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Correspondence and requests for materials should be addressed to D.R. (e-mail: ron@saturn.med.nyu.edu).

Superoxide activates mitochondrial uncoupling proteins

Karim S. Echtay*, Damien Roussel*, Julie St-Pierre*, Mika B. Jekabsons*, Susana Cadenas*, Jeff A. Stuart*, James A. Harper*, Stephen J. Roebuck*, Alastair Morrison†, Susan Pickering†, John C. Clapham‡ & Martin D. Brand*

** Medical Research Council Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 2XY, UK*
Departments of †Comparative Genomics, and ‡ Vascular Biology, GlaxoSmithKline, Harlow, Essex CM19 5AW, UK

Uncoupling protein 1 (UCP1) diverts energy from ATP synthesis to thermogenesis in the mitochondria of brown adipose tissue by catalysing a regulated leak of protons across the inner membrane[1,2]. The functions of its homologues, UCP2 and UCP3, in other tissues are debated[3,4]. UCP2 and UCP3 are present at much lower abundance than UCP1, and the uncoupling with which they are associated is not significantly thermogenic[5,6]. Mild uncoupling would, however, decrease the mitochondrial production of reactive oxygen species, which are important mediators of oxidative damage[7,8]. Here we show that superoxide increases mitochondrial proton conductance through effects on UCP1, UCP2 and UCP3. Superoxide-induced uncoupling requires fatty acids and is inhibited by purine nucleotides. It correlates with the tissue expression of UCPs, appears in mitochondria from yeast expressing UCP1, and is absent in skeletal muscle mitochondria from UCP3 knockout mice. Our findings indicate that the interaction of superoxide with UCPs may be a mechanism for decreasing the concentrations of reactive oxygen species inside mitochondria.

As coenzyme Q (CoQ) has been identified as a regulatory cofactor for proton transport by UCP1 (ref. 9), UCP2 and UCP3 (ref. 10) in liposomes, we tested the effect of CoQ in isolated mitochondria. CoQ increased proton conductance in rat kidney (but not liver) mitochondria that were oxidizing succinate. This increase required fatty acids and was prevented by GDP. CoQ activated proton conductance only when it was likely to be reduced to $CoQH_2$. Activation was abolished by superoxide dismutase, indicating that CoQ might mediate uncoupling through the production of superoxide[11]. To explore this possibility, we replaced CoQ by xanthine plus xanthine oxidase—an exogenous system that generates superoxide. Proton conductance increased, indicating that CoQ acted in mitochondria through the production of superoxide.

Incubating rat skeletal muscle mitochondria with xanthine plus xanthine oxidase to generate superoxide increased proton conductance (Fig. 1a). This is seen as an increased rate of proton leak at each membrane potential, resulting in a curve that is deflected upwards. This increase was fully inhibited either by superoxide dismutase, indicating that it was dependent on superoxide, or by 500 μM GDP (Fig. 1a). GDP had no effect on control mitochondria (data not shown), confirming previous results[12].

The superoxide effect was abolished by bovine serum albumin (BSA), which binds fatty acids (Fig. 1b), but restored by adding palmitic acid in the presence of BSA (Fig. 1c), indicating that activation by superoxide requires fatty acids. Proton conductance that is activated by fatty acids and sensitive to GDP is characteristic of uncoupling by UCP1 in brown adipose tissue (BAT) mitochondria[1,2], suggesting that the uncoupling caused by superoxide in skeletal muscle mitochondria (which lack UCP1 and -2 (ref. 13) but contain UCP3) was mediated by UCP3.

We also investigated skeletal muscle mitochondria from starved rats. Starvation for 24 h doubles the concentration of UCP3 protein without affecting the basal level of proton conductance[12]. Superoxide stimulated proton conductance twice as strongly in mitochondria from starved rats (Fig. 1d) as in mitochondria from fed rats (Fig. 1a). The same was true in the presence of BSA plus palmitate (data not shown). This correlates with a near doubling of UCP3 protein in starved rats, which was confirmed by western blot (data not shown), and implicates UCP3 in the superoxide effect.

Confirmation of the role of UCP3 was obtained using skeletal muscle mitochondria isolated from UCP3 knockout mice, which had the same basal proton conductance as the controls. Muscle mitochondria from wild-type mice showed the same GDP-sensitive stimulation of proton conductance as those from rats (Fig. 1e) and the same dependence on fatty acids (data not shown). However, superoxide had no effect on mitochondria from the skeletal muscle of UCP3 knockout mice (Fig. 1f), showing that superoxide uncoupled wild-type mitochondria by interacting with UCP3. We verified that the lack of effect of xanthine plus xanthine oxidase in UCP3 knockouts was not caused by lack of superoxide by a direct assay of superoxide production using superoxide dismutase and a homovanillic acid/horseradish peroxidase fluorescence assay[14] (data not shown). Thus, the fatty-acid-dependent, GDP-sensitive increase in proton conductance caused by xanthine plus xanthine oxidase

was caused by an effect of superoxide acting through UCP3.

We investigated whether UCP2 was also activated by superoxide. Because UCP2 is expressed widely[4,13], we checked the superoxide effect on basal proton conductance in mitochondria from several tissues that do not express UCP3 (ref. 4). Superoxide increased proton conductance in mitochondria from rat kidney in a GDP-sensitive manner (Fig. 2a). This activation required fatty acids (Fig. 2b, c). But in liver and heart mitochondria, which lack UCP2 (ref. 13), basal proton leak was not stimulated by superoxide (Fig. 2d, e). Mitochondria from spleen[13] and pancreatic β cells[15] do contain UCP2, and superoxide increased the proton conductance in the absence, but not the presence, of GDP in these mitochondria (Fig. 2f, g). These results indicate that superoxide probably acts through UCP2 in mitochondria from the kidney, spleen and pancreatic β cells.

To determine whether UCP1 is also activated by superoxide, we examined BAT mitochondria from warm-adapted rats. These mitochondria contain relatively low amounts of UCP1, which allows coupling to be measured in the absence of GDP. BAT mitochondria had low endogenous proton conductance through UCP1, which was reduced to the basal level by adding GDP (Fig. 3a). The endogenous UCP1 activity was stimulated by superoxide, and this stimulation was prevented by superoxide dismutase. GDP abolished both the endogenous activity and the superoxide-stimulated activity, returning proton conductance to the basal level.

Thus, BAT mitochondria showed the same response to superoxide as skeletal muscle and kidney mitochondria, but it was superimposed on the normal GDP-sensitive uncoupling mediated by UCP1. Unlike the effects in other tissues, superoxide was able to uncouple BAT mitochondria even in the presence of BSA, indicating either that UCP1 has a higher affinity for fatty acids than UCP2 and -3, or that BAT mitochondria have more contaminating free fatty acids than other tissues. These results suggest that UCP1 also facilitates superoxide uncoupling; however, BAT mitochondria may also contain both UCP2 and -3 (ref. 4), which complicates the interpretation.

To establish whether UCP1 was responsible for the superoxide effects in BAT mitochondria, we expressed mouse UCP1 in the yeast *Saccharomyces cerevisiae*, which has been previously shown to provide a convenient model for studying UCP1 function[16–18]. Mitochondria from control yeast hardly responded to superoxide or GDP (Fig. 3b); in contrast, mitochondria from yeast expressing modest concentrations of UCP1 (~1 μg per mg protein)[18] had greater proton conductance than control yeast mitochondria, and this extra proton conductance was completely sensitive to GDP

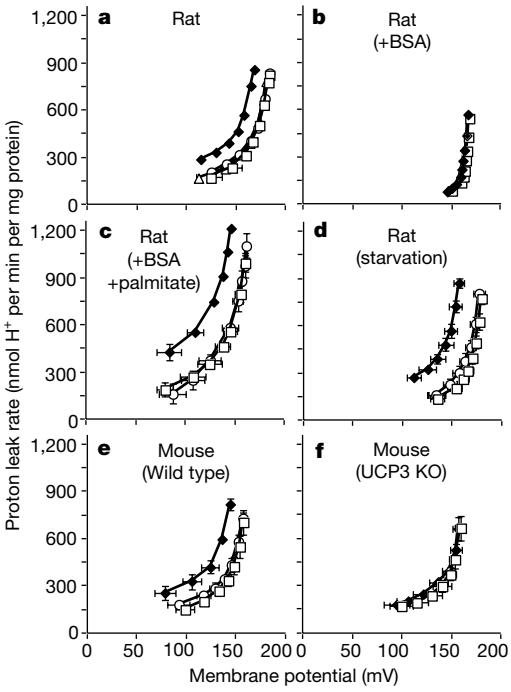

Figure 1 Effect of superoxide on the proton conductance of skeletal muscle mitochondria: superoxide activation of UCP3. Graphs show rate of proton leak as a function of its driving force (the mitochondrial membrane potential) as the potential was varied by titration with succinate. Open squares, control; filled diamonds, 50 μM xanthine plus xanthine oxidase (0.01 U per 3.5 ml) added before $TPMP^+$; open circles, xanthine plus xanthine oxidase and 500 μM GDP added before $TPMP^+$; open triangles, xanthine plus xanthine oxidase and 12 U ml^{-1} superoxide dismutase added before $TPMP^+$. Mitochondria were from control rats fed *ad libitum* (**a**); control rats, in medium supplemented with 0.3% defatted BSA (**b**); control rats, in medium with 0.3% BSA and 300 μM palmitic acid (**c**); rats starved for 24 h (**d**); wild-type mice (**e**); and littermate homozygous UCP3 knockout mice (**f**). Data are the mean ± s.e.m. of three independent experiments each performed in duplicate.

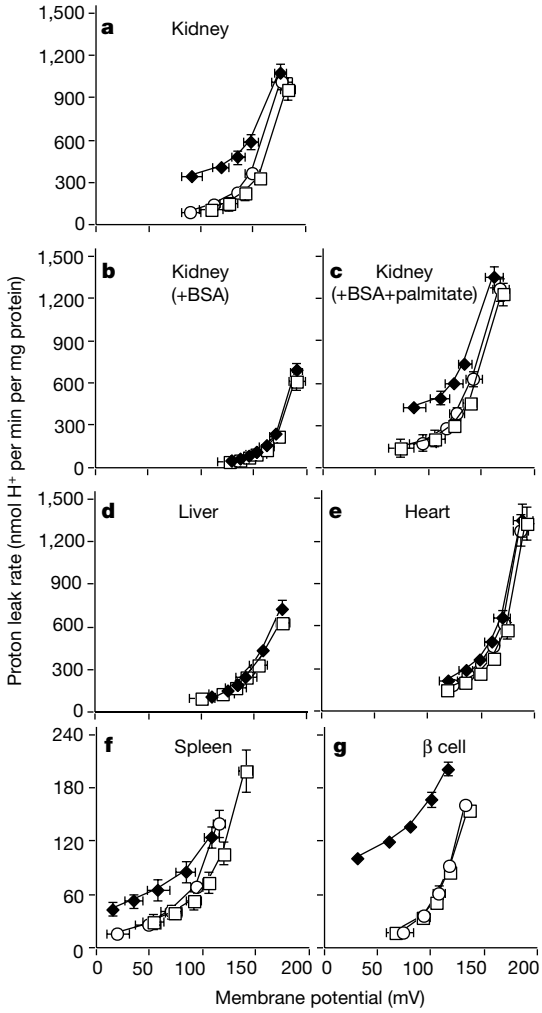

Figure 2 Effect of superoxide on the proton conductance of mitochondria from different tissues: superoxide activation of UCP2. The potential was varied by adding cyanide (up to 100 μM) using 4 mM succinate as the substrate. Open squares, control; filled diamonds, 50 μM xanthine plus xanthine oxidase (0.01 U per 3.5 ml) added before $TPMP^+$; open circles, xanthine plus xanthine oxidase and 500 μM GDP added before $TPMP^+$. Mitochondria were from rat kidney (**a**); rat kidney, in medium with 0.3% BSA (**b**); rat kidney, in medium with 0.3% BSA and 300 μM palmitic acid (**c**); rat liver (**d**); rat heart (**e**); rat spleen (**f**); mouse min6 pancreatic β cells (**g**). In **a**–**f**, data are the mean ± s.e.m. of three independent experiments each performed in duplicate; in **g**, data are the mean ± range of duplicate experiments using a single preparation.

(Fig. 3c). Thus, mouse UCP1 showed native uncoupling behaviour in yeast at these levels of expression. Superoxide stimulated the proton conductance and GDP returned it to the basal level (Fig. 3c). These results clearly show that superoxide increases GDP-sensitive proton conductance through UCP1.

UCP1 accepts many nucleotides but has a strong preference for purine nucleoside diphosphates and triphosphates[2]. We determined the nucleotide specificity and binding affinity of the inducible proton conductance catalysed by UCP2 (kidney mitochondria) and -3 (skeletal muscle mitochondria). These proteins had similar nucleotide specificity, with potent inhibition by purine but not pyrimidine nucleoside diphosphates and triphosphates (Fig. 4a). One difference was that CTP, CDP and perhaps UTP partially inhibited UCP2 but not -3. Nucleoside monophosphates had no effect on either protein. AMP had a separate stimulatory effect on skeletal muscle (but not kidney) mitochondrial proton conductance, which is mediated through the adenine nucleotide translocase, as reported previously[19]. The inhibition by GDP was found to follow simple saturation kinetics (Fig. 4b). Superoxide activation was inhibited by 50% with 17 ± 0.9 μM GDP in kidney and 8.5 ± 0.5 μM in skeletal muscle mitochondria.

Stimulation of proton conductance by xanthine plus xanthine oxidase was insensitive to catalase (Fig. 4a), showing that it was not caused by hydrogen peroxide. Kidney or liver mitochondria incubated with glucose plus glucose oxidase to generate exogenous H_2O_2 had increased basal proton conductance that was insensitive to GDP (data not shown), suggesting that peroxide or its by-products damaged the membrane and increased its proton permeability but did not activate UCPs.

Stimulation by superoxide was not inhibited by glybenclamide, a K_{ATP} channel blocker; by bongkrekate or cyclosporin A, inhibitors of the mitochondrial permeability transition; or by carboxyatractylate or bongkrekate, inhibitors of the adenine nucleotide translocase (Fig. 4a). Activation by superoxide required a few minutes to become maximal (possibly because superoxide must react or be translocated before uncoupling). The superoxide effect was fully reversible, because the proton conductance was the same as controls after a 10-min incubation of mitochondria with xanthine plus xanthine oxidase followed by the addition of superoxide dismutase.

Activation in kidney mitochondria was less at pH 6.5 and greater at pH 7.8 than at pH 7.2 (data not shown), perhaps because of increased protonation and dismutation (and hence destruction) of superoxide at more acid pH (ref. 20). GDP sensitivity was abolished at pH 7.8, however, indicating that nucleotide inhibition of UCP2 may be very sensitive to pH, as it is in UCP1 (ref. 2). Oxygen consumption by kidney mitochondria incubated with xanthine, xanthine oxidase and myxothiazol was very low, showing that superoxide does not generate its effects by causing electron flow to bypass complex III of the respiratory chain.

We conclude that superoxide interacts with UCP1, -2 and -3, which leads to an increase in proton conductance that requires fatty acids and is inhibited by purine nucleotides. This extends previous findings that reactive oxygen species (ROS) also cause fatty-acid-dependent, ATP-inhibited uncoupling in plant mitochondria[21,22]. Because our studies were performed in isolated mitochondria in the presence of high concentrations of exogenous superoxide, we do not know whether superoxide-stimulated UCP-mediated uncoupling occurs in cells or in intact organisms. This will need to be addressed by future experiments.

Two types of model could explain our results: the effects of superoxide might be direct, or might take place through some product (other than peroxide). In type I models, superoxide induces proton transport by allosterically activating the proton transport mechanisms previously proposed for UCP1 (refs 2, 23). In type II models, UCPs use the mitochondrial membrane potential to export endogenously produced superoxide anions from the matrix to the

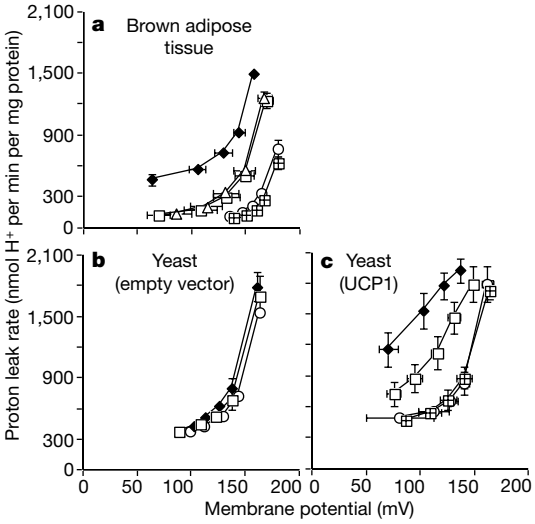

Figure 3 Effect of superoxide on the proton conductance of mitochondria from brown adipose tissue and transgenic yeast: superoxide activation of UCP1. Open squares, control; crossed squares, control plus 500 μM GDP added before $TPMP^+$; filled diamonds, 50 μM xanthine plus xanthine oxidase (0.01 U per 3.5 ml) added before $TPMP^+$; open circles, xanthine plus xanthine oxidase and 500 μM GDP added before $TPMP^+$; open triangles, xanthine plus xanthine oxidase and 12 U ml^{-1} superoxide dismutase added before $TPMP^+$. Proton leak in mitochondria from brown adipose tissue (**a**); yeast containing control empty vector (**b**); yeast expressing mouse UCP1 from pBF307 vector[18] (**c**). Data are the mean ± s.e.m. (or range) of 2–3 independent experiments each performed in duplicate.

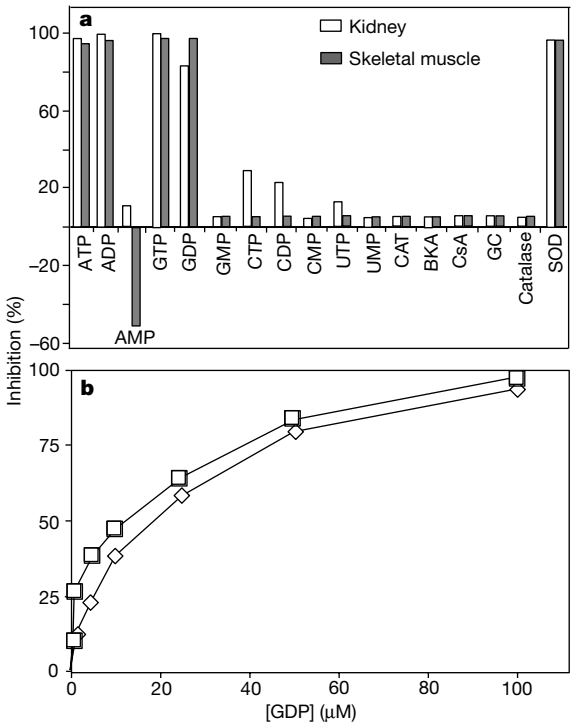

Figure 4 Nucleotide specificity and affinity of UCP2 (kidney) and UCP3 (skeletal muscle). Inhibition of proton conductance in standard medium is presented as the percentage inhibition of superoxide-activated H^+ transport rates at defined membrane potential (130 mV, interpolated from proton leak curves). **a**, Nucleotides were added at 100 μM, carboxyatractylate (CAT) at 1.5 μM, bongkrekate (BKA) at 1.5 μM, cyclosporin A (CsA) at 1 μM, glybenclamide (GC) at 1 μM, catalase at 3 U ml^{-1} and superoxide dismutase (SOD) at 12 U ml^{-1}. **b**, Dependence of inhibition on concentration of GDP. Open squares, skeletal muscle; open diamonds, kidney. Data are the mean and range of two determinations.

intermembrane space where they can either undergo more rapid dismutation because of the more acid pH or be scavenged by cytochrome *c*, CoQ or other antioxidant defences. Uncoupling by exogenous superoxide would be the result of protonation in the intermembrane space (the pK is 4.8)[20], followed by hydroperoxyl-radical diffusion into the matrix and export of superoxide anions by UCP—a similar pathway to one proposed previously[24]. In this model, UCPs would normally export superoxide produced in the matrix and would only show up as an uncoupling pathway when the matrix was flooded with superoxide being produced exogenously and continuously (for example, by xanthine oxidase).

In both types of model, UCPs can also decrease ROS production by mild uncoupling, perhaps as a feedback response to the over-production of ROS by the electron transport chain. The models can explain the increased production of matrix ROS observed in mitochondria and tissues from UCP knockout mice[5,6]. The function of UCP2 and -3 might therefore be more associated with protection against ROS than with thermogenesis. This would explain the induction of UCP expression by cold in plants[25], which has been postulated to increase the production of ROS[26].

We speculate that UCP1 evolved a thermogenic role in mammals as a side pathway of an original, more general function of protection against the cold-induced production of ROS. Such a function can explain the occurrence of UCP2 and -3 in ectotherms[3], the association of UCP2 with cells of the immune system in mammals[5,13], and the observation of nucleotide-sensitive ROS production in cells expressing UCP2 (ref. 27). It also might explain why UCP2 knockout mice are resistant to infection by endoparasites[5], as they would lack a system that normally removes ROS, and suggests interpretations of the observation that these animals have altered pancreatic β-cell function[15]. □

Methods

Proton leak measurements

We measured respiration rate and membrane potential simultaneously by using electrodes sensitive to oxygen and to the potential-dependent probe triphenylmethyl phosphonium cation ($TPMP^+$)[28]. The $TPMP^+$-binding correction was assumed to be 0.4/(μl per mg protein). Proton leak rates were calculated by multiplying oxygen consumption rates by the H^+/O ratio of six.

Mitochondria were prepared essentially as described[29]. Mitochondria isolated from total hindlimb skeletal muscle (0.35 mg protein ml^{-1}), liver (0.5 mg ml^{-1}), kidney (0.35 mg ml^{-1}), heart (0.35 mg ml^{-1}), spleen (1.0 mg ml^{-1}) or β cells (0.75 mg ml^{-1}) were incubated in standard assay medium containing 120 mM KCl, 5 mM KH_2PO_4, 3 mM HEPES and 1 mM EGTA (pH 7.2 and 37 °C) with 5 μM rotenone (a complex I inhibitor to prevent oxidation of any endogenous NAD-linked substrates), 80 ng nigericin ml^{-1} (to abolish the pH gradient) and 1 μg oligomycin ml^{-1} (to prevent ATP synthesis). We calibrated the $TPMP^+$ electrode with sequential 1-μM additions of $TPMP^+$ to 5 μM. Skeletal muscle mitochondria were titrated by incremental additions of succinate to 1 mM (oxidizable substrate); other tissues were titrated by cyanide (up to 100 μM) using 4 mM succinate as the substrate. After each run, 0.2 μM FCCP (carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone) was added to release $TPMP^+$ for baseline correction. The β cell line was kindly provided by F. Gribble and grown as described[30].

Proton leak in BAT mitochondria (0.35 mg protein ml^{-1}) isolated from rats maintained at 25 °C was measured in assay medium containing 50 mM KCl, 5 mM HEPES, 1 mM EGTA, 4 mM KH_2PO_4 (pH 7.2 and 37 °C) and supplemented with 1% BSA. BAT mitochondria were titrated by cyanide (up to about 100 μM) using 10 mM α-glycero-phosphate as the substrate.

Proton leak in yeast mitochondria[18] (0.4 mg protein ml^{-1}) was measured in assay medium containing 10 mM Tris-maleate, 650 mM sorbitol, 0.5 mM EGTA, 2 mM $MgCl_2$, 10 mM K_2HPO_4 (pH 6.8 and 30 °C). The $TPMP^+$ electrode was calibrated with sequential 1-μM additions of $TPMP^+$ to 4 μM, and then oligomycin (10 μg ml^{-1}) and nigericin (100 ng ml^{-1}) in ethanol were added. Oxidation of this ethanol (0.2%) was inhibited progressively through successive steady states by adding cyanide up to about 50 μM.

Generation of UCP3 knockout mice

We constructed a targeting deletion vector containing a neomycin cassette flanked by a 3.2-kilobase (kb) *Nhe*1–*Sac*2 fragment of the mouse *ucp3* gene for 3′ homology and a 3-kb *Nhe*1–*Eco*R1 fragment for 5′ homology. The 8.7-kb targeting construct was designed to generate a 2.5-kb deletion to remove exons 1 and 2 (exon 2 contains the start codon) from the mouse *ucp3* gene. Neomycin-resistant clones were isolated and injected into C57BL/6× CBA F_2 embryos. These chimeric mice were backcrossed for six generations onto a C57BL/6 background (N6). Western blot analysis of mitochondria and northern blot analysis of RNA from skeletal muscle of wild-type and $ucp3^{-/-}$ mice confirmed that there was no UCP3 expression in the knockouts.

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Correspondence and requests for materials should be addressed to M.D.B. (e-mail: martin.brand@mrc-dunn.cam.ac.uk).