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Mitochondrial metabolism in mammalian cold-acclimation: Magnitude and mechanisms of fatty-acid uncoupling

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Abstract

Cold-acclimation did not alter uncoupling (state 4 respiration) in rat liver or skeletal muscle mitochondria. Palmitate significantly uncoupled mitochondria, but neither the magnitude of this uncoupling nor the contribution of different inner mitochondrial membrane transporters to uncoupling was altered by cold-acclimation. Guanosine diphosphate did not reduce uncoupling, suggesting no role for uncoupling protein homologues. The adenine nucleotide transporter and the permeability transition pore, either alone or in combination, appear to contribute significantly to free fatty-acid (FFA)-induced uncoupling. Evidence suggests that these two elements may be working together, as components of the same mechanism, to mediate FFA uncoupling.

Keywords: Mitochondria; Uncoupling; Fatty-acid; Cold-acclimation; Rat; Mammal; UCP; ANT; PTP

1. Introduction

When initially exposed to ambient temperatures below the thermoneutral zone, homeothermic endotherms typically shiver, stimulating ATP turnover, and flux through the electron transport chain (ETC) where most metabolic heat is produced (Hochachka, 1974). Shivering decreases rapidly within a few days of chronic cold exposure in rats (Davis et al., 1960) and thereafter non-shivering thermogenesis (NST) becomes the predominant means of heat production. The term NST is typically used to describe thermogenesis within brown adipose tissue (BAT) of eutherian mammals. When activated by β-adrenergic stimulation, uncoupling protein 1 (UCP1) within the inner mitochondrial membrane (IMM) of BAT adipocytes facilitates the translocation of protons from the intermembrane space to the mitochondrial matrix. This dissipates the mitochondrial proton motive force (Δp), stimulating ETC flux and heat production not coupled to ATP turnover (Nedergaard et al., 2001).

While BAT NST is undoubtedly crucial to cold-acclimated mammals (Harri et al., 1984; Abelenda and Puerta, 1990; Zaninovich et al., 2003), it is estimated that only about 60% of the increase of whole-animal oxygen consumption due to NST can be attributed to BAT (Foster and Frydman, 1978; Puchalski et al., 1987). This suggests that other mechanisms of NST are upregulated during cold-acclimation, perhaps in organs other than BAT. Proton "leak" and mitochondrial uncoupling in tissues other than BAT may contribute up to 20% of standard metabolic rate even in thermoneutral mammals (Rolfe and Brand, 1996). If these pathways can be altered with cold exposure, they could contribute significantly to adaptive thermogenesis.

Mitochondria isolated from the livers of cold-acclimated ducklings (birds are not known to express UCP1) show uncoupling relative to thermoneutral controls (Goglia et al., 1993). Evidence from mammalian skeletal muscle and liver suggests that cold-acclimation uncouples mitochondria in the presence (Grav and Blix, 1979), but not in the absence (Himms-Hagen et al., 1976; Zaninovich et al., 2003; Mollica et al., 2005), of free fatty-acids (FFA). FFA may uncouple mitochondria in extra BAT tissues through the action of IMM proteins capable of transporting FFA anions (Kadenbach, 2003; Nedergaard and Cannon, 2003) or FFA hydroperoxides (Esteves and Brand, 2005) from

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the mitochondrial matrix to the intermembrane space. These proteins may include homologues to UCP1, the glutamate–aspartate transporter (GAT), the adenine nucleotide translocase (ANT), and the permeability transition pore (PTP).

Short-term (24 h) cold exposure in rats leads to a dramatic increase in skeletal muscle UCP3 mRNA (Lin et al., 1998) which corresponds to a FFA-sensitive uncoupling (Simonyan et al., 2001). The contribution of UCP homologues to FFA-induced uncoupling in mammalian chronic cold-acclimation has not been examined.

Five weeks of cold exposure caused a 70% increase of ANT content in subsarcolemmal skeletal muscle mitochondria of ducks (Roussel et al., 2000). This corresponds with increased FFA-sensitive uncoupling in skeletal muscle mitochondria (Roussel et al., 1998) that is inhibited by carboxyatractyloside (catr), a specific inhibitor of ANT (Toyomizu et al., 2002). Little information regarding ANT and cold-acclimation in mammals is available, except to note that in the hibernator, *Citellus (Spermophilus) undulatus*, heart and skeletal muscle mitochondria from winter (hibernating or aroused) animals showed an uncoupling that is sensitive to catr and bovine serum albumin (BSA) which binds FFA (Brustovetsky et al., 1992).

FFA can induce opening of the PTP, allowing solutes smaller than 1500 Da (including protons) to pass through the IMM. This uncoupling is prevented by cyclosporine A (cycA), a specific inhibitor of PTP (Wieckowski and Woytczak, 1998; Furono et al., 2001). Little information is available regarding the effect of cold-acclimation on FFA-induced PTP-mediated uncoupling, except to note that arousal from hibernation in *C.* (*Spermophilus*) *undulatus* is accompanied by an uncoupling of liver mitochondria that is partially inhibited by cycA (Amerkhanov et al., 1996).

We hypothesize that FFA-induced uncoupling increases during cold-acclimation in the rat, facilitating NST in extra BAT tissues. To test this hypothesis, we measured respiration rates of liver and skeletal muscle mitochondria in the presence and absence of FFA. To assess the roles of UCP homologues, GAT, ANT, and PTP, we measured the effect of inhibitors of these proteins on FFA-induced uncoupling.

2. Materials and methods

2.1. Animals

Male Wistar rats (*Rattus norvegicus*) were obtained from Charles River (St. Constant, QC, Canada) and maintained individually in standard shoebox cages, with water and food (Prolab RMH 3000) available ad libitum. Control rats were housed at $25\pm1\,^{\circ}C$ while cold-acclimated rats were housed at $5\pm1\,^{\circ}C$ for 2–7 weeks. In both cases, the photoperiod was 12 h light: 12 h dark (lights on at 8:00 h).

2.2. Mitochondrial isolation

Animals were euthanized by an intraperitoneal injection of Euthanyl ($270\,mg\,ml^{-1}$, 0.5 ml 200 g body mass). For isolation of liver mitochondria, the liver was removed and placed in a dish containing approximately 25 ml of ice-cold homogenization medium (in mM, 75 sucrose, 225 mannitol, 1 EGTA, 10 HEPES, with 0.05% BSA w/v, pH 7.4), rinsed of blood and trimmed of extraneous tissue. The liver was minced using scissors and rinsed of blood with three washes of homogenization buffer (25 ml). The liver was then transferred to a 50 ml glass mortar and homogenized with three passes of a loose fitting (0.4 mm clearance) Teflon pestle at 150 RPM.

The homogenate was strained through one layer of cheesecloth and centrifuged twice at $1200\,g$ at $4\,^{\circ}C$ for 10 min. After each spin any floating fat layer was removed by aspiration. The supernatant was passed through four layers of cheesecloth and centrifuged at $8700\,g$ for 10 min at $4\,^{\circ}C$. The supernatant was aspirated away along with the topmost "fluffy" layer of the pellet. Any visible material adhering to the side of the centrifuge tube was removed with cotton swabs. The pellet containing mitochondria was resuspended in 40 ml of homogenization buffer and centrifuged at $8700\,g$ for 10 min at $4\,^{\circ}C$. Supernatant and lipid was removed, and this final pellet was resuspended in 1 ml of homogenization buffer and stored on ice for no more than 2 h.

For muscle mitochondria, both gastrocnemius muscles were rinsed in 25 ml of ice-cold homogenization buffer (in mM, 100 sucrose, 10 EDTA, 100 Tris–HCl, 46 KCl, 0.05% BSA, pH 7.4). After the removal of connective tissue, the muscle was minced with scissors and incubated with protease (Substilin A, Sigma) at $0.5\,mg\,ml^{-1}$ for 5 min. The tissue was then homogenized with three passes of a loose Teflon pestle at 150 RPM, and the homogenate was left to incubate on ice for a further 5 min. This was followed by homogenization with three passes of a tight pestle (0.15 mm clearance) at 150 RPM. The homogenate was passed through cheesecloth and centrifuged twice at $2000\,g$ for 10 min at $4\,^{\circ}C$ with floating lipid and pellet removed between spins. The final supernatant was centrifuged twice at $10\,000\,g$ for 10 min at $4\,^{\circ}C$ with supernatant, fluffy layer and adhering lipid removed as for the liver preparations. The final pellet was resuspended in 1 ml of assay buffer (see below).

2.3. Mitochondrial respiration

Mitochondrial oxygen consumption was determined at $37\,^{\circ}C$ using polarographic electrodes (Rank Brothers, Cambridge, UK) with data collected on a computer using Datacan (Sable Systems, Las Vegas, NV). Electrodes were calibrated with assay buffers for either liver (in mM, 50 sucrose, 225 mannitol, 10 $MgCl_2$, 10 HEPES, 2 KH_2PO_4, 1 EGTA, pH 7.4) or muscle (in mM, 70 sucrose, 230 mannitol, 0.02 EDTA, 20 Tris–HCl, 5 KH_2PO_4, pH 7.4).

Mitochondria were added to a final concentration of 0.2 (liver) or 0.05 (muscle) mg protein ml^{-1} (determined by Bradford assay with BSA as standard). Unless otherwise stated, all compounds were dissolved in the appropriate assay buffer with all concentrations reported as final concentrations in the respiration chamber.

State 2 respiration was determined in the presence of rotenone (2 μg ml^{-1}, dissolved in 95% ethanol) and 6 mM succinate. To stimulate state 3 respiration, ADP was added to 0.15 mM. When all of the ADP was converted to ATP, the mitochondria entered state 4 respiration, which was used to assess the degree of uncoupling. For each mitochondrial preparation, after state 4 was established, palmitate (dissolved in 95% ethanol) was added to a final concentration of 0.003 mM (muscle) or 0.015 mM (liver), to assess the ability of FFA to uncouple respiration. BSA was then added to a final concentration of 0.5% (w/v) and the effect on respiration observed after FFA were bound.

The contribution of different IMM proteins to palmitate-stimulated uncoupling was assessed by adding specific inhibitors and measuring the effect on respiration rates following palmitate addition. Guanosine diphosphate (GDP), known to block UCP1 and UCP3 (Talbot et al., 2004; Esteves and Brand, 2005) was added to 1.0 mM (liver) or 1.5 mM (muscle). High extra-mitochondrial glutamate blocks GAT (Samartsev et al., 1997), and was added to 8 mM. Catr blocks ANT and was added to 0.0125 mM. Cyc A was added to 0.0017 mM (liver) and 0.0051 mM (muscle) to inhibit the opening of PTP. Cyc A and catr were also added in combination.

2.4. Statistical analysis

The effects of FFA and cold-acclimation on state 4 respiration rates were assessed using two-way ANOVA and Student–Newman–Keuls all-pairwise multiple comparison calculated by SigmaStat (SPSS Inc.). Respiration rates in the presence of inhibitors were expressed as a percentage of basal state 4 respiration prior to palmitate addition. The ability of these inhibitors to reduce palmitate-induced uncoupling, and the effect of cold-acclimation on this ability, was assessed using two-way ANOVA with Dunnett's multiple comparison with the palmitate treatment as control.

3. Results

Initial determinations of respiration rates in states 2, 3, and 4 were performed with no BSA added to the assay buffers so that the effects of FFA could be assessed. This resulted in relatively high states 2 and 4 rates and low respiratory control ratios (data not shown), compared with studies employing BSA, especially in liver. Nonetheless, addition of palmitate significantly uncoupled mitochondria (increased state 4 respiration) in both liver and muscle by approximately 50% (Fig. 1). In muscle, the effect of palmitate was completely abolished by the addition of BSA (Fig. 1B). The addition of BSA after palmitate to liver mitochondria significantly reduced respiration rate to levels approximately 30% below the initial basal state 4 determined in the absence of BSA (Fig. 1A). When respiratory control ratios were calculated from state 4 with BSA, they were comparable to those reported elsewhere for rat liver mitochondria (Rolfe et al., 1994). In neither tissue did cold-acclimation significantly affect state 4 respiration nor the uncoupling effect on palmitate (Fig. 1).

In Fig. 2, individual preparations were analyzed to illustrate the effects of various compounds on palmitate-induced uncoupling. This analysis shows that in liver mitochondria BSA reduced respiration below original state

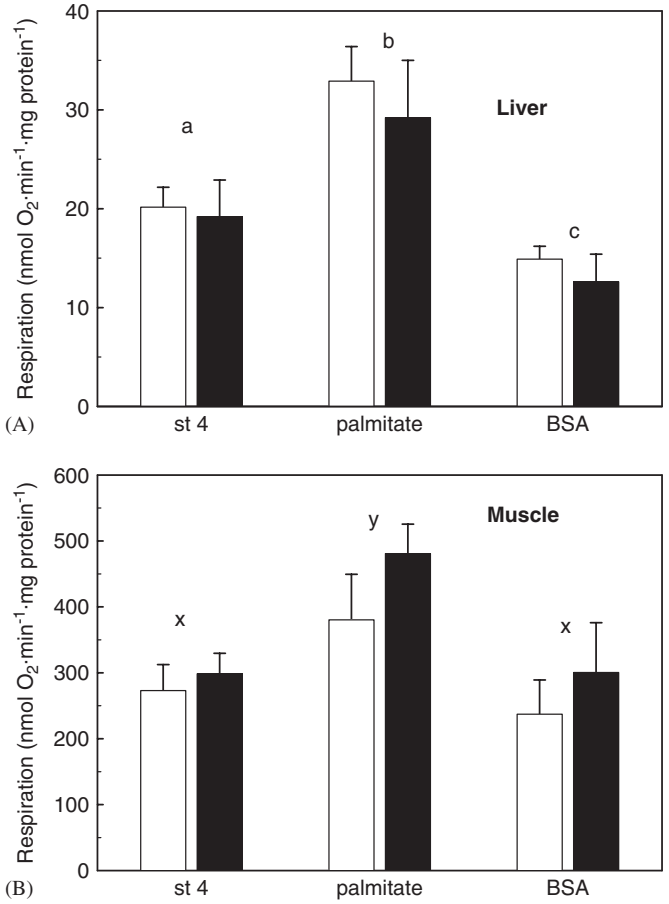

Fig. 1. Respiration of isolated liver (A) and gastrocnemius muscle (B) mitochondria from rats acclimated to 25 °C (white bars, $N = 6$) or 5 °C (black bars, $N = 5$). Values are mean ± SEM. Groups with different labels are significantly different from each other (two-way ANOVA). Respiration was measured with succinate as substrate in the presence of rotenone (see Section 2). The effect of FFA on respiration was determined by adding palmitate. BSA was then added to bind palmitate. In liver (A), palmitate significantly uncoupled mitochondria (increased state 4 respiration). BSA significantly reduced respiration rates below initial state 4 values, suggesting FFA contamination of the liver mitochondrial preparation. In muscle (B), palmitate-induced uncoupling was completely abolished by BSA addition with no further suppression of respiration. In neither tissue did cold-acclimation significantly change state 4 nor the effects of palmitate on it.

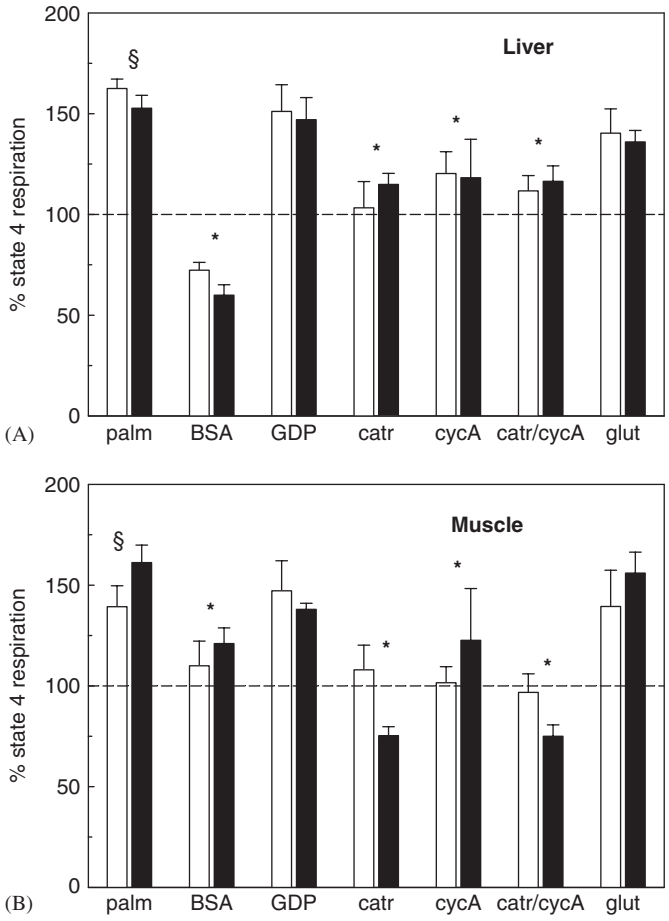

Fig. 2. Reversal of palmitate-induced uncoupling in liver (A) and muscle (B) mitochondria from rats acclimated to 25 °C (white bars) or 5 °C (black bars). Values are expressed as a percentage of state 4 respiration before addition of palmitate, and are mean ± SEM. Palmitate significantly increased respiration rate (indicated by §). Asterisks indicate significant difference from the palmitate treatment (two-way ANOVA with Dunnett's multiple comparison test).

4 values, but there was no significant difference between the control and cold-acclimated conditions. In liver mitochondria, catr and cycA, either alone or in combination, significantly decreased palmitate-induced uncoupling, but there was no significant effect of acclimation temperature. Neither GDP nor glutamate significantly reduced FFA-induced uncoupling.

In muscle mitochondria, BSA, catr, cycA, and the combination of cycA and catr all significantly decreased palmitate-induced uncoupling. There was no significant effect of cold-acclimation on any of these conditions. Again, neither GDP nor glutamate significantly affected respiration in the presence of palmitate.

4. Discussion

Cold-acclimation may reduce mitochondrial coupling and elevate state 4 respiration in bird liver (Goglia et al., 1993), but data from mammalian studies are equivocal. A few mammalian studies suggest increased uncoupling in liver and skeletal muscle (Hoch, 1992; Bravo et al., 2001), but most show no change in basal state 4 respiration (Himms-Hagen et al., 1976; Ballantyne and George, 1977; Zaninovich et al., 2003). In fact, a very recent study suggests that basal proton leak in rat liver may actually decrease after 15 days of cold exposure (Mollica et al., 2005). Our data agree with these latter studies, as there is no significant increase in state 4 respiration in either liver or gastrocnemius following cold-acclimation, at least when measured in the absence of added FFA.

Cold-acclimation increases FFA-induced uncoupling in bird skeletal muscle (Toyomizu et al., 2002). It has also been suggested that FFA uncoupling in extra BAT tissues contributes to NST in mammalian cold-acclimation because BSA significantly reduced uncoupling in muscle mitochondria isolated from cold-exposed fur seals, but not warm-acclimated animals (Grav and Blix, 1979). Our data do not support any increased sensitivity of mammalian mitochondria to uncoupling by FFA in cold-acclimation. Palmitate does significantly increase state 4 respiration in both liver and skeletal muscle mitochondria, but the magnitude of this uncoupling does not differ between animals acclimated to 25 or 5 °C (Fig. 1). BSA (0.5%) completely abolished the palmitate uncoupling in muscle and reduced respiration rate below state 4 in liver. This suggests that, despite efforts to remove lipid during isolation, there was some FFA contamination in the liver mitochondrial preparation. It seems likely, therefore, that the apparently increased FFA uncoupling in cold-acclimated fur seal muscle (Grav and Blix, 1979) may have been due to higher FFA levels in the mitochondrial preparation rather than increased mitochondrial sensitivity to FFA. A recent study showed no recoupling effect of 0.4% BSA in rat liver mitochondria, but basal rates were measured in the presence of 0.1% BSA, which would have bound most FFA (Mollica et al., 2005).

In rat liver, expression of a UCP2-like protein peaks after 15 days of exposure to 5 °C corresponding to an increase in state 4 respiration (Bravo et al., 2001). We found no increase in state 4 respiration over a similar time course of cold exposure (Fig. 1A). Furthermore, GDP did not significantly decrease liver state 4 respiration in the presence of palmitate, regardless of acclimation temperature (Fig. 2A), arguing against a significant role for UCP2 in cold-acclimation or FFA-induced uncoupling.

In skeletal muscle mitochondria, our data suggest no role for UCP homologues in uncoupling. GDP did not alter palmitate-stimulated state 4 respiration, regardless of acclimation temperature (Fig. 2B). In rats, UCP3 levels triple after 24 h of cold exposure, and this corresponds with an apparent increase in FFA-sensitive uncoupling (Simonyan et al., 2001). This effect may be short-lived, however, and not related to chronic cold-acclimation. After 1 day of cold exposure, UCP3 mRNA levels fall, and from 3 to 15 days they do not differ from thermoneutral controls (Larkin et al., 1997; Lin et al., 1998; Mollica et al., 2005). Moreover, proton leak is significantly reduced after 15 days

of cold exposure in rats (Mollica et al., 2005). UCP3 mRNA in skeletal muscle of hibernating ground squirrels is 3-fold higher than non-hibernating controls (Boyer et al., 1998), but there is no apparent uncoupling, at least in the absence of FFA (Barger et al., 2003). It would be interesting to assess the effect of FFA on extra-BAT uncoupling during arousal from hibernation when thermogenesis is dramatically increased.

Increases in ANT expression with cold-acclimation correspond with catr-sensitive FFA-induced uncoupling in birds (Roussel et al., 1998, 2000; Toyomizu et al., 2002). These studies inspired us to examine a potential role of ANT in rat cold-acclimation. Palmitate-stimulated uncoupling was significantly reduced by catr in both liver and skeletal muscle, but there was no significant effect of cold-acclimation (Fig. 2). Our results support other data from mice and rats showing no increase (Simonyan et al., 2001) or a significant decrease (Mollica et al., 2005) in the ability of catr to recouple FFA treated mitochondria after cold-acclimation, and no increase in tissue ANT content after cold-acclimation (Mollica et al., 2005).

The PTP may be opened directly by interaction with FFA (Wieckowski and Woytczak, 1998; Sultan and Sokolove, 2001), and it may play a role in fatty-acid-induced mitochondrial uncoupling (Brustovetsky et al., 1993; Vercesi et al., 1997; Bodrova et al., 2000; Sultan and Sokolove, 2001). Our data show that in both liver and muscle mitochondria cycA significantly reduced palmitate-induced uncoupling, but there was no effect of acclimation temperature (Fig. 2).

It has been suggested that some ANT molecules form core components of the PTP assembly within the IMM, and therefore PTP and ANT may function as two parts of a single mechanism (Wieckowski and Woytczak, 1998; Bodrova et al., 2000). To test this hypothesis, we compared the effects of catr and cycA when added separately and in combination. If these were acting on completely separate systems, one would predict that their combined effects would be additive. This is not the case, and the recoupling effect of the combination of the two inhibitors closely resembled their individual effects (Fig. 2), even in liver mitochondria where BSA reduced respiration below basal state 4 levels. This suggests that ANT and PTP function as part of the same system, at least in regard to FFA-induced uncoupling.

A recent study found that glutamate significantly reversed the ability of palmitate to decrease membrane potential in rat liver and skeletal muscle mitochondria, suggesting an uncoupling role for GAT (Mollica et al., 2005). Our data showed that glutamate did not reverse the uncoupling effects of palmitate on liver or muscle mitochondrial respiration (Fig. 2). Taken together, the results of these two studies suggest that subtle changes in proton leak kinetics may alter membrane potential, but not significantly affect respiration rates.

Our data does not support the hypothesis that cold-acclimation in eutherian mammals results in a change in mitochondrial quality with regard to FFA-induced uncoupling. It should be noted, however, that in rats, total liver mitochondrial content doubles after 4–5 weeks of cold exposure (Horwitz, 1976). Oxygen consumption of hepatocytes isolated during the summer from cold-acclimated (but not hibernating) ground squirrels (*Spermophilus lateralis*) is significantly higher than thermoneutral controls (Staples and Hochachka, 1998). After 4 weeks of cold exposure in rats, the mass of tibialis muscle significantly increases, while mass-specific citrate synthase activity remains constant (Deveci and Egginton, 2002). So, although the sensitivity of individual mitochondria to FFA may not be altered by cold-acclimation, an increase in total mitochondria would augment thermogenic capacities from FFA-induced uncoupling. In addition, cold exposure induces an increase in circulating FFA (Heldmaier and Seidl, 1985) that could further increase uncoupling in these tissues. We suggest that a better model for studying potential mitochondrial mechanisms of NST might be marsupial mammals. Although there is evidence for UCP2 and UCP3 expression in marsupials (Kabat et al., 2004), they are not known to have BAT or express UCP1 (Rose et al., 1999). Despite this, marsupials do exhibit NST (Rose et al., 1999), and 3–5 weeks of cold exposure increased the expression of a homologue to eutherian UCP3 in skeletal muscle (Schaeffer et al., 2005), a similar pattern to that observed in chickens (Raimbault et al., 2001).

In summary, this study found that cold-acclimation did not alter state 4 respiration in rat liver or skeletal muscle mitochondria. The addition of palmitate significantly uncoupled mitochondria (increased state 4 respiration), but the magnitude of this uncoupling, or the contribution of different IMM transporters to uncoupling, was not altered by cold-acclimation. Neither GDP nor glutamate reduced this uncoupling in skeletal muscle or liver, suggesting that UCP homologues and GAT do not contribute to FFA-induced uncoupling or cold-acclimation. The ANT and/or PTP appear to contribute significantly to FFA-induced uncoupling. Evidence suggests that these two elements may be working together to mediate FFA-induced uncoupling.

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