RNA was isolated from skeletal muscle of A. flavipes and used for first-strand cDNA synthesis (SUPERSCRIPT II, GIBCO/BRL) according to the manufacturer’s protocol. ANT1 primers were deduced from the genome of Monodelphis domestica (http://www.ensembl.org) and obtained from MWG Biotech (Ebersberg, Germany; sense: 5’-ATG GAT GGC CCT GCT GTC TTC TGC GCC AAC CTG GAC AAT GCT; antisense: 5’-CCG TCA CGG TCT GCC CGA TCA TCC AGC TCA). Twenty micrograms of RNA were electrophoresed in a 1% denaturing agarose gel (5% formaldehyde, 0.02 M MOPS, 5 mM sodium acetate, 1 mM disodium EDTA, pH 8.9), transferred overnight in 10× SSC to a nylon membrane (Hybond N, Amersham), and UV cross-linked. The UCP3 cDNA was random prime-labeled with [α-32P]dCTP (Rediprime DNA labeling system, Amersham) and hybridized to the nylon membrane, as described previously (Jastroch et al. 2004b). After hybridization, the blots were washed with 2 × SSC/0.1% SDS for 20 min, 1 × SSC/0.1% SDS for 10 min, and 0.5 × SSC/0.1% SDS for 10 min at room temperature. Signal intensities were then monitored by exposure to a PhosphorScreen (Molecular Dynamics). The hybridized probes were then detected by phosphor imaging (Storm 860, Molecular Dynamics), and signal intensities were quantified using ArrayVision 7.0 (Imaging Research). Ethidium bromide staining of total RNA served to normalize gel loading and was quantified densitometrically (Scion Image Software 4.0.2).

Statistical Analysis

Values presented are means ± SEM. Unpaired Student’s t-tests and one-way ANOVA were performed with the level of significance set to P < 0.05.

Results

COX Activity

COX activity is generally used to estimate oxidative capacity and represents a marker for mitochondrial density in tissues. Measuring crude skeletal muscle tissue homogenates of Antechinus flavipes, we found a tendency toward higher oxidative capacity in CA individuals (106.2 ± 7.3 [WA] vs. 135.3 ± 13.3 [CA] μatoms O min⁻¹ g⁻¹ wet weight; Fig. 1).

State 4 Respiration and Basal Proton Conductance of Liver and Skeletal Muscle Mitochondria

We measured oxygen consumption of isolated mitochondria and compared the values of resting (state 4) respiration in isolated skeletal muscle and liver mitochondria of WA and CA A. flavipes. When respiring on succinate, state 4 respiration was not significantly different in liver (WA: 46.14 ± 5.46 nmol O min⁻¹ mg⁻¹; CA: 48.80 ± 1.53 nmol O min⁻¹ mg⁻¹) and skeletal muscle (WA: 69.78 ± 7.59 nmol O min⁻¹ mg⁻¹; CA: 71.74 ± 2.34 nmol O min⁻¹ mg⁻¹). We next compared the full kinetic response of proton leak (monitored as oxygen con-
Figure 1. Cytochrome c oxidase activity in skeletal muscle of cold-acclimated and warm-acclimated Antechinus flavipes. Filled bar displays individuals maintained at 24°C, whereas open bar displays individuals exposed to 10°C. Bars are means ± SEM of three (warm exposed) or four (cold exposed) independent experiments/individuals, each performed in duplicate.

sumption rate) at 35°C to stepwise changes in its driving force, membrane potential, in skeletal and liver mitochondria from WA and CA A. flavipes (Fig. 2). In response to cold, the kinetic response of proton leak was slightly but not significantly increased in both tissues of CA animals. As known for eutherian mammals, liver basal proton conductance was lower than skeletal muscle proton conductance (about 50% when compared at the highest common potential).

Hydroxynonenal-Induced Proton Conductance of Skeletal Muscle Mitochondria Is Sensitive to Carboxyatractylate but Not GDP

Proton conductance can be changed by activation and inhibition of mitochondrial carriers with an uncoupling function. Induced proton leakage is reflected in a shift of the basal proton leak curve upward. In rodents, uncoupling proteins and the ANT catalyze mild uncoupling after activation by HNE. Prevention of HNE activation by GDP indicates the involvement of a UCP (Echtay et al. 2003), whereas inhibition by CAT indicates the involvement of the ANT.

In this study, we incubated isolated skeletal muscle mitochondria with 35 μM HNE 5 min before energization to induce proton leak (Fig. 3). HNE clearly shifted the proton leak curve of CA A. flavipes upward, demonstrating higher conductance (Fig. 3A), while HNE had only a minor effect in WA skeletal muscle mitochondria (Fig. 3B). The administration of CAT before HNE prevented the induction by HNE. We next quantified the induced proportion of proton leak. Because proton motive force is the driving force of the proton leak, to make comparisons between the two acclimation temperatures we compared the oxygen consumption driving proton leak at a common membrane potential. At the common potential of 150 mV (Fig. 3C), HNE treatment in the CA group results in a 40% increase of proton leak (basal: 41.87 ± 2.28 nmol O min⁻¹ mg⁻¹ protein; HNE induced: 58.57 ± 3.18 nmol O min⁻¹ mg⁻¹ protein; P<0.05), which could be fully prevented by the administration of CAT (43.25 ± 3.29 nmol O min⁻¹ mg⁻¹ protein). This effect was not apparent in WA animals (basal: 38.29 ± 1.14 nmol O min⁻¹ mg⁻¹ protein; HNE induced: 46.86 ± 3.23 nmol O min⁻¹ mg⁻¹ protein; CAT + HNE: 46.38 ± 4.04 nmol O min⁻¹ mg⁻¹ protein). GDP (1 mM) had no inhibitory effect on HNE activation in either the WA or the CA group (Fig. 3C). Palmitate (100 μM) had no additional effect on proton conductance on either basal conditions or HNE-induced conditions (data not shown).

HNE showed a greater effect on proton conductance in skeletal muscle mitochondria of CA A. flavipes. These effects may be reflected in gene expression of ANT1 and UCP3. Western blot analysis using a commercially available antirodent ANT1 antibody did not cross-react with ANT1, as judged by comparing the expected size (deduced from the nucleotide sequence) with the band pattern. Pertaining to UCP3, a new mouse UCP3 antibody in our lab kindly provided by R. Porter could not unambiguously detect marsupial UCP3.

We cloned an ANT fragment from A. flavipes (GenBank accession no. EF450126) that could be classified as ANT1 using phylogenetic inference as performed previously (data not shown; Jastroch et al. 2004b). Our Northern blot experiments revealed highest ANT1 mRNA expression in skeletal muscle (Fig. 4A) with a similar pattern to tissue specificity of UCP3 mRNA, as demonstrated previously (Jastroch et al. 2004b). We found no difference in ANT1 and UCP3 mRNA levels between the CA and WA group (Fig. 4B, 4C).

Figure 2. Measurement of basal proton conductance. The graph shows the full kinetic response of proton leak from mitochondria isolated from warm- (filled symbols) and cold- (open symbols) exposed Antechinus flavipes. Upper curves display the proton leak of skeletal muscle (diamonds), lower curves display the proton leak of liver (squares). Data are means ± SEM of three (warm exposed) or four (cold exposed) independent experiments/individuals, each performed in duplicate.
Figure 3. Inducible proton conductance in skeletal muscle mitochondria of *Antechinus flavipes*. A, Time course showing the addition of activators and inhibitors of proton conductance before energization. B, C, Full kinetic response of proton leak under basal conditions (diamonds), after addition with 4-hydroxynonenal (HNE; squares), and after addition of HNE and CAT (crossed filled squares). B shows the proton leak kinetics of the warm-acclimated group (filled symbols), C shows the cold-acclimated group (open symbols). D, Comparison of oxygen consumption driving proton leak at a common membrane potential of 150 mV. Data are means ± SEM of three (warm exposed) or four (cold exposed) independent experiments/individuals, each performed in duplicate. Asterisk indicates HNE versus control/HNE/CAT.

Discussion

We investigated the role of proton conductance in myotubular mitochondria during cold stress in the small marsupial *Antechinus flavipes*. Previously, the lack of BAT in marsupials led to suggestions of a thermogenic role for skeletal muscle (Kabat et al. 2003; May 2003; Schaeffer et al. 2003, 2005). The upregulation of UCP3 in *Monodelphis domestica* in response to cold mimics the regulation of UCP1, suggesting that UCP3 may uncouple myotubular mitochondria and produce heat, thereby compensating for the lack of BAT. We demonstrated that basal proton conductance of skeletal muscle mitochondria of *A. flavipes* plays no role in cold-adaptive heat generation, as found for BAT. Instead, the induction of mild uncoupling by HNE in CA *A. flavipes* suggests a propensity for the regulated increase of proton conductance to protect the skeletal muscle cells from lipid peroxidation during cold exposure.

A tendency toward increased oxidative capacity, measured as COX activity, in response to cold demonstrated adaptive metabolic changes in skeletal muscle of *A. flavipes*, as found for *M. domestica* (Schaeffer et al. 2003). Although cold exposure triggered an increase in oxidative capacity of skeletal muscle in marsupials (*A. flavipes* and *M. domestica*), one cannot distinguish between higher demand for ATP or inefficient mitochondrial coupling. A higher demand for ATP may be dependent on increased locomotion or shivering (May 2003; Schaeffer et al. 2005), whereas uncoupling of the mitochondria would increase nonshivering heat dissipation directly.

Here, we demonstrated that basal proton conductance of *A. flavipes* mitochondria was unchanged despite cold exposure. An adaptive thermogenic role for skeletal muscle proton conductance and marsupial UCP3 is unlikely when compared with the situation of eutherian BAT mitochondria: a high UCP1 concentration (reaching up to 5% of mitochondrial protein; Ricquier et al. 1984) leads to an elevation of basal leak respi-
ration, which can be diminished only by addition of UCP1-inhibiting purine nucleotides such as GDP (Nicholls and Locke 1984). The high proportion of uncoupled respiration by UCP1 provides the basis for NST. Notably, UCP1 uncoupling activity can be induced by addition of palmitate. Palmitate induction is also indicative of a thermogenic function because the cascade leading to dramatic free fatty acid release in BAT is triggered by cold (Lowell and Spiegelman 2000). In our experiments, the addition of 100 μM palmitate had no effect on basal proton conductance (data not shown).

While a slight elevation of basal proton conductance was found in myotubular mitochondria from CA skeletal muscle, it could not be related directly to UCP3 since a similar elevation was found in liver mitochondria (containing no UCP3).

High proton motive force increases the generation of superoxides that subsequently produce carbon-centered radicals in the cellular system (Brand 2005). HNE is a well-characterized metabolite of lipid peroxidation. It has been hypothesized that superoxides and HNE themselves mitigate de novo production in a feedback mechanism that lowers proton motive force because the cascade leading to dramatic free fatty acid release in BAT is triggered by cold (Lowell and Spiegelman 2000). In our experiments, the addition of 100 μM palmitate had no effect on basal proton conductance of the myotubular mitochondria (data not shown).

Figure 4. Analysis of ANT1 and UCP3 gene expression. Total RNA (20 μg) isolated from selected tissues was hybridized with an ANT1 700–base pair cDNA fragment or a UCP3 fragment, as described previously (Jastroch et al. 2004b). A Northern blot analysis of ANT1 expression in multiple tissues of the Antechinus flavipes. B, C, Quantitative analysis of ANT1 and UCP3 mRNA levels between cold-acclimated and warm-acclimated groups.

could be explained by upregulation of the proposed feedback mechanism. Under challenging physiological conditions, increased HNE induction was previously provoked in rodents during fasting (Echtay et al. 2002). During fasting, the organism is forced to save energy instead of wasting energy by inefficient ATP production. Mild uncoupling occurs despite unfavorable energy dissipation, perhaps demonstrating the importance of controlling free radicals in cells. The primary role of mild uncoupling in A. flavipes may therefore be the prevention of mitochondrial superoxide production. Here, we show that cold is a physiological stimulus that recruits a propensity for mild uncoupling too.

A secondary role in heat production is questionable, since mild uncoupling most likely does not produce sufficient heat under physiological concentrations of superoxide and HNE. The HNE concentration used by others and in this study was not sufficient to measure mild uncoupling activity of mitochondrial carriers in the proton leak assay.

We further examined the molecular mechanism underlying mild uncoupling in A. flavipes and found prevention by CAT demonstrating the involvement of the ANT. In our experiments, we did not investigate the effect of CAT on nonstimulated proton conductance, and we therefore cannot completely exclude the involvement of other mitochondrial carrier proteins. The eponymous function of the ANT is the exchange of ADP/ATP between cytosol and the mitochondrial compartment. Further functions have been suggested, such as the involvement in the permeability transition pore and mild uncoupling (Halestrap et al. 1997; Echtay et al. 2003). We showed that A. flavipes ANT1 (GenBank accession no. EF450126) was most prominent in skeletal muscle and likely to explain induced uncoupling in myotubular mitochondria. As found for UCP3, ANT1 mRNA levels did not differ between the WA and CA group. Immunological detection using the only commercial available ANT1 antibody failed in the marsupial A. flavipes. Whether higher HNE sensitivity during cold exposure was caused by higher ANT protein concentration or allosteric changes or whether other mitochondrial proteins may be involved requires further investigation. Considering that HNE also reacts with proteins and lipids in mitochondria, different concentrations of downstream metabolites between WA and CA mitochondria may be responsible for increased ANT uncoupling activity in the cold.

In contrast to rodents, we found no evidence for an involvement of UCP3 in mild uncoupling, since no inhibition in the presence of 1 mM GDP was observed. These experiments suggested that marsupial UCP3 was not involved in HNE-induced uncoupling activity. However, the nonphysiological concentrations of HNE, necessary to observe uncoupling in vitro, may have activated UCP3 constitutively and inhibited GDP binding. However, studies in the UCP1-knockout mouse show that mitochondrial proton leak is GDP insensitive despite elevated levels of UCP2 and UCP3 (Monemdjou et al. 1999). Alternatively, HNE may not activate UCP3 in marsupials.

Most recently, the major proportion of HNE-induced proton conductance was ascribed to the ANT in mouse and rat skeletal
muscle, while only a minor proportion was attributable to UCP3 (Parker et al. 2008), supporting our data in *A. flavipes*. Heat produced from mild uncoupling may be of benefit during thermogenesis, but such increases in proton conductance may be a by-product due to increased lipid peroxidation during cold exposure rather than a true thermogenic contribution.

**Acknowledgments**

This research was supported by a Centre for Systems Biology, University of Southern Queensland grant (to K.W.W. and M.J.) and by the Deutsche Forschungsgemeinschaft DFG KL973/7 (to M.K.). We thank Adele Jones for excellent technical assistance.

**Literature Cited**


