# Production of endogenous matrix superoxide from mitochondrial complex I leads to activation of uncoupling protein 3

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**Abstract Superoxide generated using exogenous xanthine oxidase indirectly activates an uncoupling protein (UCP)-mediated proton conductance of the mitochondrial inner membrane. We investigated whether endogenous mitochondrial superoxide production could also activate proton conductance. When respiring on succinate, rat skeletal muscle mitochondria produced large amounts of matrix superoxide. Addition of GDP to inhibit UCP3 markedly inhibited proton conductance and increased superoxide production. Both superoxide production and the GDP-sensitive proton conductance were suppressed by rotenone plus an antioxidant. Thus, endogenous superoxide can activate the proton conductance of UCP3, which in turn limits mitochondrial superoxide production. These observations provide a departure point for studies under more physiological conditions.**


*Key words:* Mild uncoupling; Reactive oxygen species; Superoxide; Uncoupling protein; Complex I

## 1. Introduction

Uncoupling protein 1 (UCP1) is found exclusively in brown adipose tissue and mediates adaptive thermogenesis in mammals [1,2]. UCP1 homologues, UCP2 and UCP3, are present in several mammalian tissues, with UCP3 characteristic of skeletal muscle mitochondria. Whether the homologues can also catalyse proton conductance under physiological conditions is debated, but it is clear that neither UCP2 nor UCP3 contributes to the basal proton leak that is a feature of all mitochondria [3,4].

UCP1, UCP2 and UCP3 all catalyse a superoxide-inducible proton conductance [5]. Exogenous superoxide generated in the surrounding medium using xanthine and xanthine oxidase leads to increased proton transport in a reaction that is potently inhibited by nucleoside di- and triphosphates, including GDP. The absence of this reaction in mitochondria from UCP3 knockout mice [4] shows that it is mediated by UCP3 in muscle. Artificially generated superoxide activates UCPs from the matrix side of the inner membrane, since activation is prevented by the mitochondrially targeted antioxidant mitoQ but occurs when mitoQ acts as a superoxide generator in the matrix [6,7]. The proton conductance of the UCPs can also be activated by reactive aldehydes such as 4-hydroxy-2-nonenal [8] and by a carbon-centred radical generator, 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) [9]. A mitochondrially targeted carbon-centred radical quencher, mitoPBN ([4-[4-[[(1,1-dimethylethyl)oxidoimino]methyl]phenoxy]-butyl]triphenylphosphonium), prevents activation by superoxide and AAPH, but not by hydroxynonenal, indicating that exogenous superoxide activates UCPs through a pathway of carbon-centred radical production, lipid peroxidation and reactive aldehyde production [9].

An important function of this superoxide-activated pathway may be to cause mild uncoupling in response to any overproduction of superoxide by the mitochondrial electron transport chain, leading to a small decrease in mitochondrial membrane potential and a consequent strong reduction in endogenous superoxide production [6,7]. However, activation of UCPs by superoxide generated endogenously in the mitochondrial matrix by the electron transport chain has yet to be demonstrated experimentally. Indications that it may occur come from observations that incubation of UCP2-containing mitochondria with GDP induces an increase in hydrogen peroxide generation [10]. Also, superoxide production and markers of oxidative damage are higher in mitochondria from UCP2- and UCP3-knockout mice [11–13] and the proton conductance of mitochondria in intact thymocytes is higher when UCP2 is present and active [14].

Most of the superoxide produced in cells is generated by the mitochondrial electron transport chain. The relative importance of complex I and complex III is debated [15–18], but it is matrix superoxide production that will activate UCPs, and most of this is from complex I [19,20]. Superoxide production from this source is maximal when complex II substrates are oxidised in the absence of complex I inhibitors, so that reverse electron flow occurs [15–18]. Reverse electron transport and complex I superoxide production are highly dependent on mitochondrial proton motive force, which drives the electrons from complex II thermodynamically uphill back into complex I [6,20–22].

This study investigates whether superoxide from endogenous sources in muscle mitochondria can activate mild uncoupling that is mediated by UCP3 (and hence sensitive to GDP). If our model is correct, this activation would serve to attenuate mitochondrial superoxide production, so a second aim was to measure whether inhibition of UCP3 by GDP can

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*Abbreviations:* UCP, uncoupling protein; SOD, superoxide dismutase; AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride; $TPMP^+$, triphenylmethyl phosphonium cation; PHPA, *p*-hydroxyphenyl acetic acid

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cause an increase in the rate of endogenous superoxide production.

## 2. Materials and methods

### 2.1. Isolation of skeletal muscle mitochondria

Skeletal muscle mitochondria were isolated at 4°C as in [23]. Female Wistar rats (6–8 weeks old) were stunned and killed by cervical dislocation. Skeletal muscle from the hindlimbs was dissected 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 tissue 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) bovine serum albumin (BSA), 2.1 U $ml^{-1}$ protease (subtilsin), 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. Protein concentration was determined using the biuret method [24].

### 2.2. Measurement of mitochondrial hydrogen peroxide production

In the presence of exogenous and matrix superoxide dismutase (SOD), mitochondrial superoxide production can be measured as the appearance of hydrogen peroxide in the medium. Hydrogen peroxide generation rates were determined fluorometrically by measurement of oxidation of *p*-hydroxyphenyl acetic acid (PHPA) coupled to the enzymatic reduction of $H_2O_2$ by horseradish peroxidase. The increase in fluorescence at 400 nm with excitation at 320 nm was followed on a computer-controlled spectrofluorimeter with appropriate correction for background and standard curve as in [19]. Mitochondria were incubated at 0.35 mg mitochondrial protein $ml^{-1}$ in assay buffer containing 120 mM KCl, 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA, 4 mM succinate, 1 mg $ml^{-1}$ oligomycin, 80 ng $ml^{-1}$ nigericin, 50 mg $ml^{-1}$ PHPA, 4 U $ml^{-1}$ horseradish peroxidase, 2 μM triphenylmethyl phosphonium cation ($TPMP^+$) and 30 U $ml^{-1}$ SOD (pH 7.2 and 37°C). When used, 2 μM rotenone, 500 μM GDP and 2 μM mitoVitamin E were added.

### 2.3. Measurement of proton conductance

The respiration rate of mitochondria, in the presence of oligomycin to inhibit ATP synthesis, is proportional to the rate at which protons leak across the 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 with electron transport chain inhibitors [23,25,26]. Respiration rate and membrane potential were determined simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe $TPMP^+$ [27].

Mitochondria (0.35 mg protein $ml^{-1}$) were incubated at 37°C in 3.5 ml of assay medium identical to the medium for $H_2O_2$ detection. The $TPMP^+$ electrode was calibrated with sequential additions of up to 2 μM $TPMP^+$. Four mM succinate was added to start the reaction. Where indicated, $TPMP^+$ was replaced with identical concentrations of mitoVitamin E to measure the mitochondrial membrane potential in the presence of this mitochondrially targeted antioxidant. Respiration and potential were inhibited progressively through successive steady states by additions of malonate up to 3 mM. To avoid generating the maximum membrane potential and to limit superoxide production, where indicated in Fig. 2d, the first two aliquots of malonate (total 0.35 mM) were added before succinate. At the end of each run 0.4 μM carbonylcyanide *p*-trifluoromethoxy phenylhydrazone (FCCP) was added to dissipate the membrane potential and release all $TPMP^+$, or mitoVitamin E, back into the media, allowing correction for any small electrode drift. The $TPMP^+$(and mitoVitamin E) binding correction factor was taken as 0.35 μl $mg^{-1}$ of protein. This is probably an underestimate for mitoVitamin E, resulting in overestimation of the membrane potential in Fig. 2c, however, comparisons made between conditions both using mitoVitamin E to measure membrane potential will be valid. Oxygen consumption was mea-

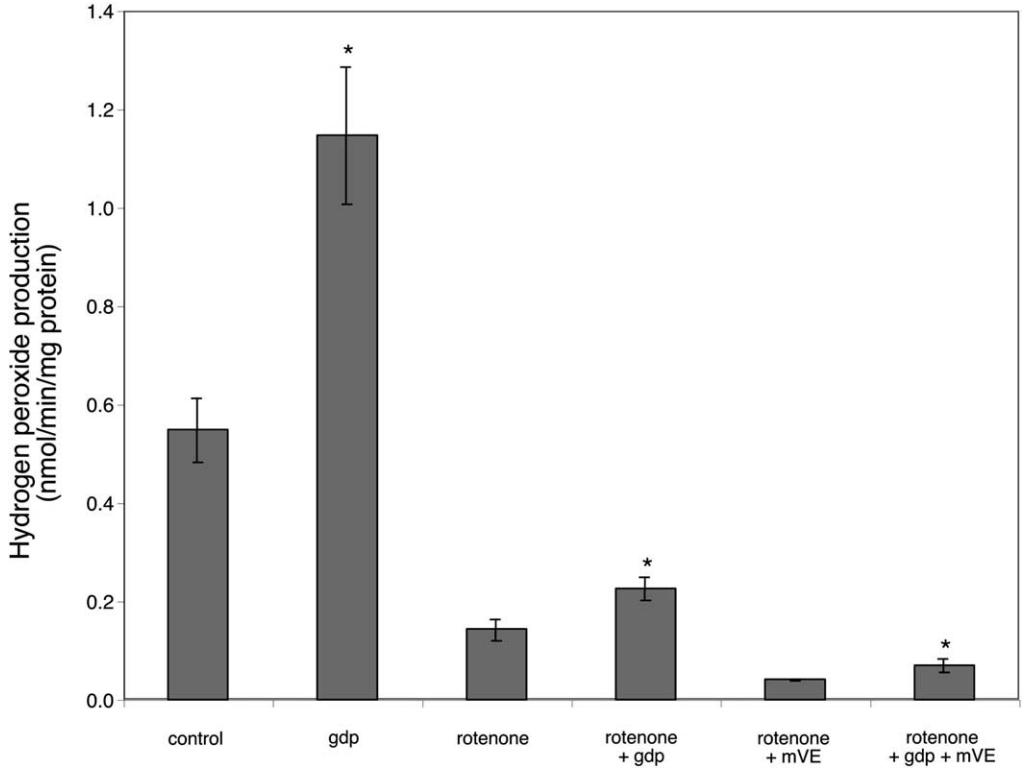


Fig. 1. $H_2O_2$ production by muscle mitochondria. For details see Section 2. Rat skeletal muscle mitochondria (0.35 mg protein $ml^{-1}$) were incubated in assay buffer (120 mM KCl, 3 mM HEPES, 1 mM EGTA, 0.3% (w/v) BSA, 4 mM succinate, 1 mg $ml^{-1}$ oligomycin, 80 ng $ml^{-1}$ nigericin, 50 mg $ml^{-1}$ PHPA, 4 U $ml^{-1}$ horseradish peroxidase, 2 μM $TPMP^+$ and 30 U $ml^{-1}$ SOD, pH 7.2 and 37°C). 2 μM rotenone, 500 μM GDP and 2 μM mitoVitamin E (mVE) were added where indicated. Hydrogen peroxide generation rates were determined fluorometrically. Data are means ± S.E.M. of three to six independent experiments, each performed in duplicate. $*P < 0.05$, compared to the same condition in the absence of GDP by unpaired Student's *t*-test.

sured in the same vessel using a Clark-type oxygen electrode calibrated with air-saturated assay medium, which was assumed to contain 406 nmol O $ml^{-1}$ at 37°C [28]. Electrode linearity from 100–0% air saturation was checked routinely by following uncoupled respiration with 0.4 μM FCCP.

## 3. Results and discussion

We first determined whether isolated skeletal muscle mitochondria produced superoxide at significant rates when respiring in the absence of respiratory inhibitors. Reverse electron flow into complex I of the respiratory chain of rat brain, heart and liver mitochondria, and of submitochondrial particles from rat brain and bovine heart, can produce large amounts of superoxide and hydrogen peroxide [15–18]. The same was true for mitochondria isolated from rat skeletal muscle (Fig. 1). With the complex II substrate succinate, large rates of hydrogen peroxide production (about 0.5 nmol $min^{-1}$ $mg^{-1}$ protein) were detected, indicating large rates of superoxide production. Addition of 2 μM rotenone to inhibit complex I reduced the rate of hydrogen peroxide production by approximately 75%, demonstrating that most of the superoxide was produced at complex I as a result of reverse electron flow from succinate dehydrogenase into complex I. This superoxide production from complex I is on the matrix side of the inner membrane [15–18,20].

Since UCPs are stimulated by superoxide produced exogenously using xanthine plus xanthine oxidase, we investigated whether the endogenous superoxide produced from reverse electron transport into complex I was sufficient to activate the proton conductance of UCP3. Fig. 2a demonstrates that under identical conditions to those used to measure superoxide production in Fig. 1 there was indeed enough superoxide produced to activate a UCP3-mediated mild uncoupling of the mitochondria. There was significant GDP-sensitive proton conductance under these conditions: oxygen consumption driving the proton leak was greater at any given membrane potential than it was when UCP3 was inhibited by GDP. Consequently, when UCP3 was activated by endogenous superoxide, the membrane potential in state 4 was 10 mV lower than when GDP was present and UCP3 was inhibited (Fig. 2a).

Fig. 1 shows that addition of rotenone strongly (but not completely) depressed superoxide production. Fig. 2b shows that in the presence of rotenone the GDP-sensitive proton conductance was attenuated, but it was not abolished, particularly at higher potentials. Even the lowered rate of superoxide production in the mitochondrial matrix in the presence of rotenone appeared to be sufficient to activate UCP3 at high potential.

To further decrease the concentrations of matrix superoxide and other activatory species, we used the mitochondrially targeted antioxidant, mitoVitamin E. This compound abolishes activation of UCP2 by exogenously generated superoxide [6,7]. Fig. 1 shows that when rotenone and mitoVitamin E

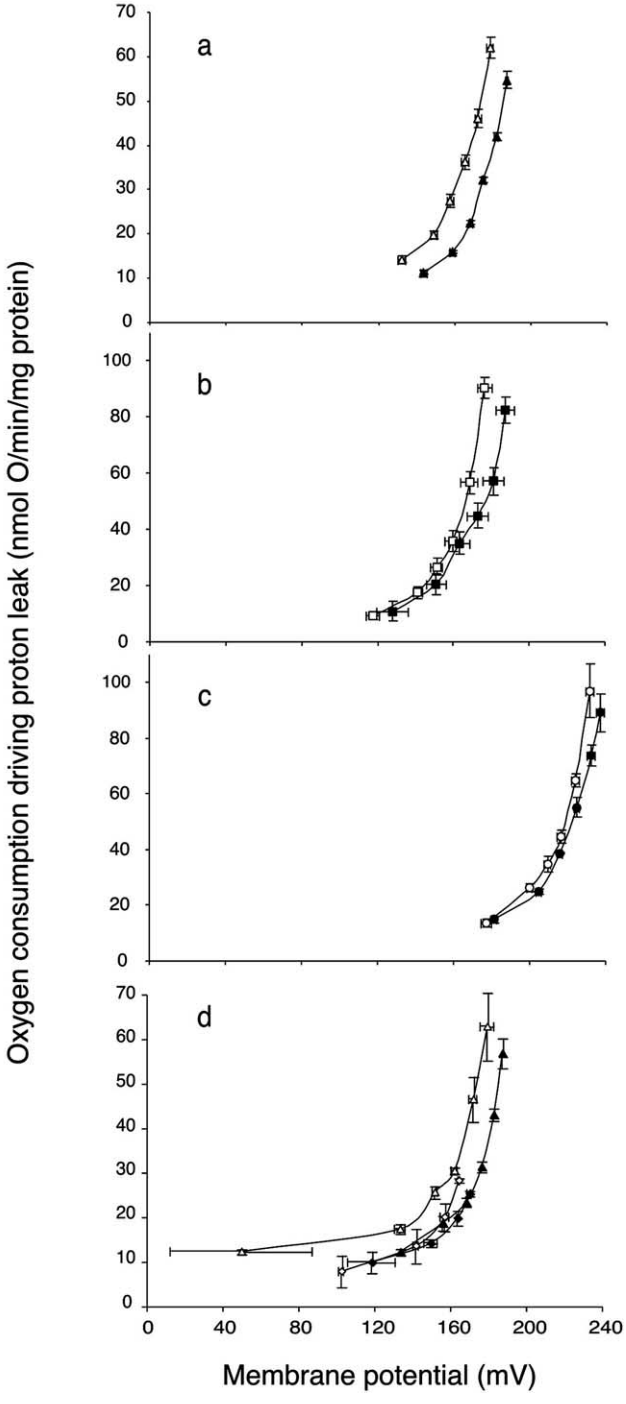


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Fig. 2. Effect of superoxide produced endogenously from complex I on the proton conductance of rat skeletal muscle mitochondria. Rat skeletal muscle mitochondria (0.35 mg protein $ml^{-1}$) were incubated at 37°C in assay buffer exactly as in Fig. 1. The kinetics of the mitochondrial proton leak were obtained by simultaneous measurement of membrane potential and oxygen consumption, using succinate as a substrate and varying the potential with sequential additions of malonate up to 3 mM. a: Sensitivity to 500 μM GDP; △ control; ▲ plus 500 μM GDP. b: Reduction of GDP-sensitivity in the presence of 2 μM rotenone; □ control; ■ plus 500 μM GDP. c: Loss of GDP-sensitivity in the presence of 2 μM rotenone and 2 μM mitoVitamin E; ○ control; ● plus 500 μM GDP. d: Effect of initial high potential on subsequent GDP-sensitivity at lower potential; △ control; ▲ plus 500 μM GDP; ◇ control with the first two malonate aliquots (0.35 mM) added before succinate; ◆ plus 500 μM GDP with 0.35 mM malonate added before succinate. Data are means ± S.E.M. (or range) of seven (a), three (b,c) or two (d) independent experiments, each performed in duplicate. The state 4 (i.e. highest) membrane potential was significantly increased by GDP in a and b but not in c; $P < 0.05$ by paired Student's *t*-test.

were both present, superoxide production by muscle mitochondria was minimal. Fig. 2c shows that this small rate of endogenous superoxide production was insufficient to activate UCP3, as the GDP-sensitive component of the proton conductance seen in Fig. 2a,b was almost totally abolished. The curves in Fig. 2c are shifted to the right compared to Fig. 2a,b, because of an underestimate of the mitoVitamin E binding correction factor which results in an overestimation of the membrane potential, but does not affect comparisons between curves within Fig. 2c. Previous investigations of endogenous activation of UCP3 were carried out with rotenone present to prevent complications due to build-up of oxaloacetate and inhibition of succinate dehydrogenase, which may explain why no nucleotide-sensitive basal proton conductance was observed [5].

Superoxide production by reverse electron transport from complex I is very sensitive to mitochondrial membrane potential [6,20–22], yet the endogenous activation of UCP3 persisted even after titration of the potential to low values, where superoxide production will have ceased (Fig. 2a). Superoxide activates UCPs indirectly, through lipid peroxidation products [8], so the activation at low potential in Fig. 2a is probably caused by the continued presence of these downstream activators after superoxide production has been abolished by the decrease in membrane potential. The lack of such activation in Fig. 2b at low membrane potentials would reflect decreased accumulation of downstream activators after smaller initial superoxide production (Fig. 1). To test whether superoxide production at high membrane potential (170–180 mV) during the first few points of the titration in Fig. 2a led to production of other more persistent activators, we repeated the experiment but added the first two aliquots of malonate (0.35 mM) before adding the substrate, so that very high potentials were never reached, and superoxide and its downstream products could not be formed (Fig. 2d). Under these conditions, endogenous GDP-sensitive activation was greatly attenuated at the highest potential reached (165 mV), and abolished at lower potentials, supporting the suggestion that the persistent activation at lower potentials in Fig. 2a was indeed caused by longer-lived downstream activators formed only when large amounts of superoxide were generated at the highest potentials.

Proton transport by UCP1 requires free fatty acids [2], so the UCP1 homologues might also require free fatty acids if they perform a similar function. This is the case when the proton conductances of UCP2 and UCP3 are activated by exogenous superoxide from xanthine oxidase [5]. However, when superoxide was produced in the matrix of UCP2-containing mitochondria by using mitoQ [7], or when UCPs were directly activated using hydroxynonenal [8], a GDP-sensitive proton conductance was observed even in the presence of BSA, a fatty acid chelator. All the experiments in this study were performed in the presence of BSA, with no added fatty acid, yet we observed GDP-sensitive proton conductance. Therefore, when superoxide from the matrix activates the proton conductance of UCP2 or UCP3, added fatty acids are not required, suggesting that fatty acids may only be required for the entry of superoxide into the matrix when it is generated externally.

These findings show that the proton conductance of UCP3 in skeletal muscle mitochondria can be activated indirectly by superoxide generated endogenously by the mitochondrial electron transport chain. The activation diminished as superoxide production was attenuated, but even quite low rates of endogenous superoxide production were sufficient to activate UCP3 and cause a mild uncoupling of the mitochondria. The presence and significance of this activation in intact cells have yet to be demonstrated.

We have proposed that activation of mild uncoupling by UCPs is a mechanism to decrease endogenous superoxide production. In the second part of this study we investigated whether inhibition of UCP3 prevented this feedback and released the control it is postulated to exert over superoxide production. Fig. 1 shows that inhibition of UCP3 by GDP did indeed allow higher rates of superoxide production from the electron transport chain: in the absence of rotenone, addition of 500 μM GDP, to fully inhibit UCP3, augmented superoxide production by about 75%. This enhanced superoxide production can be explained by the 10 mV increase in membrane potential seen in Fig. 2a when UCP3 was inhibited by GDP, due to the steep dependence of superoxide production on membrane potential [20–22]. Even when complex I was inhibited using rotenone, addition of GDP to abolish superoxide activation of UCP3 still resulted in significant increases in membrane potential (Fig. 2b) and in the rate of superoxide production (Fig. 1).

These data show that the mitochondrial electron transport chain can produce sufficient superoxide to activate the proton conductance of UCP3. Activation of UCP3 causes mild uncoupling, which lowers $\Delta p$ and decreases reverse electron transport into complex I, resulting in decreased superoxide production from complex I and other sites in the respiratory chain. UCPs can thus act as part of a feedback mechanism to limit the rate of endogenous superoxide production in the mitochondrial matrix, and so act to limit oxidative damage. These observations provide a departure point for studies under more complex conditions, including the presence of physiological concentrations of purine nucleotides.

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## References

[1] Nedergaard, J., Golozoubova, V., Matthias, A., Asadi, A., Jacobsson, A. and Cannon, B. (2001) Biochim. Biophys. Acta 1504, 82–106.
[2] Nicholls, D.G. and Rial, E. (1999) J. Bioenerg. Biomembr. 31, 399–406.
[3] Cadenas, S., Buckingham, J.A., Samec, S., Seydoux, J., Din, N., Dulloo, A.G. and Brand, M.D. (1999) FEBS Lett. 462, 257–260.
[4] Cadenas, S., Echtay, K.S., Harper, J.A., Jekabsons, M.B., Buckingham, J.A., Grau, E., Abuin, A., Chapman, H., Clapham, J.C. and Brand, M.D. (2002) J. Biol. Chem. 277, 2773–2778.
[5] Echtay, K.S., Roussel, D., St-Pierre, J., Jekabsons, M.B., Cadenas, S., Stuart, J.A., Harper, J.A., Roebuck, S.J., Morrison, A., Pickering, S., Clapham, J.C. and Brand, M.D. (2002) Nature 415, 96–99.
[6] Brand, M.D., Buckingham, J.B., Esteves, T.C., Green, K., Lambert, A.J., Miwa, S., Murphy, M.P., Pakay, J.L., Talbot, D.A. and Echtay, K.S. (2004) Biochem. Soc. Symp. 71, in press.
[7] Echtay, K.S., Murphy, M.P., Smith, R.A., Talbot, D.A. and Brand, M.D. (2002) J. Biol. Chem. 277, 47129–47135.
[8] Echtay, K.S., Esteves, T.C., Pakay, J.L., Jekabsons, M.B., Lambert, A.J., Portero-Otin, M., Pamplona, R., Vidal-Puig, A., Wang, S., Roebuck, S.J. and Brand, M.D. (2003) EMBO J. 22, 4103–4110.

[9] Murphy, M.P., Echtay, K.S., Blaikie, F.H., Asin-Cayuela, J., Cocheme, H.M., Green, K., Buckingham, J.A., Taylor, E.R., Hurrell, F., Hughes, G., Miwa, S., Cooper, C.E., Svistunenko, D.A., Smith, R.A.J. and Brand, M.D. (2003) J. Biol. Chem. 278, 48534–48545.
[10] Negre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M., Salvayre, R., Penicaud, L. and Casteilla, L. (1997) FASEB J. 11, 809–815.
[11] Arsenijevic, D., Onuma, H., Pecqueur, C., Raimbault, S., Manning, B.S., Miroux, B., Couplan, E., Alves-Guerra, M.C., Goubern, M., Surwit, R., Bouillaud, F., Richard, D., Collins, S. and Ricquier, D. (2000) Nat. Genet. 26, 435–439.
[12] Brand, M.D., Pamplona, R., Portero-Otin, M., Requena, J.R., Roebuck, S.J., Buckingham, J.A., Clapham, J.C. and Cadenas, S. (2002) Biochem. J. 368, 597–603.
[13] Vidal-Puig, A.J., Grujic, D., Zhang, C.Y., Hagen, T., Boss, O., Ido, Y., Szczepanik, A., Wade, J., Mootha, V., Cortright, R., Muoio, D.M. and Lowell, B.B. (2000) J. Biol. Chem. 275, 16258–16266.
[14] Krauss, S., Zhang, C.Y. and Lowell, B.B. (2002) Proc. Natl. Acad. Sci. USA 99, 118–122.
[15] Liu, Y., Fiskum, G. and Schubert, D. (2002) J. Neurochem. 80, 780–787.
[16] Hansford, R.G., Hogue, B.A. and Mildaziene, V. (1997) J. Bioenerg. Biomembr. 29, 89–95.
[17] Votyakova, T.V. and Reynolds, I.J. (2001) J. Neurochem. 79, 266–277.
[18] Turrens, J.F. and Boveris, A. (1980) Biochem. J. 191, 421–427.
[19] St-Pierre, J., Buckingham, J.A., Roebuck, S.J. and Brand, M.D. (2002) J. Biol. Chem. 277, 44784–44790.
[20] Miwa, S., St-Pierre, J., Partridge, L. and Brand, M.D. (2003) Free Radic. Biol. Med. 35, 938–948.
[21] Korshunov, S.S., Skulachev, V.P. and Starkov, A.A. (1997) FEBS Lett. 416, 15–18.
[22] Liu, S.S. (1997) Biosci. Rep. 17, 259–272.
[23] Cadenas, S. and Brand, M.D. (2000) Biochem. J. 348, 209–213.
[24] Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751–766.
[25] Brand, M.D. (1990) Biochim. Biophys. Acta 1018, 128–133.
[26] Nicholls, D.G. (1974) Eur. J. Biochem. 50, 305–315.
[27] Brand, M.D. (1995) in: Bioenergetics – A Practical Approach (Brown, G.C. and Cooper, C.E., Eds.), pp. 39–62, IRL Press, Oxford.
[28] Reynafarje, B., Costa, L.E. and Lehninger, A.L. (1985) Anal. Biochem. 145, 406–418.