Avian uncoupling protein expressed in yeast mitochondria prevents endogenous free radical damage

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The longevity of birds is surprising since they exhibit high metabolic rates and elevated blood sugar levels compared with mammals of the same body size, which presumably expose them to higher rates of free oxygen radical production, which is implicated in accelerated senescence. Uncoupling proteins (UCPs) are transporters of the inner mitochondrial membrane and their physiological activity is still a subject of debate. Avian UCP was found in birds but data on its activity are scarce. Avian UCP (*Gallus gallus*) was overexpressed in yeast and we assessed its ability to prevent mitochondrial reactive oxygen species (ROS) production by measuring ROS damage (aconitase activity) and antioxidant defences (MnSOD activity). We show that avian UCP protects yeast mitochondria against the deleterious impact of ROS, but without stimulation of superoxide dismutase activity. Avian UCP protein was specifically immunodetected and retinoic acid, which belongs to the carotenoid family, was found to trigger its activity. These data show that avian UCP basal activity protects against ROS damage. However, when activated by retinoic acid, avian UCP can also operate as the mammalian thermogenic UCP1. The hypothesis that avian UCP activities are state- and species-dependent is further discussed.

Keywords: uncoupling protein; bird; reactive oxygen species; oxidative stress ageing

1. INTRODUCTION

Evolution theory suggests that ageing, the progressive decline in an organism's internal condition, is caused by a lifelong accumulation of damage in somatic cells (Kirkwood & Austad 2000). Highly reactive molecules derived from oxygen, reactive oxygen species (ROS), are continuously produced during normal cellular respiration in the mitochondria (Raha & Robinson 2000), which partially explains the accumulation of deleterious change in macromolecules during ageing (free radical theory or oxidative stress theory of ageing; Beckman & Ames 1998; Hughes & Reynolds 2005). Both invertebrate and vertebrate animals show life span extension concomitant with oxidative stress resistance (Migliaccio *et al.* 1999) or enhanced antioxidant defences (Melov *et al.* 2000), thus providing some evidence for the impact of oxidative stress on ageing.

Birds are relevant models for studying the ageing process given that most species live dramatically longer than mammals of equal size, despite specific physiological characteristics which should lead to an overall higher exposure to oxidative stress (Holmes & Austad 1995). There are two basic mechanisms by which birds could have evolved to cope with free radical damage: (i) a more effective antioxidant defence, and (ii) reduced ROS production (Papa & Skulachev 1997). Comparative studies conducted on amphibians, mammals and birds found that reduced free radical production near critical

targets such as mitochondrial DNA could be a major factor contributing to the low avian rate of ageing (Perez-Campo *et al.* 1998).

Production of ROS occurs in mitochondria during oxidative phosphorylation. Mitochondrial ROS are mainly produced during the resting state of the respiratory chain (state 4: no ATP production), the elevated proton gradient ($\Delta\mu\text{H}^+$) leading to an increased probability for electrons to react directly with dioxygen and to form superoxide (Papa & Skulachev 1997). Because ROS production is low when $\Delta\mu\text{H}^+$ is reduced (state 3), one might hypothesize that a small uncoupling of oxidative phosphorylation during state 4—which could dissipate the proton gradient without having protons pass through the ATP synthase—would prevent ROS accumulation within mitochondria (Miwa & Brand 2003). A well-known example of such uncoupling was first discovered in brown adipose tissue of rodents, where a mitochondrial transporter of the inner membrane—the uncoupling protein 1 (UCP1)—dissipates the energy of substrate oxidation as heat (Nicholls & Locke 1984). UCP1 homologues (UCP2 and 3) have been identified in mammals (reviewed in Ricquier & Bouillaud 2000) and were implicated, among other functions, in the control of oxidative stress (Arsenijevic *et al.* 2000; Echtay *et al.* 2002).

In 2001, a mitochondrial UCP homologue was discovered in birds (avian UCP; Raimbault *et al.* 2001; Vianna *et al.* 2001) that shared 70% homology with mammalian UCP2 and 3. Avian UCP gene expression was subsequently found in several species (Talbot *et al.* 2003) but its biological function is still a subject of debate. It was

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initially suggested that avian UCP was involved in non-shivering thermogenesis, based on its predominant distribution in skeletal muscle, thyroid hormone control and glucagon/cold-induced gene expression (Raimbault *et al.* 2001; Vianna *et al.* 2001; Collin *et al.* 2003c). However, a conflicting finding is that avian UCP gene expression is increased during fasting, when energy expenditure is reduced (Evock-Clover *et al.* 2002). In addition, the regulation of avian UCP gene expression appears to be related to free fatty acid (FFA) utilization (Collin *et al.* 2003a) and free radical metabolism (St. Pierre *et al.* 2002; Talbot *et al.* 2003).

Thus, whether avian UCP is involved in thermogenesis, lipid utilization or oxidative stress remains to be defined. To date, no study has focused on the impact of avian UCP in a recombinant model using yeast (but see Vianna *et al.* 2001). In this paper, we present the first results recorded for avian UCP (*Gallus gallus*) protein activity in the prevention of ROS damage in isolated yeast mitochondria.

2. MATERIAL AND METHODS

(a) Expression of avian UCP in yeast

Recombinant expression vectors for avian UCP have been described previously (Fleury *et al.* 1997) and were constructed using the avian UCP transcript obtained by Raimbault *et al.* (2001) and expressed in *Saccharomyces cerevisiae*. The expression of avian UCP in yeast mitochondria was probed in Western blot experiments, as described by Pecqueur *et al.* (2001), using anti-human UCP2-605 antibodies (diluted 1/5000).

(b) Isolation of mitochondria

Precultures of yeast transformed with the pYedp-avian UCP constructs were grown overnight in SP media (0.17% yeast nitrogen base, 0.5% (NH₄)₂SO₄, 2% glucose, 0.1% casamino acids, 20 mg l⁻¹ tryptophan and 40 mg l⁻¹ adenine, pH 6.5) to an A₆₀₀ of 2–3. Yeasts were then transferred to an induction medium of galactose (2% D-galactose, 0.17% yeast nitrogen base, 0.5% (NH₄)₂SO₄, 2% glucose, 0.1% casamino acids, 20 mg l⁻¹ tryptophan and 40 mg l⁻¹ adenine, pH 6.5) at a 1/100 dilution for overnight growth. Mitochondria were isolated from yeast cultures, as previously described (Arechaga *et al.* 1993). Mitochondrial protein content was assayed by the bicinchoninic acid method according to the manufacturer's protocol.

(c) Mitochondrial respiration and membrane potential measurement

Respiration and proton conductance were measured at 20 °C immediately after mitochondrial isolation. Mitochondria were suspended in respiration buffer (0.65 M mannitol, 10 mM Tris-maleate, 0.5 mM EGTA, 2 mM MgCl₂, 10 mM K₂HPO₄–KH₂PO₄ and 0 or 0.1% bovine serum albumin, BSA; pH 6.8) at 0.25 mg of protein per millilitre in a 1.5 ml oxygraph chamber (Hansatech). To initiate respiration, 3 mM NADH was added. Oligomycin (1 µg ml⁻¹) was added to inhibit the ATP synthase, so that all oxygen consumption was attributable to the leak pathway and not to ATP synthesis. Nigericin (100 ng ml⁻¹) was added to clamp the pH gradient across the inner membrane. Simultaneous recording of membrane potential was done with a homemade TPP⁺ electrode (tetraphenylphosphonium), according to published procedures (Locke *et al.* 1982).

Figure 1. Immunodetection of avian UCP. Mitochondria were prepared either from yeast containing the pYedp or the pYedp-avian UCP plasmid (pYedp-avUCP) and 30 µg mitochondrial protein was loaded onto an SDS-9% polyacrylamide gel. Western blot analysis was performed using the hUCP2-605 antibody at 0.3 µg ml⁻¹.

Membrane potential was calculated and corrected for TPP non-specific binding (evaluated for 50 mV) as specified in Couplan *et al.* (2002). Titrations were done with cyanide (KCN) at final concentrations up to 0.5 mM.

(d) Superoxide effect on proton leak measurements

Experiments to evaluate the effect of superoxide on pYedp-avian UCP yeast mitochondria were done in the same conditions as indicated above. Exogenous superoxide was produced in an oxygen chamber by the superoxide-generating system xanthine 50 µM per xanthine oxidase 0.004 units per 1.5 ml as described by Echtay *et al.* (2002).

(e) Aconitase and fumarase, and superoxide dismutase (MnSOD) activities

Aconitase and fumarase activities were measured in three independent experiments. At the end of the titration/respiration recordings, the mitochondrial suspension in the respiration mixture was collected, centrifuged to eliminate the NADH, and the mitochondrial pellet was lysed by the addition of 400 µl Triton X-100 to a final concentration of 0.2% w/v. 200 µl aliquots of these lysed mitochondria were used for simultaneous measurements of aconitase or fumarase activities, as described previously (Couplan *et al.* 2002). The linear rate was used to evaluate the enzymatic activities. The aconitase/fumarase ratio was expressed as the ratio of the respective rates of optical density increase.

Mitochondria of six independent preparations of transformed yeasts (75 mg of protein for each blot) were run under non-denaturing, non-reducing conditions by electrophoresis in the presence of 0.1 mg ml⁻¹ riboflavin, as described by Wong *et al.* (1995). MnSOD activity was revealed with nitro-blue-tetrazolium for 30 min.

(f) Statistics

Means were compared using ANOVA after testing for normality (Shapiro–Wilk test).

3. RESULTS

(a) Expression of avian UCP

The expression of avian UCP in mitochondria isolated from yeast was verified by Western blot using the antibody directed against human UCP2 (hUCP2-605) already used for UCP2 immunodetection (Pecqueur *et al.* 2001). Figure 1 shows the protein detection in six independent experiments, confirming that a protein with a molecular

Figure 2. Effects of superoxide on the proton leak kinetics of avian UCP transfected yeasts. Yeast mitochondria were isolated as described in §2. Respiratory activity and membrane potential were recorded simultaneously in the presence of NADH and oligomycin at 20 °C using electrodes sensitive to oxygen and the potential-dependent probe cation TPP⁺. Titration was performed with increasing concentrations of cyanide. Curves were obtained for avian UCP mitochondria with BSA (circles), without BSA and in the presence of 3 mM GDP (others). Dashed circles indicated the state 4 values for both avian UCP and pYedp mitochondria (ANOVA, $F < 7.04$, $p < 0.038$). The histogram in the inset shows the maximal values reached for membrane potential and oxygen consumption in state 4. Xanthine and xanthine oxidase generating superoxide were incubated with mitochondria for 10 min before the addition of NADH (b; no BSA present). Data obtained in the presence of BSA were similar (not shown).

weight close to 30 kDa reacting with anti-UCP2 antibodies was expressed only in pYedp-avian UCP yeasts.

(b) Higher state 4 oxygen consumption for avian UCP in the absence of BSA

When mitochondrial proton leak was estimated using the plots of oxygen consumption versus membrane potential in the presence of BSA, no difference was found between control and avian UCP mitochondria. However, in the absence of BSA, avian UCP mitochondria showed an increased oxygen consumption in state 4 (figure 2a; dashed circles). Addition of GDP had no influence on this state 4 rate or on membrane potential (figure 2a).

These measurements were performed following the incubation of mitochondria with the superoxide

generating system (xanthine plus xanthine oxidase) given that treatment of mitochondria with a superoxide generating system was suggested to trigger UCP2 activity (Echtay *et al.* 2002). However, the proton conductance across the inner membrane of avian UCP mitochondria did not change in the presence of superoxide (figure 2b).

(c) Retinoic acid activates avian UCP

This lack of significant difference between avian UCP and control isolated mitochondria under basal conditions (BSA) might be due to the lack of the proper activator for proton leakage via avian UCP. Thus, known activators of UCPs had to be tested. These include fatty acids, retinoic acid and pH (Rial *et al.* 1999). Oxygen consumption was recorded simultaneously in two oxygen chambers for control and avian UCP yeasts. Experiments were done

Figure 3. Effect of lauric, palmitic (FFA) and retinoic acids on the respiratory rate of mitochondria isolated from yeast expressing pYedp, UCP1 or avian UCP. (a) Respiratory rate of yeast mitochondria incubated at 20 °C in the presence of NADH and oligomycin. FFAs or retinoic acid was added progressively during oxygen consumption recordings. Data represent the mean value $\pm$ s.e.m. of three (FFAs) or four (retinoic acid) independent experiments performed at least in duplicate. *Differences are significant from 50–70 μ M and ANOVA for correlated samples, $F < -2.15$, $p < 0.05$. Mean value for state 3 of respiration (in the presence of ADP) of avian UCP mitochondria are indicated. (b) Simultaneous recording of membrane potential changes of isolated mitochondria expressing avian UCP with values indicated above the trace and oxygen consumption (Ox) rates (nmol O₂ min⁻¹ mg⁻¹) in the presence of all-*trans* retinoic acid (subsequent addition of 25 μ M). Retinoic acid caused a decrease in membrane potential and a stimulation of respiration that was not reversed by addition of 3 mM GDP.

in the presence of 0.1% BSA (16 μ M). Subsequent addition of palmitic acid or lauric acid (up to 150 μ M) did not induce an increase in the oxygen consumption of the transfected yeasts, contrary to the sharp rise observed with mitochondria prepared from a rat UCP1-expressing yeast strain (figure 3a) showing a considerable increase in the sensitivity of mitochondria to FFA when UCP1 is present (Rial *et al.* 1999). Titration with all-*trans* retinoic acid showed a significantly higher sensitivity of avian UCP mitochondria to this compound when compared with control (pYedp mitochondria). No difference was observed in the oxygen consumption when the experiments were

done at pH 6.8, 7 or 7.5. Membrane potential and oxygen consumption both depend upon membrane conductance. Therefore, the simultaneous recording of membrane potential showed that retinoic acid addition resulted in an immediate membrane potential decrease followed by an increase in oxygen consumption, demonstrating that the increase in respiratory rate is explained by an increase in membrane conductance of protons (figure 3b). Following the accepted procedures used to study activation and inhibition of UCP1, we explored the possibility that GDP could inhibit the proton transport catalysed by avian UCP activated by retinoic acid. However, several additions of

3 mM GDP were not able to reverse the activation of avian UCP by retinoic acid. This indicates functional differences between avian UCP and UCP1. In fact, it has not been shown that GDP could reverse activation of UCP2 or of avian UCP, but was only able to prevent activation if the GDP was added first (Talbot *et al.* 2003). Therefore, the apparent contradiction in the effect of GDP seems to stem from a poor sensitivity of avian UCP (UCP2) to this inhibitor which renders it ineffective once activation has occurred.

(d) Avian UCP protects mitochondria against oxidative stress

Oxygen radicals inactivate the Krebs cycle enzyme aconitase whereas fumarase, another Krebs cycle enzyme, is insensitive to these radicals. Therefore, the aconitase/fumarase ratio is a convenient measure of the oxidative damage to mitochondria (Li *et al.* 2001). MnSOD activity can be used as a reflection of the strength of antioxidant defences in mitochondria. Prepared avian UCP isolated mitochondria display a slightly but significantly higher initial aconitase/fumarase ratio (figure 4a; isolated mitochondria). When the aconitase/fumarase ratio was determined in mitochondria incubated for 20 min in state 4 (the approximate time used for a cyanide titration during proton leak measurements at different membrane potentials), the ratio was halved compared with the aconitase/fumarase ratio observed for mitochondrial preparations. No further difference was then observed between avian UCP and control mitochondria. After the titration experiments with cyanide (KCN) used to plot figure 4, the aconitase/fumarase ratio was further reduced. In control mitochondria the ratio was reduced by a factor of 6.8 (compared with state 4), while for avian UCP mitochondria the ratio was reduced only by a factor of 2.3. Exposure to xanthine plus xanthine oxidase before cyanide titration lowered the ratio even further, confirming that more extensive oxidative damage was created by this superoxide-generating system. No further difference was seen between avian UCP and control mitochondria in this case. In these experiments it was also shown that GDP addition has no effect.

MnSOD activity measured in six independent mitochondrial preparations is presented in figure 4b. For each paired pYedp/pYedp-avian UCP mitochondria, MnSOD activity was always slightly higher in the control strain, leading to a mean MnSOD activity ratio avian UCP/pYedp of 0.620 ± 0.094 (ANOVA, $F = -3.57$, $p = 0.008$). This result tallies with the aconitase/fumarase data obtained on isolated mitochondria, suggesting that avian UCP confers on isolated yeast mitochondria an improved ability to prevent oxygen radical production and a lesser need to upregulate antioxidant defence mechanisms.

4. DISCUSSION

(a) Avian UCP and oxidative stress

In this study, yeast mitochondria containing avian UCP differed by a significantly increased state 4 of respiration. However, a detailed analysis (figure 2) showed that the explanation for this is not an increased permeability of the inner membrane to protons. Actually, the presence of avian UCP seemed to result in a higher activity of the respiratory chain in mitochondria without uncoupling. A first conclusion is that the presence of avian UCP has no

Figure 4. Response of matrix aconitase activity to mitochondria isolation, respiration, KCN treatment and exogenous superoxide, and MnSOD activity in avian UCP mitochondria. (a) Aconitase activity was measured spectrophotometrically in isolated mitochondria of yeasts transfected with avian UCP plasmid. Mitochondria were taken before, during and after cyanide titration in the presence or absence of the superoxide-generating system (xxo). The results are means $\pm$ s.e.m. of three correlated paired experiments undertaken in duplicate, ANOVA for correlated samples; *isolated mitochondria $F = -3.33$, $p = 0.029$; **KCN $t = -7.25$, $p = 0.005$. Measurements undertaken in the presence of GDP (grey bars) during KCN or KCN⁺ superoxide titrations did not modify the aconitase activity in avian UCP yeasts compared with xxo samples (ANOVA for correlated samples, $F = -1.83$, $p = 0.14$). (b) MnSOD activity was measured on six independent preparations of isolated mitochondria of either pYedp or avian UCP yeasts, as described in §2. MnSOD quantifications were normalized for each track by the maximal respiration rate that is proportional to the mitochondrial protein quantity and then MnSOD activity for pYedp was normalized at 1. Inset: two examples of MnSOD activity measured on native gel. *MnSOD activity in avian UCP mitochondria was significantly reduced (0.620 ± 0.094 versus 1; ANOVA for correlated samples, $F = -3.57$, $p = 0.008$).

deleterious effect on yeast mitochondria. State 4 is associated with enhanced ROS production owing to the high membrane potential (Papa & Skulachev 1997). Based on the reduced damage owing to ROS (figure 4), our data support the possibility of a control of oxidative stress by avian UCP when expressed in yeast mitochondria. This result can be related to the induced expression of avian UCP by the utilization of lipid as fuel substrate, during fasting or rewarming after torpor or long diving events (Vianna *et al.* 2001; Evock-Clover *et al.* 2002;

Collin *et al.* 2003b), which can be interpreted as an increased exposure to ROS production (St. Pierre *et al.* 2002). However, since this damage prevention is not a result of uncoupling, the mechanism of action of avian UCP remains undefined. Indeed, avian UCP uncouples only in the presence of retinoic acid (figure 3), a strong activator of both transcription and activity of the thermogenic UCP1 and of UCP2 (Rial *et al.* 1999). It is notable that the increase in respiration obtained in the presence of retinoic acid compares well with the state 3 rate in the presence of a saturating amount of ADP. This means that with the expression level found in this model, activation of avian UCP leads to a large increase in energy expenditure and in heat production. Therefore, this uncoupling activity could confer on avian UCP the potential to be also a thermogenic protein when animals face cold stress (Talbot *et al.* 2004).

Because we did not observe any induced uncoupling activity or a greater protection against exogenous superoxide (figure 4), we conclude that avian UCP is not activated after incubation with xanthine/xanthine oxidase when expressed in yeast mitochondria. This result contrasts with the superoxide activation of penguin UCP obtained from skeletal muscle (Talbot *et al.* 2003, 2004), but a direct involvement of avian UCP was not demonstrated in these papers. A lack of superoxide-induced uncoupling activity was also observed in yeast expressing UCP3 (Harper *et al.* 2002). Recent studies have proposed that superoxide activation of UCPs is achieved by superoxide-induced lipid peroxidation of the mitochondrial membrane (Echtay *et al.* 2003). These lipids, generated after attack on mitochondrial phospholipids by superoxide, are expected to differ whether they are from yeast or mammalian mitochondria. Such products, which share structural properties with retinoic acid, could therefore be different in mammals and yeast, thus potentially explaining the lack of stimulation of avian UCP by superoxide observed in our study. Our non-effect of GDP on avian UCP is not surprising when activation by retinoic acid of mammalian UCP2 was also not reversed by GDP (Rial *et al.* 1999). Indeed, whether UCPs are regulated via the same general mechanism has not been definitively proven and remains a point of controversy (Couplan *et al.* 2002).

Carotenoids provide coloration for sexual signals (Olson & Owens 1998), promote immune function (Blount *et al.* 2003b) and are used as antioxidant molecules. The organism must therefore divide its carotenoid pool between these functions. In the case of oxidative stress, the balance in carotenoid investment is disrupted, thereby inducing a cost for the animal in terms of sexual selection or reduced life expectancy (Blount *et al.* 2003a). The specific stimulation of avian UCP by retinoic acid, which derives from β -carotene, could confer on carotenoids a new role in oxidative defence, by directly controlling mitochondrial ROS production. As stressed by von Schantz *et al.* (1999), one way to reduce oxidative stress is to minimize the production of free radicals. In this case, avian uncoupling protein may be a key protein, which, by its prevention of ROS damage, may also play an ecological and evolutionary role.

An interesting finding in previous studies was that avian UCP gene expression rose in the heart of hummingbirds when they are in torpor (i.e. their body temperature is reduced to 15 °C) and in rewarming (Vianna *et al.* 2001).

Such a phenomenon can be related to the ischaemia–reperfusion process, since during rewarming or reperfusion oxygen consumption and metabolic activity are sharply increased. A recent study demonstrated that when UCP1 is expressed in mammalian heart muscle, it protects against oxidative damage initiated by the high concentration of oxygen during reperfusion (Hoerter *et al.* 2004). Hummingbird avian UCP is expressed at high levels in heart muscle, which represents 2% of the total body mass (0.6% in mammals), and helps sustain the higher working frequency in vertebrates (1250 bpm; Lasiewski 1964). Thus, avian UCP may provide effective control of ROS production in the hummingbird during rewarming. This control process could also have a physiological significance during long migratory flights of this species, fat being the principal fuel (Suarez *et al.* 1986) and probably increasing free radical production.

(b) *State-dependent roles for avian UCP*

Previous studies of gene expression of avian UCP supported the view that avian UCP uncoupling activity may be triggered only temporarily when its mRNA levels are enhanced to face a particular situation (i.e. thermogenesis; Vianna *et al.* 2001). To date, the latter study presented the only data recorded in yeast cells, and suggested that transient expression of hummingbird avian UCP induces a decrease in membrane potential. In the present paper, a deeper bioenergetic analysis was conducted comparing yeast mitochondria with or without avian UCP. The presence of avian UCP resulted in mitochondria having a stronger state 4 rate of respiration, although membrane permeability to protons was unchanged. Addition of retinoic acid led to mitochondrial uncoupling, reproducing the situation observed with mammalian UCP2. Finally, it was shown that the presence of avian UCP resulted in a lower oxidative damage *in vivo* (judged from the aconitase to fumarase ratio), whereas oxidative defences were lowered (MnSOD). Interestingly, using isolated mitochondria it was observed that the presence of avian UCP did not protect against oxidative damage when mitochondria were maintained in state 4, whereas protection was afforded when inhibition of the respiratory chain occurred. In these experiments, the uncoupling activity of avian UCP was not triggered by retinoic acid and therefore remained undetectable. While we cannot exclude the possibility that marginal uncoupling escaped our attention, this may also question the hypothesis that all the protective effect of avian UCP comes from this marginal uncoupling, which *in vivo* could be replaced by an equally marginal ATP production. On the other hand, the observation reported here that in our model, the protection appeared to be restricted to situations of inhibition of the respiratory chain may have important implications because one expects organisms to face exposure to such poisons, which exist naturally, whereas a blockade of mitochondria in strict state 4 appears very unlikely.

Since the discovery of UCP1, uncoupling proteins have been found in a great variety of organisms from plants to mammals (reviewed in Jezek *et al.* 2001). Regarding the evolutionary process, organisms have been progressively confronted with the presence of dioxygen and of its deleterious effects on cells, before euthermy appeared. Therefore, uncoupling proteins and avian UCP may have first been selected to cope with oxygen and its unwanted

oxidation reactions, with their thermogenic role being acquired subsequently. Thus, one might hypothesize that avian UCP may ensure species- and state-specific roles, depending on the organism's transient needs (thermogenesis or protection against ROS damage). The specific immunodetection of avian UCP opens up the field of *in vivo* study of the role of this protein in birds.

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REFERENCES

- Arechaga, J., Raimbault, S., Prieto, S., Levi-Megruis, C., Zaragoza, P., Miroux, B., Ricquier, D., Bouillaud, F. & Rial, E. 1993 Cysteine residues are not essential for uncoupling protein function. *Biochem. J.* **296**, 693–700.
- Arsenijevic, D., *et al.* 2000 Disruption of the uncoupling protein-2 gene in mice reveals a role in immunity and reactive oxygen species production. *Nat. Genet.* **26**, 435–439.
- Beckman, K. B. & Ames, B. N. 1998 The free radical theory of ageing matures. *Physiol. Rev.* **78**, 548–571.
- Blount, J. D., Metcalfe, N. B., Arnold, K. E., Surai, P. F., Devevey, G. L. & Monaghan, P. 2003a Neonatal nutrition, adult antioxidant defences and sexual attractiveness in the zebra finch. *Proc. R. Soc. B* **270**, 1691–1696. (doi:10.1098/rspb.2003.2411)
- Blount, J. D., Metcalfe, N. B., Birkhead, T. R. & Surai, P. F. 2003b Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science* **300**, 125–127.
- Collin, A., Buyse, J., Van As, P., Darras, V. M., Malheiros, R. D., Moraes, V. M. B., Reyns, G. E., Taouis, M. & Decuypere, E. 2003a Cold-induced enhancement of avian uncoupling protein expression, heat production, and triiodothyronine concentrations in broiler chicks. *Gen. Comp. Endocrinol.* **130**, 70–77.
- Collin, A., Malheiros, R. D., Moraes, V. M. B., Van As, P., Darras, V. M., Reyns, G. E., Taouis, M., Decuypere, E. & Buyse, J. 2003b Effects of dietary macronutrient content on energy metabolism and uncoupling protein mRNA expression in broiler chickens. *Br. J. Nutr.* **90**, 261–270.
- Collin, A., Taouis, M., Buyse, J., Ifuta, N. B., Darras, V. M., Van As, P., Malheiros, R. D. & Decuypere, E. 2003c Thyroid status, but not insulin status, affects expression of avian uncoupling protein mRNA in chicken. *Am. J. Physiol. Endocrinol. Metab.* **284**, E771–E777.
- Couplan, E., Gonzalez-Barroso, M. D. M., Alves-Guerra, M.-C., Ricquier, D., Goubern, M. & Bouillaud, F. 2002 No evidence for a basal, retinoic, or superoxide-induced uncoupling activity of the uncoupling protein 2 present in spleen or lung mitochondria. *J. Biol. Chem.* **277**, 26268–26275.
- Echtay, K. S., *et al.* 2002 Superoxide activates mitochondrial uncoupling proteins. *Nature* **415**, 96–99.
- Echtay, K. S., *et al.* 2003 A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. *EMBO J.* **22**, 4103–4110.
- Evock-Clover, C. M., Poch, S. M., Richards, M. P., Ashwell, C. M. & McMurtry, J. P. 2002 Expression of an uncoupling protein gene homolog in chickens. *Comp. Biochem. Physiol. A* **133**, 345–358.
- Fleury, C., *et al.* 1997 Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. *Nat. Genet.* **15**, 269–272.
- Harper, J. A., Stuart, J. A., Jekabsons, M. B., Roussel, D., Brindle, K. M., Dickinson, K., Jones, R. B. & Brand, M. D. 2002 Artifactual uncoupling by uncoupling protein 3 in yeast mitochondria at the concentrations found in mouse and rat skeletal-muscle mitochondria. *Biochem. J.* **361**, 49–56.
- Hoerter, J., Gonzalez-Barroso, M. D. M., Couplan, E., Mateo, P., Gelly, C., Cassard-Doulcier, A.-M., Diulez, P. & Bouillaud, F. 2004 Mitochondrial uncoupling protein 1 expressed in heart of transgenic mice protects against ischemic reperfusion damage. *Circulation*.
- Holmes, D. J. & Austad, S. N. 1995 Birds as animal models for the comparative biology of aging: a prospectus. *J. Gerontol.* **50A**, B59–B66.
- Hughes, K. A. & Reynolds, R. M. 2005 Evolutionary and mechanistic theories of aging. *Annu. Rev. Entomol.* **50**, 421–445.
- Jezeq, P., Borecky, J., Zackova, M., Costa, A. D. T. & Arruda, P. 2001 Possible basic and specific functions of plant uncoupling proteins (pUCP). *Biosci. Rep.* **2**, 237–245.
- Kirkwood, T. B. L. & Austad, S. N. 2000 Why do we age? *Nature* **408**, 233–238.
- Lasiewski, R. C. 1964 Body temperature, heart and breathing rate, and evaporative water loss in hummingbirds. *Physiol. Zool.* **37**, 212–223.
- Li, Q. Y., Pedersen, C., Day, B. J. & Patel, M. 2001 Dependence of excitotoxic neurodegeneration on mitochondrial aconitase inactivation. *J. Neurochem.* **78**, 746–755.
- Locke, R. M., Rial, E., Scott, I. D. & Nicholls, D. G. 1982 Fatty acids as acute regulators of the proton conductance of hamster brown-fat mitochondria. *Eur. J. Biochem.* **129**, 373–380.
- Melov, S., *et al.* 2000 Extension of life-span with superoxide dismutase/catalase mimetics. *Science* **289**, 1567–1569.
- Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfranccone, L. & Pelicci, P. G. 1999 The p66^{shc} adaptor protein controls oxidative stress response and life span in mammals. *Nature* **402**, 309–313.
- Miwa, S. & Brand, M. D. 2003 Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. *Biochem. Soc. Trans.* **31**, 1300–1301.
- Nicholls, D. G. & Locke, R. M. 1984 Thermogenic mechanisms in brown fat. *Physiol. Rev.* **64**, 1–64.
- Olson, V. A. & Owens, I. P. F. 1998 Costly sexual signals: are carotenoids rare, risky or required? *Trends Ecol. Evol.* **13**, 510–514.
- Papa, S. & Skulachev, V. P. 1997 Reactive oxygen species, mitochondria, apoptosis and aging. *Mol. Cell. Biochem.* **174**, 305–319.
- Pecqueur, C., Alves-Guerra, M.-C., Gelly, C., Levi-Meyrueis, C., Couplan, E., Collins, S., Ricquier, D., Bouillaud, F. & Miroux, B. 2001 Uncoupling protein 2, *in vivo* distribution, induction upon oxidative stress, and evidence for translational regulation. *J. Biol. Chem.* **276**, 8705–8712.
- Perez-Campo, R., Lopez-Torres, M., Cadenas, S., Rojas, C. & Barja, G. 1998 Longevity and antioxidant enzymes, non-enzymatic antioxidants and oxidative stress in the vertebrate lung: a comparative study. *J. Comp. Physiol. B* **168**, 149–158.
- Raha, S. & Robinson, B. H. 2000 Mitochondria, oxygen free radicals, and apoptosis. *Trends Biochem. Sci.* **25**, 502–508.
- Raimbault, S., *et al.* 2001 An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds. *Biochem. J.* **353**, 441–444.
- Rial, E., Gonzalez-Barroso, M. D. M., Fleury, C., Iturizaga, S., Sanchis, D., Jimenez-Jimenez, J., Ricquier, D., Goubern, M. & Bouillaud, F. 1999 Retinoids activate proton transport by the uncoupling proteins UCP1 and UCP2. *EMBO J.* **18**, 5827–5833.

- Ricquier, D. & Bouillaud, F. 2000 The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *Biochem. J.* **345**, 161–179.
- St. Pierre, J., Buckingham, J. A., Roebuck, S. J. & Brand, M. D. 2002 Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem.* **277**, 44 784–44 790.
- Suarez, R. K., Brown, G. S. & Hochachka, P. W. 1986 Metabolic sources of energy for hummingbird flight. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **251**, R537–R542.
- Talbot, D. A., Hanuise, N., Rey, B., Rouanet, J.-L., Duchamp, C. & Brand, M. D. 2003 Superoxide activates a GDP-sensitive proton conductance in skeletal muscle mitochondria from king penguin (*Aptenodytes patagonicus*). *Biochem. Biophys. Res. Commun.* **312**, 983–988.
- Talbot, D. A., Duchamp, C., Rey, B., Hanuise, N., Rouanet, J.-L., Sibille, B. & Brand, M. D. 2004 Uncoupling protein and ATP/ADP carrier increase mitochondrial conductance after cold adaptation of king penguins. *J. Physiol.* **558**, 123–135.
- Vianna, C. R., *et al.* 2001 Cloning and characterization of an uncoupling homolog in hummingbirds. *Physiol. Genomics* **5**, 137–145.
- von Schantz, T., Bensch, S., Grahm, M., Hasselquist, D. & Wittzell, H. 1999 Good genes, oxidative stress and condition-dependent sexual signals. *Proc. R. Soc. B* **266**, 1–12. (doi:10.1098/rspb.1999.0597)
- Wong, P. C., Pardo, C. A., Borchelt, D. R., Lee, M. K., Copeland, N. G., Jenkins, N. A., Sisodia, S. S., Cleveland, D. W. & Price, D. L. 1995 An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* **14**, 1105–1116.

As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.