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Uncoupling protein 3 protects aconitase against inactivation in isolated skeletal muscle mitochondria

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Abstract

Mitochondrial uncoupling proteins only catalyse proton transport when they are activated. Activators include superoxide and reactive alkenals, suggesting new physiological functions for UCP2 and UCP3: their activation by superoxide when protonmotive force is high causes mild uncoupling, which lowers protonmotive force and attenuates superoxide generation by the electron transport chain. This feedback loop acts to prevent excessive mitochondrial superoxide production. Superoxide inactivates aconitase in the mitochondrial matrix, so aconitase activity provides a sensitive measure of the effects of UCPs on matrix superoxide. We find that inhibition of UCP3 in isolated skeletal muscle mitochondria by GDP decreases aconitase activity by 25% after 20 min incubation. The GDP effect is absent in skeletal muscle mitochondria from UCP3 knockout mice, showing that it is mediated by UCP3. Protection of aconitase by UCP3 in the absence of nucleotides does not require added fatty acids. The purine nucleoside diphosphates and triphosphates cause aconitase inactivation, but the monophosphates and CDP do not, consistent with the known nucleotide specificity of UCP3. The IC_{50} for GDP is about 100 μM. These findings support the proposal that UCP3 attenuates endogenous radical production by the mitochondrial electron transport chain at high protonmotive force.

Keywords: Aconitase; UCP3; Superoxide; Reactive oxygen species; Mitochondria

1. Introduction

Mitochondria are the major cellular site of single electron reduction of oxygen to form the reactive and toxic oxygen radicals, superoxide and its protonated form, hydroperoxyl radical. These in turn generate other species, including hydroxyl radicals. Hydroperoxyl and hydroxyl radicals can attack membrane phospholipids and produce reactive alkenals such as hydroxynonenal. The uncoupling proteins (UCPs) are a family of mitochondrial inner membrane proteins that are part of a larger family of mitochondrial transporters [1]. UCPs catalyse proton transport across the mitochondrial inner membrane, uncoupling the oxidation of reduced substrates from the phosphorylation of ADP to ATP. However, they are tightly regulated to prevent unnecessary loss of energy, and in the presence of physiological concentrations of nucleotides, they only uncouple in the presence of appropriate activators [2–5]. The physiological activator of UCP1 has long been thought to be free fatty acids, and the physiological function of UCP1 is primarily adaptive thermogenesis [6,7]. Recently, we discovered that superoxide [8] and hydroxynonenal [9] are potent activators of UCP1, UCP2 and UCP3, as well as plant [10] and avian UCPs [11].

This discovery led to the proposal that a major physiological function of UCP2 and UCP3 is to attenuate superoxide production by the electron transport chain, and so protect against oxidative damage [2–4,9,12]. Specifically, superoxide/hydroperoxyl radical would lead to the generation of carbon-centred radicals on the fatty acid side chains of membrane phospholipids. Peroxidation of these radicals would lead to the production of reactive alkenals that would activate the proton conductance of the UCPs, leading to mild uncoupling. The consequent decrease in protonmotive force (and local oxygen tension [13,14])

Abbreviations: UCP, uncoupling protein; BSA, bovine serum albumin

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would attenuate superoxide production, completing a protective negative feedback loop.

There is good evidence in isolated mitochondria for each of the steps in the alkenal feedback model. Superoxide and products of the oxidation of membrane phospholipids, such as hydroxynonenal, activate the proton conductance of the UCPs, causing mild uncoupling. Proton conductance can be activated by generating carbon-centred radicals, and prevented by quenching such radicals [12]. Mild uncoupling strongly decreases superoxide production [13,15–19]. These observations are all consistent with a role of the UCPs in keeping superoxide production low by causing mild uncoupling.

There is also much evidence that is consistent with the alkenal feedback model in vivo. Mitochondria isolated from UCP3 knockout mice have higher oxidative damage than those from controls: they have decreased aconitase activity [20] and increased markers of oxidative damage [21]. Similarly, UCP2 knockout mice are resistant to infection by the intracellular parasite *Toxoplasma gondii* through a mechanism that may involve increased reactive oxygen species production by macrophages [22], and their pancreatic islet cells have increased production of reactive oxygen species [23].

Importantly, endogenous radical production in mitochondria appears to be able to activate the protective function of UCPs. Inhibition of UCPs by GDP increases both protonmotive force and the mitochondrial production of reactive oxygen species [24,25]. Inhibition of UCP3 causes an increase in superoxide, and a greater proton conductance of the mitochondrial inner membrane [26].

To assess more fully the role UCP3 plays in maintaining a low matrix superoxide concentration, we have used aconitase as a sensitive target of superoxide [27]. The catalytic function of this citric acid cycle enzyme is dependent upon a cubane $[4Fe–4S]^{2+}$ cluster, in which only three of the four irons are ligated to cysteine residues. The fourth iron is exposed to the solvent and open to attack from superoxide, which causes a one-electron oxidation of the iron-sulphur cluster, releasing the exposed iron in the ferrous state and inactivating the enzyme. Superoxide is not the only oxidant that can inactivate aconitase, but the inactivation is reasonably specific since the second-order rate constant for the reaction of superoxide with substrate-free aconitase is approximately 10^4, 10^5 and 10^2 times greater than those for hydrogen peroxide, oxygen and peroxynitrite [27]. The inactivation of aconitase by superoxide can be rapidly reversed both in vivo and in vitro [28,29]. In cultured mammalian cells, conditions that increase superoxide production inactivate aconitase, but the enzyme is reactivated upon removal of the superoxide source [30]. Thus, the level of aconitase activity within mitochondria will be determined by the relative rates of inactivation by superoxide or other oxidants, and the rate of reactivation by reduction and restoration of iron to the cluster. At any given flux of superoxide, the dynamic inactivation and reactivation of aconitase will reach a steady state in which the fraction of active enzyme will reflect the concentration of superoxide in the mitochondrial matrix [31]. We have exploited this property to investigate the effects of UCP3 inhibition and ablation on matrix superoxide. The results provide strong support for the protective feedback loop function of UCP3.

2. Materials and methods

2.1. Isolation of mitochondria

Skeletal muscle and liver mitochondria were isolated at 4 °C as previously described [32,33]. Female Wistar rats (6–8 weeks old), *UCP3*$^{-/-}$ mice (from Michael Cawthorne and Steven Wang, University of Buckingham, UK) or control wild type mice were stunned and killed by cervical dislocation. Skeletal muscle from the hind limbs was dissected (from one rat or four mice) and placed in ice-cold CP-1 medium (containing 100 mM KCl, 50 mM Tris/HCl and 2 mM EGTA, pH 7.4 at 4 °C). Muscle was shredded with a razor blade, minced with scissors, rinsed with CP-1 medium, stirred for 4 min in CP-2 medium (CP-1 plus 1 mM ATP, 5 mM $MgCl_2$, 0.5% (w/v) BSA, 2.1 U ml^{-1} protease (subtilisin), pH 7.4 at 4 °C) and homogenised in CP-2 medium using a polytron tissue homogeniser. The homogenate was stirred in CP-2 for 6 min, then mitochondria were isolated using differential centrifugation and resuspended in CP-1 medium. Liver from rats was rapidly excised and immersed in ice-cold STE isolation buffer (containing 250 mM sucrose, 5 mM Tris/HCl and 2 mM EGTA, pH 7.4 at 4 °C). The tissue was chopped and homogenised in a Teflon/glass homogeniser, then mitochondria were separated by differential centrifugation and resuspended in STE. Protein concentration was determined using the biuret method [34].

2.2. Mitochondrial aconitase activity

Mitochondrial aconitase activity was measured by following the appearance of NADPH at 340 nm [27] in a stirred glass cuvette open to the air. Mitochondria from skeletal muscle (0.1 mg protein ml^{-1}) or liver (0.5 mg protein ml^{-1}) were incubated for 20 min at 37 °C in 3.5 ml assay buffer (120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.4 mM NADP, 2 U isocitrate dehydrogenase and 5 mM citrate, pH 7.2) containing 5 μM rotenone and 4 mM succinate. After 20 min, superoxide dismutase (45 U) was added to remove any superoxide and the reaction was started by the addition of 0.12% (v/v) Triton-X 100 to allow access of the added reagents to the matrix aconitase. Where indicated, nucleotides were added at 500 μM unless stated otherwise and BSA was added at 0.3% (w/v). Where indicated, exogenous superoxide was generated using xanthine (50 μM) and xanthine oxidase

(3 mU per ml). Stocks of xanthine at 0.35 mM and xanthine oxidase at 2 U ml^{-1} were prepared in assay medium.

2.3. Calculation of free GDP concentration

The free concentration of GDP in media containing $MgCl_2$ was calculated as the average of the values given by three different computer programmes (EqCal from Biosoft, Sliders from Max Chelator [35] and Bound and Determined [36]). The apparent stability constant for Mg-GDP binding was taken from [37].

2.4. Statistical analysis

Values are means ± S.E.M. Significance was tested in Microsoft Excel by unpaired Student's *t*-test or, for multiple samples, one-way ANOVA, with $P < 0.05$ considered significant.

3. Results

3.1. Inactivation of aconitase by GDP in skeletal muscle mitochondria

If UCP3 limits production of superoxide from the mitochondrial electron transport chain, then inhibition of UCP3 activity should lead to higher levels of matrix superoxide and greater inactivation of matrix aconitase.

Fig. 1 shows a typical example of the spectrophotometric determination of aconitase activity in isolated skeletal muscle mitochondria, measured as the appearance of absorbance due to NADPH. Mitochondria were incubated in the presence of succinate for 20 min under different conditions, to allow endogenous superoxide or other radical production to exert any effects on aconitase. At 20 min, they were lysed by adding triton-X 100 and the activity of matrix aconitase was assayed between 22 and 25 min. The control trace shows that there was little NADPH formation during the 20-min preincubation, but rapid formation after lysis, due to the exposed matrix aconitase activity.

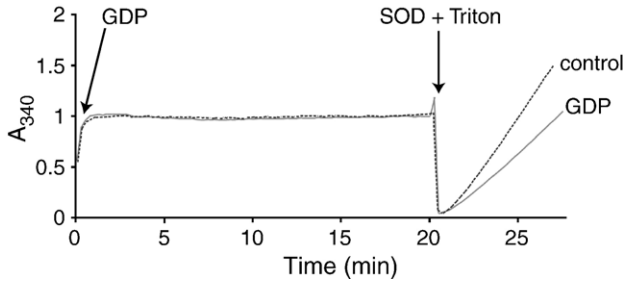

Fig. 1. Measurement of aconitase activity. Rat skeletal muscle mitochondria were incubated at 0.1 mg protein/ml in assay buffer (120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.4 mM NADP, 2 U isocitrate dehydrogenase and 5 mM citrate, pH 7.2) containing 5 μM rotenone and 4 mM succinate at 37 °C, in the absence (dotted line) or presence (solid line) of 500 μM GDP. At 20 min, superoxide dismutase (SOD, 45 U) was added to remove any superoxide and the assay of aconitase was started by adding 0.12% (v/v) Triton-X 100. Aconitase activity was measured as the rate of NADPH production, calculated from the rate of change of A_{340} between 22 min and 25 min. Representative traces are shown. In subsequent figures, the value for each condition in each experiment was the average of 2–4 runs carried out as shown here.

When 500 μM GDP was added at 0 min to inhibit UCP3 throughout the 20-min incubation, there was a lower rate of NADPH formation after lysis at 20 min (Fig. 1), indicating lower aconitase activity and therefore higher aconitase inactivation. This was not due to any direct inhibitory effect of GDP in the assay, as GDP had no effect if the mitochondria were lysed at 0 min and the aconitase was assayed immediately (not shown).

The experiment was also performed in skeletal muscle mitochondria in the absence of the complex I inhibitor, rotenone, to increase matrix superoxide production by reverse electron flow into complex I [19]. As expected, this resulted in a lower aconitase activity at 20 min than in the presence of rotenone, and this low activity was decreased still further by the addition of GDP at 0 min (not shown). However, the relative magnitude of the GDP effect was greatest when rotenone was present, so subsequent experiments used skeletal muscle mitochondria in the presence of rotenone as shown in Fig. 1.

Fig. 2a shows the averaged results of 16 experiments: inhibition of UCP3 by GDP during the 20-min incubation period resulted in a significant 25% reduction in the activity of aconitase compared to paired controls with GDP absent.

3.2. Effect of BSA on aconitase damage

It is well established that the proton conductance of UCP1 is stimulated by free fatty acids. The activation of UCP2 and UCP3 by exogenous superoxide also requires the presence of fatty acids [8]. To test whether the protection of aconitase by UCP3 requires free fatty acids, the 20-min incubation of skeletal muscle mitochondria was repeated in the presence of BSA. Fig. 2b shows that aconitase activity was unaffected by inclusion of BSA in the incubation, and that inhibition of UCP3 by GDP still decreased aconitase activity under these conditions, illustrating that removal of free fatty acids had no effect on the ability of UCP3 to protect aconitase in the mitochondrial matrix. Thus, fatty acids are required for activation of the proton conductance of UCPs by exogenous superoxide [8], but not for UCP3 to protect aconitase against endogenously produced superoxide or other reactive species (Fig. 2b).

3.3. Nucleotide specificity of aconitase inactivation

Purine nucleoside di- and triphosphates (GDP, GTP, ADP and ATP) are good inhibitors of UCPs, whereas purine nucleoside monophosphates and pyrimidine nucleotides are not [8]. Nucleotide specificity therefore provides a useful tool for mapping a measured behaviour to the UCPs. By

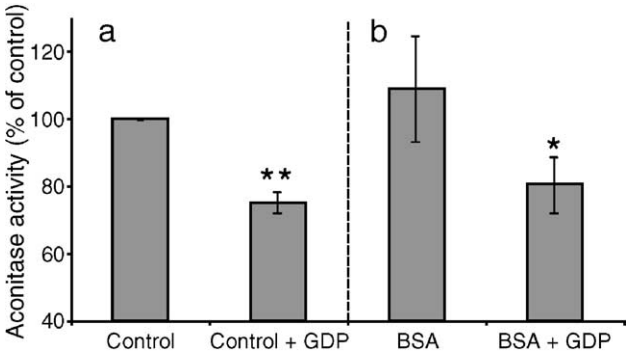

Fig. 2. Effect of GDP on aconitase activity in skeletal muscle mitochondria. Aconitase activity was measured in rat skeletal muscle mitochondria after 20 min incubation in the absence and presence of 500 μM GDP, (a) in the absence and (b) in the presence of 0.3% (w/v) BSA. Aconitase activity in the control without BSA was 900±60 mU per mg mitochondrial protein. Results (expressed as percent of control in the same preparation) are means±S.E.M. of 16 (a) and 3 (b) independent experiments. *$P<0.05$ and **$P<0.01$ by paired Student's *t*-test compared to control (a) or BSA (b) without GDP.

replacing GDP with other nucleotides, we found that the pattern of aconitase inhibition fitted well with the known specificity of nucleotide effects on UCPs (Fig. 3). Incubation of skeletal muscle mitochondria with 500 μM GDP, GTP, ADP or ATP resulted in significant aconitase inactivation relative to the no-nucleotide control. However, 500 μM GMP, CMP, CDP or UMP had no effect, consistent with the contention that the effect of the nucleotides on matrix aconitase activity was caused by their inhibition of UCP3.

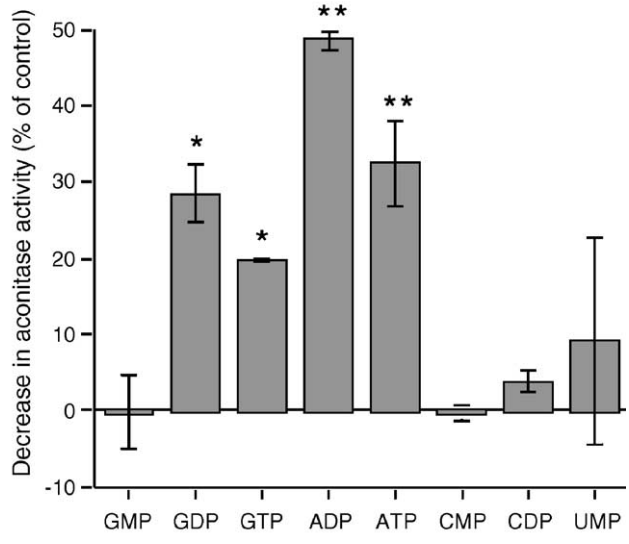

Fig. 3. Nucleotide specificity of aconitase inactivation in skeletal muscle mitochondria. Aconitase activity was measured in rat skeletal muscle mitochondria after 20 min incubation in the presence of different nucleotides at 500 μM. Results (expressed as percent decrease in activity compared to control without nucleotide in the same preparation) are means±S.E.M. of 3–4 independent experiments. *$P<0.05$ and **$P<0.01$ by ANOVA compared to control without nucleotide.

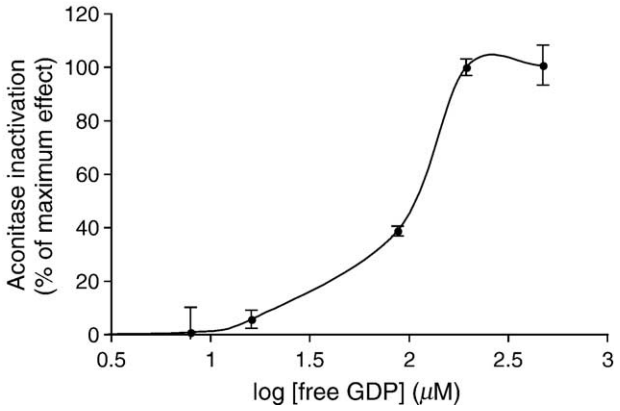

Fig. 4. Concentration-dependence of the inactivation of aconitase by GDP in skeletal muscle mitochondria. Aconitase activity was measured in rat skeletal muscle mitochondria after 20 min incubation in the presence of different concentrations of free GDP. Due to Mg^{2+} binding, free GDP concentrations were calculated using three different computer programmes and the average value was taken. Results (expressed as percent of the inhibition given by 500 μM GDP in the same preparation) are means±S.E.M. of 3–6 independent experiments. The concentration of free GDP that gave half of the maximum effect (IC_{50}) was interpolated from the curve (fitted by the smoothing function of Microsoft Excel) to be about 100 μM.

3.4. Concentration-dependence of the inactivation of aconitase by GDP

The free GDP concentration that caused half-maximal inactivation of aconitase was determined. Fig. 4 shows that inactivation of aconitase by free GDP was saturable, with an IC_{50} for free GDP of about 100 μM.

3.5. Absence of GDP-induced aconitase inactivation in mitochondria from tissues lacking UCP

Liver mitochondria normally lack both UCP protein [38] and GDP-sensitive, superoxide-stimulated proton conductance, indicating the absence of functional UCPs [8]. Fig. 5

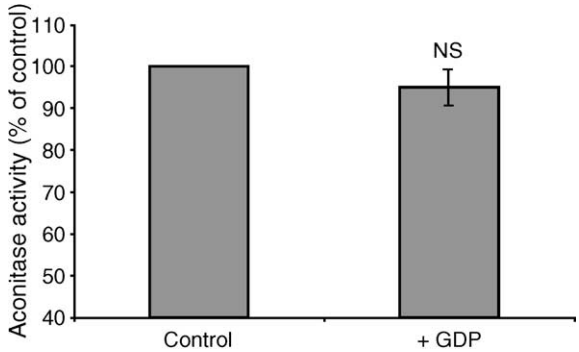

Fig. 5. Effect of GDP on aconitase activity in liver mitochondria. Aconitase activity was measured in rat liver mitochondria (0.5 mg protein/ml) after 20 min incubation in the presence and absence of 500 μM GDP. Aconitase activity in the control was 83±5 mU per mg mitochondrial protein. Results (expressed as percent of control using the same preparation) are means ± S.E.M. of 3 independent experiments. NS, not significantly different by paired Student's *t*-test compared to no GDP.

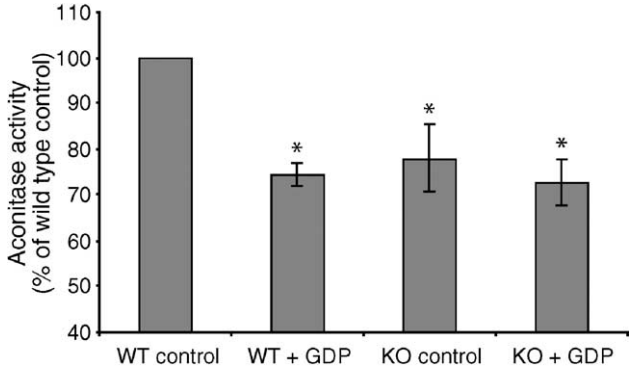

Fig. 6. Effect of GDP on aconitase activity in skeletal muscle mitochondria from wild type and UCP3 knockout mice. Aconitase activity was measured in mouse skeletal muscle mitochondria from wild type (WT) or *UCP3*$^{-/-}$ (KO) mice after 20 min incubation in the presence and absence of 500 μM GDP. Aconitase activity in the wild type control was 400±60 mU per mg mitochondrial protein. Results (expressed as percent of paired wild type control without GDP) are means±S.E.M. of 4 independent paired experiments. *$P<0.05$ by ANOVA compared to wild type control, but not significantly different from each other.

shows that GDP had no effect on aconitase activity in rat liver mitochondria.

A more stringent test of UCP3 involvement was provided by experiments using skeletal muscle mitochondria from *UCP3*$^{-/-}$ mice. Fig. 6 shows that GDP addition led to inactivation of aconitase in skeletal muscle mitochondria from wild type mice, to about the same extent as it did in mitochondria from rats (Fig. 2). However, the aconitase activity of mitochondria from UCP3 knockout mice was lower than controls even in the absence of GDP, and GDP had no further inactivatory effect (Fig. 6), demonstrating that the ability of GDP to cause inactivation of aconitase depended on the presence of UCP3 in the mitochondrial membrane.

4. Discussion

The thermogenic function of UCP1 is well-established [6,7]. The functions of UCP2 and UCP3 are more uncertain, although regulation of the production of reactive oxygen species by the electron transport chain (to prevent damage and as a metabolic signal) is currently the most attractive of the several candidates that have been put forward [4]. In this model [2,3,9,12], at high protonmotive force, the electron transport chain produces superoxide and other radicals that activate the proton conductance of UCPs, leading to mild uncoupling that lowers the protonmotive force and attenuates radical production. There is considerable evidence for the individual steps of this model in isolated mitochondria [2–4,26]. There is supporting evidence from *UCP3*$^{-/-}$ mice, which yield muscle mitochondria with lower aconitase activity [20] and greater oxidative damage [21] than controls. In the present study, we directly address the ability of UCP3 to protect matrix aconitase against the inactivatory effects of endogenous reactive oxygen species under well-controlled conditions. The results show that UCP3 can function in isolated mitochondria to protect matrix aconitase against endogenous radicals (presumably superoxide) produced by the electron transport chain, as predicted by the functional model described above. In contrast, there is little unambiguous experimental support for other functional models of UCP3 that propose its involvement in fatty acid export [4], and the standard versions of such models would have difficulty in explaining the inhibition of aconitase observed in the present study.

Unlike the proton transporting mechanism of UCP1 in brown adipose tissue mitochondria [6] and the activation of UCPs by externally generated superoxide [8], protection of aconitase by UCP3 is not dependent on the presence of BSA or the addition of free fatty acids (Fig. 2). While there is a general assumption that all UCP-related activity requires fatty acids, several studies have recently suggested that this is not the case. Activation of the proton conductance of UCP2 in mitochondria, either directly by external hydroxynonenal [9] or by quinone derivatives under conditions in which they cause radical production in the matrix [38], occurs in the presence of BSA without added fatty acids. Similarly, the activation of UCP3 by endogenously produced superoxide in muscle mitochondria does not require free fatty acids [26]. Therefore, the data presented here are consistent with the observations that activation of UCP2 and UCP3-mediated proton conductance by alkenals or superoxide in the mitochondrial matrix does not require added free fatty acids. In this respect UCP2 and UCP3 appear to differ from UCP1. It may be that the proton conductance catalysed by UCP2 and UCP3 has no requirement for fatty acids, that the endogenous levels of fatty acids in isolated mitochondria are high enough, even in the presence of BSA, to cause full activation, or that fatty acids are not in fact activators, but merely displace inhibitory nucleotides, as they may do with UCP1 [39,40].

Our observation that only purine nucleoside diphosphates and triphosphates inhibit UCP3 and cause aconitase inactivation is consistent with the known specificity with which nucleotides inhibit UCPs [8,41]. However, the IC_{50} was higher than expected from studies of GDP binding to reconstituted UCP2 or UCP3 [41,42] or GDP inhibition of the proton conductance of UCPs [8,43]. There could be several reasons for this, including a non-linear relationship between GDP inhibition of UCP3 and the eventual increase in matrix superoxide production, or a decrease in the affinity for inhibitory nucleotides caused by endogenous fatty acids.

We conclude that UCP3 can function in intact isolated mitochondria under defined conditions to protect matrix aconitase from inactivation by superoxide or other radical-derived species produced endogenously by the electron transport chain. This observation supports models suggesting that UCP3 normally functions to attenuate mitochondrial radical production by causing mild uncoupling, and so protects skeletal muscle against potentially damaging

mitochondrial superoxide production when substrate and oxygen concentrations are high.

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