J Bioenerg Biomembr (2010) 42:345–353
DOI 10.1007/s10863-010-9303-x

Identification and characterization of uncoupling protein in heart and muscle mitochondria of canary birds

Malgorzata B. Slocinska · Zakaria Ali Moh. Almsherqi · Francis E. Sluse · Rachel Navet · Yuru Deng

Received: 24 April 2010 / Accepted: 16 June 2010 / Published online: 5 August 2010

Abstract An uncoupling protein (cUCP) was identified in heart and skeletal muscle mitochondria of canary birds. cUCP was immunodetected using polyclonal antibodies raised against murine UCP2. Its molecular mass was similar to those of mammalian UCPs (32 kDa). The activity of cUCP was stimulated by palmitic acid (PA) and inhibited by GTP mainly in state 3 respiration. Additions of PA augmented state 4 respiration and lowered the ADP/O ratio. Thus, the activity of cUCP diverted energy from oxidative phosphorylation in state 3 respiration. cUCP in heart and skeletal muscles of canary birds might have implications in thermogenesis as well as protection against free radical production.

Keywords Mitochondria · Uncoupling protein · Heart · Skeletal muscle · Canary birds

Abbreviations

cUCP	uncoupling protein in canary birds
avUCP	uncoupling protein in birds
V_{UCP}	contribution of UCP activity in state 3 respiration
$V_{ATP\ synthase}$	contribution of ATP synthase in state 3 respiration
V_3 (Jo)	respiratory rate in state 3 respiration
Jp	rate of ATP synthesis
FFA	free fatty acids
PA	palmitic acid
GTP	guanosine triphosphate
BSA	bovine serum albumin
CATR	carboxyatractylozide
RCR	respiratory control ratio
CQ	coenzyme Q

Introduction

In 1961, Peter Mitchell has proposed that electron transport and ATP synthesis are coupled by a proton gradient across the inner mitochondrial membrane (Mitchell 1979). According to this hypothesis, transfer of electrons through the respiratory chain leads to the pumping of protons from the matrix to the cytosolic site of the inner mitochondrial membrane. Protons ejected from mitochondria return through the ATP synthase, driving ATP synthesis. However, some of the protons backflow to the matrix bypassed the ATP synthase. This proton leak was originally considered as an isolation artifact (Skulachev 1998). Since the discovery of brown adipose tissue (BAT) in mammals, it has been gradually recognized that a factor that uncouples BAT mitochondria is an uncoupling protein (Nicholls and Locke 1984; Cannon and Nedergaard 1985). This protein, UCP1,

Malgorzata B. Slocinska and Zakaria Ali Moh Almsherqi contributed equally to this work

M. B. Slocinska (✉)
Department of Animal Physiology and Development,
Adam Mickiewicz University,
Poznań, ul.,
Umultowska 89, Poland
e-mail: slocina@amu.edu.pl

Z. A. M. Almsherqi · Y. Deng (✉)
Department of Physiology, Yong Loo Lin School of Medicine,
National University of Singapore,
Kent Ridge, Singapore
e-mail: phsdy@nus.edu.sg

F. E. Sluse · R. Navet
Laboratory of Bioenergetics, Center of Oxygen Research and Development, Department of Life Sciences,
Institute of Chemistry B6c, University of Liege,
Sart Tilman,
Liege, Belgium

present in the inner mitochondrial membrane, plays a crucial role in thermogenesis and is believed to be restricted to newborn, cold-acclimated and hibernating animals (Klingenberg 1990; Giacobino 2001). However, other uncoupling proteins in non-thermogenic tissues of mammals, such as UCP1, UCP2, UCP3, UCP4 and UCP5, have been identified (Ricquier and Bouilland 2000; Boss et al. 2000). The detectable amounts of UCP2 were found ubiquitously in different tissues (Fleury et al. 1997; Gimeno et al. 1997); UCP3 is predominantly expressed in the skeletal muscles (Boss et al. 1997), whereas UCP4 and UCP5 mainly are expressed in the central nervous system (Sanchis et al. 1998; Mao et al. 1999). UCP-like proteins have been also discovered in plants (Vercesi et al. 1995; Maia et al. 1998), fishes (Stuart et al. 1999), protists (Jarmuszkiewicz et al. 1999), fungi (Jarmuszkiewicz et al. 2000b), insects (Fridell et al. 2004) and nematodes (Iser et al. 2005). The cloning of avian UCP (avUCP) (Vianna et al. 2001, Raimbault et al. 2001) has added a new protein to the rapidly growing number of UCPs found throughout *Eucaryota*. It has been reported that avDNA encodes a protein highly homologous with mammalian UCP2 and UCP3 (around 70 %) and only 55% identical to UCP1 and is primarily expressed in the skeletal muscles (Vianna et al. 2001, Raimbault et al. 2001). However, high levels of avUCP have been also detected in heart and liver of hummingbirds (Vianna et al. 2001).

The action of UCPs belonging to the large mitochondrial anion carriers family is to mediate a protonophoretic cycle activated by free fatty acids (FFA) and inhibited allosterically by purine nucleotides (i.e. GDP, GTP, ADP or ATP) (Klingenberg 1990; Nicholls and Locke 1984). UCPs dissipate the H^+ electrochemical gradient built up by the respiratory chain thereby uncoupling the respiration from phosphorylation. A direct consequence of their activity is a decrease in ATP synthesis per oxygen consumed (yield of oxidative phosphorylation) and an increase in electron flux at the expense of reducing substrates (Skulachev 1996)

Although the role of UCP1 in heat generation is indisputable, the exact role of UCP2 and UCP3 still remains unclear. It has been shown that in mammals, UCPs decrease reactive oxygen species (ROS) production *in vivo* (Maxwell et al. 1999) and *in vitro* (Kowaltowski et al. 1998; Jarmuszkiewicz et al. 2000a); therefore, it can be considered an additional antioxidant system preventing oxidative damage of the cells at the expense of oxidative phosphorylation yield (Jarmuszkiewicz et al. 1998). Moreover, UCPs might be implicated in numerous physiological and pathological phenomena, like adaptive thermogenesis, body weight regulation, fatty acid metabolism, inflammation and heart failure (Samec et al. 1998a, b; Himms-Hagen and Harper 2001; Skulachev 1998; Echtay et al. 2002; Ježek et al. 2004). The upregulation of mammalian UCP2 and UCP3 mRNA levels has been observed during starvation (Samec et al. 1998a, b), cold (Boss et al. 1997) and heart failure (Essop et al. 2004)

In birds, the physiological role of UCP still waits for disclosure. Because of the absence of brown adipose tissue (Johnston 1971) and increased amounts of UCPs in birds in response to food deprivation and cold (Raimbault et al. 2001; Vianna et al. 2001; Talbot et al. 2004), avUCP may play a thermogenic role similar to UCP1 in BAT. However, its close molecular relationship to mammalian UCP2 and UCP3 (Vianna et al. 2001; Raimbault et al.; Bicudo et al. 2002) may indicate that physiological meaning of avUCP is more related to UCP2 and UCP3.

The features of long-lived canary birds (maximum life span= 24 years) (Altman and Dittmer 1972) with high metabolic rate and low mitochondrial free radical leak (Barja et al. 1994), in addition to the lacking of brown adipose tissue, make them a good model to explore the potential roles of uncoupling proteins in lowering ROS level and its contribution to slow aging. Moreover, the unique zigzag cristae in their heart mitochondria have been observed (Slautterback 1965). Although their functional significance remains obscure, the similar zigzag crista has been reported in the mitochondria of starved amoeba *Chaos carolinense* (Deng and Mieczkowski 1998) and has been suggested to play a protective role in response to oxidative stress (Deng et al. 2002).

Our studies are the first demonstration of the existence of uncoupling activity of UCP's homolog in the heart and skeletal muscle mitochondria of canary birds. In this report, we highlight the presence of canary UCP (cUCP) in the isolated heart and skeletal muscle mitochondria depleted of endogenous free fatty acids, through their sensitivity to palmitic acid (PA) and GTP. In addition, we investigated whether PA-induced UCP activity is able to divert energy from oxidative phosphorylation and that GTP inhibits UCP activity in a way that could be modulated by the redox state of coenzyme Q (CoQ).

Materials & methods

Animals

Canary birds (*Serinus canaries*) with age of 2–3 years old were obtained from local commercial suppliers in Singapore. Animal ethics were approved by the National University of Singapore in compliance with WHO International Guiding Principles for Animal Research.

Isolation of mitochondria

Animals were sacrificed and the heart and skeletal muscles were quickly collected in ice-cold isolation medium containing 100 mM KCl, 50 mM Tris-HCl pH 7.4, 1 mM K_2HPO_4,

100 mM sucrose, 50 mM EGTA and 0.2% bovine serum albumin (BSA) and chopped into small pieces within 1 minute. Afterwards, rings of fat and connective tissue were removed. The rinsed pieces of muscles were homogenized in isolation medium with a Polytron homogenizer set at 14.000 RPM for 3 s. The upper part of the homogenate was collected and homogenized with a Teflon-glass homogenizer at 1,000 RPM for 4 strokes. Muscle homogenate was centrifuged at 500 x g for 10 min at 4 °C and the obtained supernatant was centrifuged at 10,000x g for 10 min. The pellet containing mitochondria was washed in isolation medium and centrifuged at 6,000 x g for 10 min. The final mitochondrial pellet was suspended in the medium containing 0.2 M mannitol, 0.1 M sucrose, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, and stored on ice for further assay.

All above-mentioned procedures were performed at 4 °C. The presence of BSA in isolation medium allowed chelating of free fatty acids (FFA) from the mitochondrial suspension and the obtained mitochondria were fully depleted from FFA. Mitochondrial protein concentration was determined by the Bradford method (Bradford 1976).

The mitochondria from the heart and muscle of mice were isolated following the same protocol and procedures.

Mitochondrial oxygen consumption

Oxygen consumption of heart and muscle mitochondria was measured respectively at 25 °C in a water-thermostatized incubation chamber with a Clark-type O_2 electrode (Oxytherm, Hansatech) in 1.3 ml of incubation medium containing 0.2 M mannitol, 75 mM sucrose, 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 10 mM K_2HPO_4, and 10 mM KCl. All measurements were made in the presence of 10 mM succinate, 80 μM ATP, and 5 μM rotenone. ATP in final concentration of 80 μM did not affect UCP activity and was added to ensure complete activation of succinate dehydrogenase.

The ADP/O ratio was determined by an ADP pulse method with 175–250 nmoles of ADP and succinate as oxidizable substrate in the presence of rotenone to block electron input from Complex I. The total amount of oxygen consumed during state 3 respirations (Jo) was used for calculating the ADP/O ratio. ADP/O ratio and Jo were used to calculate the rate of ADP phosphorylation (Jp = Jo x ADP/O). The pre-pulse of ADP was applied before the main pulse of ADP to activate succinate dehydrogenase by the produced ATP. Proton leak was determined in state 3 phosphorylation by using increasing concentrations of n-butylmalonate.

Immunoblotting of UCPs

Mitochondrial proteins were separated in 12% polyacrylamide gels and electro-blotted onto nitrocellulose membranes. Blots were blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 30 min and probed overnight at 4 °C with affinity purified UCP2 and UCP3 antibodies (Chemicon, AB 3040 and AB 3046) in 1:500 dilutions. The blots were washed with TBS-T and incubated with affinity-purified goat anti-rabbit IgG (H&L) horseradish peroxidase-conjugated secondary antibodies (Chemicon) for 1 h at room temperature. The blots were washed again and developed using a standard ECL detection kit (Amersham). Blot results were quantitatively analysed by Scan Pack 3.0

Results

Immunological detection of UCP in canary heart and skeletal muscle

Polyclonal antibodies developed against murine UCP2 and human UCP3 were used to evidence the presence of UCP (cUCP) in canary's heart and muscle mitochondria, respectively. A single band with molecular weight of 32 kDa was detected by anti-UCP2 and anti-UCP3 antibodies (Fig. 1A, B) showing high specificity towards UCP2 and UCP3. Cross-reactivity of mammalian UCP2 and UCP3 with a cUCP homolog confirmed the close sequence homology between these proteins in birds and mammals.

Respiratory rates measurements in canary heart and skeletal muscle mitochondria

Figure 2 shows oxygen consumption in resting state 4 with succinate (plus oligomycin) as oxidizable substrate in heart and skeletal muscle mitochondria of canary birds. The successive, increasing concentrations of PA were added (up to 30 μM) to reach maximum stimulation of state 4 respiration by PA. After addition of 20 μM PA, state 4 respiration rate increased from 70 to 229 nmoles of oxygen/min/mg of mitochondrial proteins for the skeletal muscle and from 71 to 187 nmoles of oxygen/min/mg of mitochondrial proteins for the heart, respectively. The half maximum stimulation by PA was reached at 10 μM PA. PA-stimulated respiration was only weakly inhibited by purine nucleotide such as GTP. Thus, when GTP was added in the absence of BSA, almost no change in respiratory rate was observed. The addition of BSA, which adsorbs FFA, partially reversed the PA-stimulated respiration. Similar slopes of oxygen uptake after addition of BSA and before addition of PA (for heart) and higher respiratory rate after BSA compared to the initial rate, before PA addition (for muscle), confirmed the absence of endogenous FFA in the mitochondrial suspension (Fig. 2). The GTP inhibition observed in the resting respiration state was between 0–10%.

Determination of coupling parameters in heart and muscle mitochondria of canary birds

State 3 respiratory rates and ADP/O ratios were measured during ADP/O pulses in the presence or absence of 10 μM PA (Fig. 3). State 3 respiration remains unchanged in the presence of PA, whereas ADP/O and respiratory control ratio (RCR) significantly declined in both skeletal and heart muscle mitochondria (Fig. 3 and Table 1). A drop in ADP/O ratio in the presence of PA indicates a decrease in the efficiency of ATP synthesis and suggests the possible activation of cUCP protein. The effect of PA on ADP/O ratio was PA-concentration dependent.

The contribution of cUCP activity and ATP synthesis in state 3 phosphorylation were calculated from the paired measurements of ADP/O and Jo (V_3) in the absence and presence of 10 μM PA. As described by Jarmuszkiewicz et al. (1999):

$$V_{ATP\ synthase} = Jo \times (ADP/O)_{+PA}/(ADP/O)_{-PA} \quad (1)$$

and

$$V_{UCP} = Jo - V_{ATP\ synthase}. \quad (2)$$

cUCP contribution to state 3 respiration is shown as the mean calculated values obtained with a set of mitochondrial preparations, 46±3 and 51±2 nmoles of oxygen/min/mg of protein for the heart and skeletal muscle respectively. Proton leak induced by PA (that can be attributed to cUCP activity) and proton leak occurring in the absence of PA (endogenous proton leak) were illustrated in Fig. 4.

In order to determine how the participation of ATP synthase and cUCP changed during phosphorylating respiration, the rate of succinate dehydrogenase was decreased by succinate uptake limitation. Example obtained from a single mitochondrial preparation is shown in Table 2A and B. ADP/O ratios and state 3 respiratory rates were measured in the absence or presence of 10 μM PA with increasing concentrations of n-butylmalonate, which inhibits the uptake of succinate as oxidizable substrate. Figure 4 and Table 2 show that oxygen consumption in state 3 respiration gradually decreased with the increasing concentrations of n-butylmalonate up to 5 mM (in the case of heart muscle) and up to 2 mM (in the case of skeletal muscle), inhibiting respiration in state 3 phosphorylation. In the absence of PA, ADP/O remained constant, whereas in the presence of PA, ADP/O was lower and decreased with increasing the concentrations of n-butylmalonate (Table 2). Thus, the lowering electron supply to the cytochrome pathway amplified the decrease in ADP/O induced by PA.

In the absence of PA, titration of respiration with n-butyl malonate reveals a linear relationship between Jp and Jo crossing the abscissa axis on the right of origin in mitochondria depleted of FFA. This means that a part of state 3 respiration is sustained by a constant, endogenous proton leak. As was shown in Fig. 4, proton leak in muscle and heart mitochondria was very low, 6 and 12 nmoles of oxygen/ml/mg of protein, respectively, contrary to mammalian skeletal muscle (25 nmoles of oxygen/ml/mg of protein) (Jarmuszkiewicz et al. 2004).

Discussion

Our results support evidence for the activity of an UCP-like protein in heart and skeletal muscle mitochondria of canary birds. To date, the experimental evidence of avian UCPs has come only from their expression in yeast mitochondria (Klingenberg et al. 2001; Jaburek et al. 1999) and

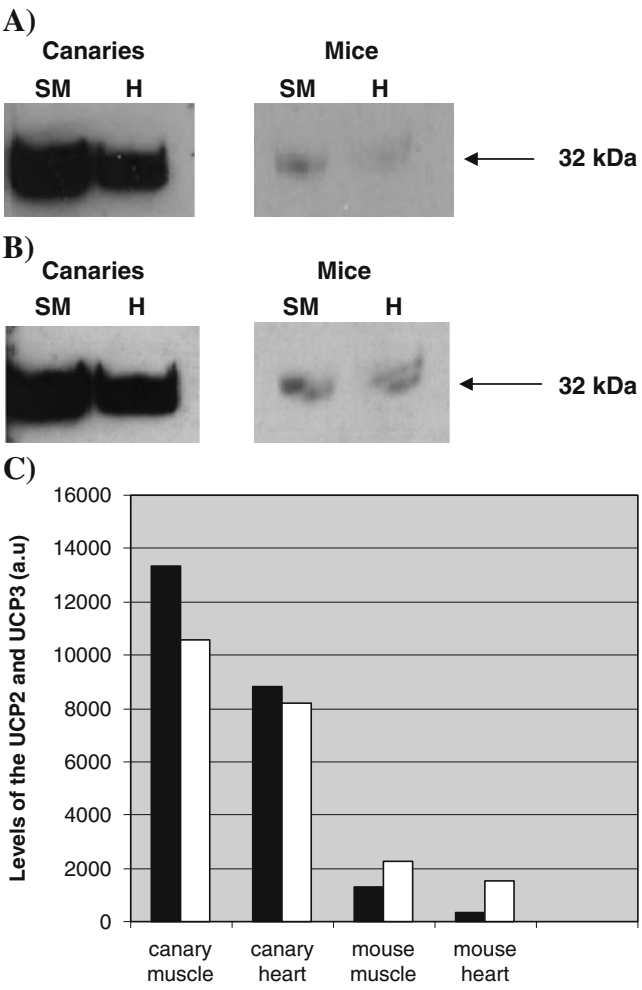

Fig. 1 UCP2 (**A**) and UCP3 (**B**) immunoreactivity in mitochondria isolated from skeletal muscle (SM) and heart (H) of canary birds and mice (32 kDa band). Mice mitochondria were used as a control. 100 μg of mitochondrial protein was added per lane. **C**) UCP protein expression level in canary and mice heart mitochondria. Immunodetection with UCP2 is labeled as ■. Immunodetection with UCP3 is labelled as □. The results were representative of at least three independent experiments

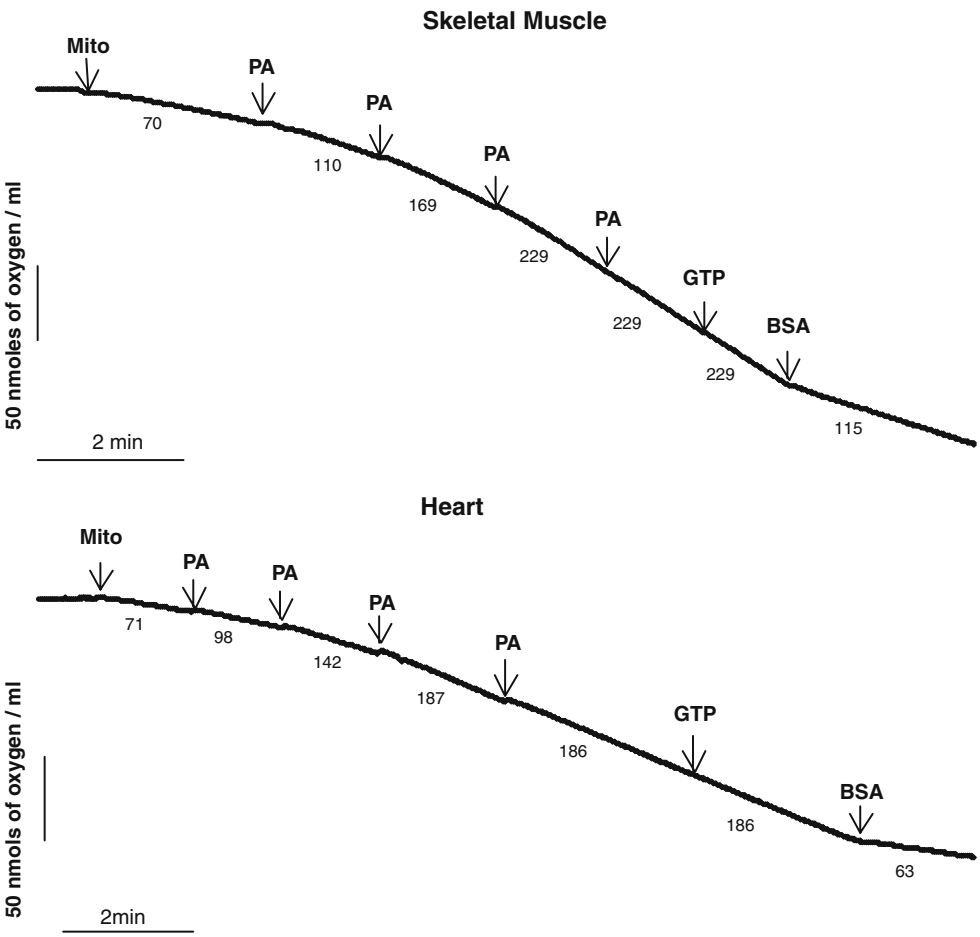

Fig. 2 The effect of PA on respiratory rates of canary heart and skeletal muscle mitochondria depleted of free fatty acids. Mitochondria were incubated as described with oligomycin (2 μg/mg of protein) to inhibit ATP synthase. Mitochondria (mito), PA (in sequence: 5 μM, 10 μM, 20 μM, 30 μM), 2 mM GTP, and 0.2% BSA were added as indicated. Numbers on the traces refer to oxygen consumption in nmoles of oxygen/ml/mg of proteins obtained from one mitochondrial preparation. The mean value for heart mitochondria (8 oxygen traces) for state 4 and state 4 plus 20 μM PA, were 70±4 and 187±8, respectively; for skeletal muscle mitochondria (5 oxygen traces), 70±6 and 229±14, respectively

reconstitution into liposomes (Žačkova et al. 2003). Here, we highlight for the first time uncoupling activity of canary UCP (cUCP) in isolated heart and skeletal muscle mitochondria through their sensitivity to free fatty acids (PA) and purine nucleotides (GTP).

We demonstrate that oligomycin-treated mitochondria from canary heart and skeletal muscles fully depleted of endogenous free fatty acids, exhibit a PA-stimulation of state 4 respiration, suggesting the existence of FFA-induced H^+ reuptake. Stimulation of state 4 by PA was also observed in the presence of CATR, an inhibitor of the adenylate nucleotide translocator (ANT). ANT thus could not participate in the FFA-induced H^+ dissipation. As was shown in Fig. 2, addition of 20 μM PA to FFA-depleted mitochondria in the resting state stimulated respiration, up to 220% in the skeletal muscle and 162% in the myocardial mitochondria. Higher stimulation of respiration by PA in state 4 respiration of skeletal muscle mitochondria compared to heart mitochondria may indicate the higher activity of UCP protein and its physiological significance in muscle mitochondria of canary birds. It has been suggested that the muscles of birds are the main sites of non-shivering thermogenesis and their body heat might be generated through mediation of uncoupling proteins (Duchamp et al. 1991). The GTP inhibition observed in the resting respiratory state was between 0–10%. The lack of GTP inhibition and insensitivity to purine nucleotides might be due to the high reduction level of CoQ in this resting respiratory state. It has been proposed by Sluse and coworkers (Jarmuszkiewicz et al. 2004; Navet et al. 2005) that the coenzyme Q redox state could be the metabolic sensor that the modulates the purine nucleotide inhibition of UCPs. This could be the same in the heart and skeletal muscle mitochondria of canary birds.

As was illustrated in Fig. 3 and Tables 1 & 2, PA did not stimulate respiratory rate in state 3 but its uncoupling effect was evidenced by a decrease in ADP/O and respiratory control ratio (RCR). Proton gradient built up by the respiratory chain that is used for ATP synthesis in the absence of PA could be shared between oxidative phosphorylation and cUCP activity in the presence of PA. The PA concentration-dependent (not shown) decrease in ADP/O ratio resulting in a significant decline in the efficiency of oxidative phosphorylation demonstrates an activation of cUCP in state 3 respiration. Above-mentioned results imply that PA-induced H^+ recycling can divert energy from oxidative phosphorylation in state 3 respiration even if state 3 is not increased.

In animal mitochondria, not only UCPs but also several other mitochondrial anion carriers like the ATP/ADP anti-

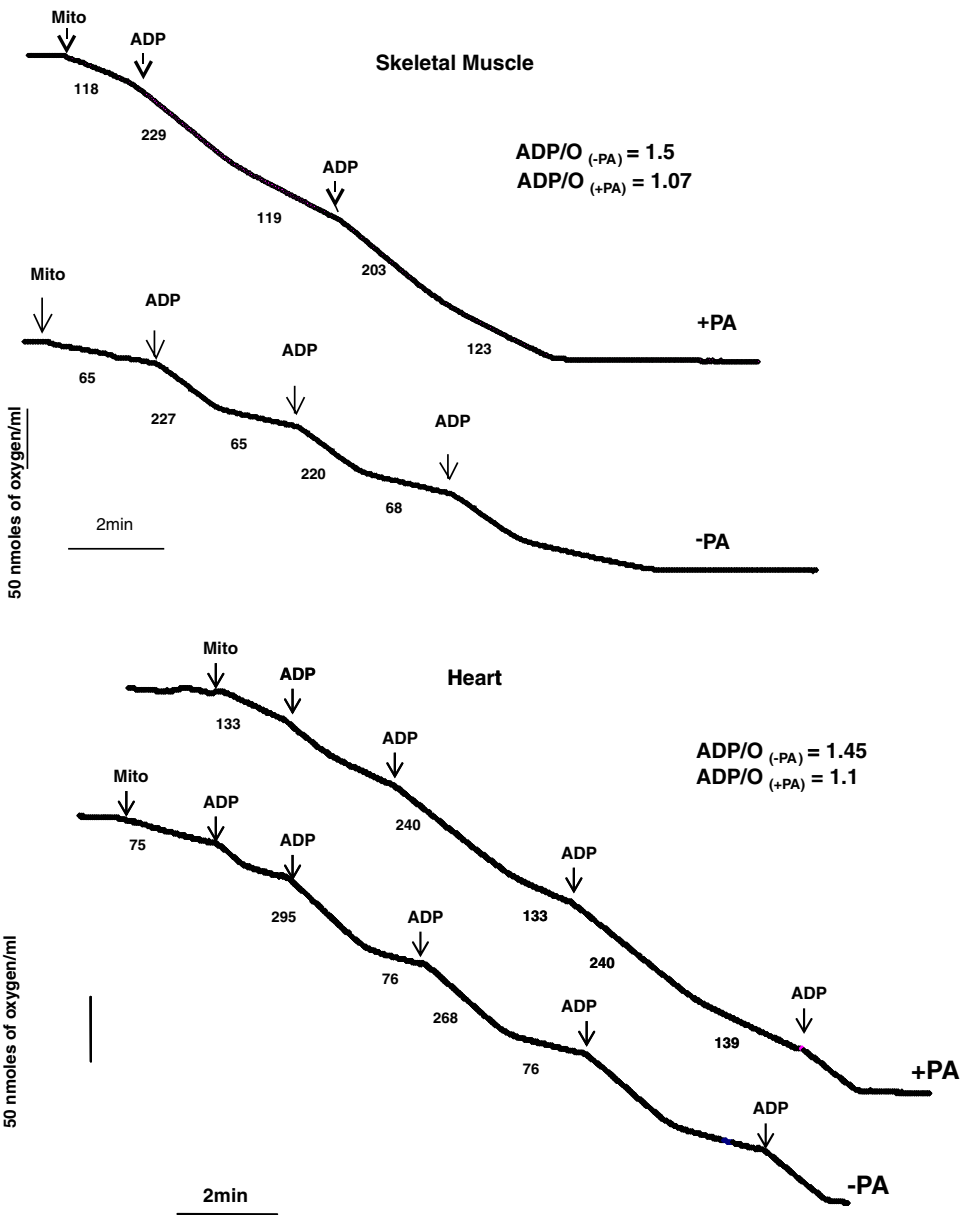

Fig. 3 The effect of PA on coupling parameters of canary heart and skeletal muscle mitochondria. Mitochondria were incubated as described, in the presence or absence of 10 μM PA. A representative example of respiratory rates from one of 8 and 5 mitochondrial preparations, for heart and skeletal muscles, respectively

porter, and phosphate or dicarboxylate carrier are considered to mediate FFA-induced uncoupling (Andreyew et al. 1989; Žačkova et al. 2000; Wieckowski and Wojtczak 1997). However it seems unlikely that such mitochondrial uncoupling takes place during state 3 phosphorylating respiration, by the carriers that are mainly engaged in ADP, succinate, or phosphate import. Thus, the first candidate to catalyze the PA-induced H^+ re-recycling observed in canaries' skeletal and heart muscle mitochondria is uncoupling protein.

The measurement of ADP/O ratio (Table 1) was used to determine the contribution of dissipating pathway (PA-stimulated proton leak) and cytochrome pathway leading to ATP synthesis in canary's heart and skeletal muscle mitochondria during state 3 phosphorylation. The ADP/O calculations can be done only when isolated mitochondria are well coupled and stable during the time of experiment. According to the equations (1) and (2), $V_{ATP\ synthase}$ represents 73% and V_{UCP} represents 27% of state 3 respiration at 10 μM PA for the skeletal muscle and 83% and 17% for the heart, respectively. These numbers indicate the higher activation and/or a higher content of cUCP in the mitochondria of skeletal muscle compared to heart. Our immunoblotting results support these observations. So far, most of the studies on UCPs have been based on the detection of mRNA levels by using Northern blot techniques (Fleury et al. 1997; Gimeno et al. 1997; Vianna et al. 2001). We found detectable amounts of UCP protein with western blotting in intact isolated mitochondria under normal physiological conditions (Fig. 1A, B). The amount of UCP protein in canary skeletal muscle was slightly

Table 1 Effect of PA on coupling parameters in heart and skeletal muscle mitochondria of canary birds

	ADP/O	RCR
HEART		
−PA	1.45±0.01	3.9±0.4
+PA	1.2±0.1	1.8±0.1
MUSCLE		
−PA	1.5±0.03	3.0±0.05
+PA	1.07±0.3	1.4±0.03

higher than in canary heart mitochondria and significantly higher than those of mice skeletal muscle mitochondria (Fig. 1A, B). Immunodetection of cUCP with antibodies raised against murine UCP2 and human UCP3 protein confirmed the close molecular relationship between UCPs of birds and mammals. In conclusion, anti-UCP antibodies cross-reacting widely not only with mammalian UCPs, indicate that this protein is well conserved throughout the species.

ADP/O calculations show how the fluctuations in the level of cellular FFA concentrations (caused by some physiological conditions such as fasting, diabetes or ischemia) may decrease the efficiency of oxidative phosphorylation through the activation of UCPs. However, the contribution of the dissipating pathway, thus UCP activity in canary's heart and skeletal muscle mitochondria in state 3 respiration is not so significant as compared to the other organisms. For example, in amoeba *Acanthamoeba castellani* and tomato, UCP-like protein can dissipate around 50% of redox energy (Jarmuszkiewicz et al. 1998; Jarmuszkiewicz et al. 2000a).

Table 2 Effect of n-butylmalonate titration on ADP/O ratio and state 3 respiration in the presence and absence of 10 μM PA in canary heart (A) and skeletal muscle (B) mitochondria. Assay conditions as described in Materials and Methods. The mean values deal with a single preparation

n-butyl (mM)	Jo (state 3)		ADP/O	
	−PA	+PA	−PA	+PA
A) HEART				
0	267	240	1.45	1.20
0.5	227	222	1.45	1.12
1	189	200	1.44	1.10
2	148	175	1.40	1.02
5	84	–	1.31	–
B) MUSCLE				
0	206	182	1.5	1.07
0,3	165	175	1.5	1.07
0.5	159	163	1.5	1.07
0.7	137	154	1.5	0.96
1	128	137	1.5	0.91
2	117	130	1.5	0.84

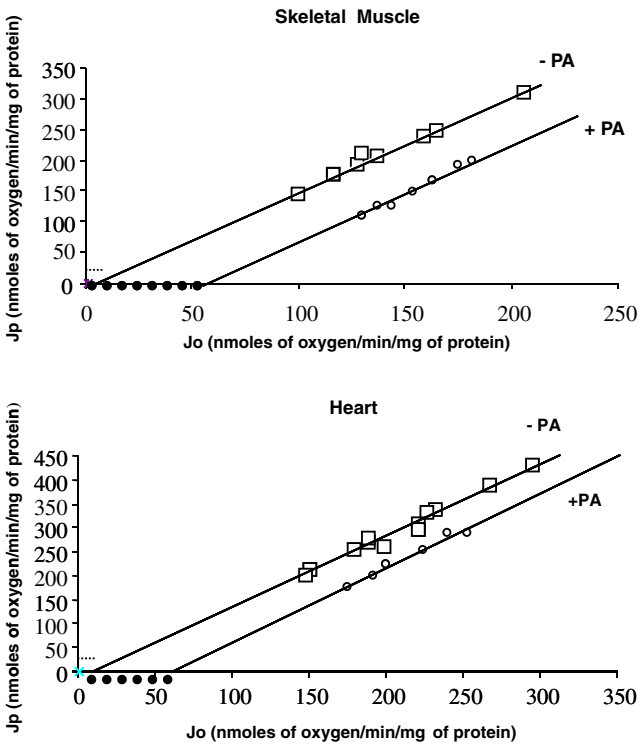

Fig. 4 The relationship between the rate of ATP synthesis (Jo x ADP/O) and the rate of oxygen uptake in state 3 respiration (Jo) in the absence of GTP. Assay conditions are as described. Oxidation rate of succinate was gradually decreased by increasing concentrations of n-butylmalonate in the absence (□) and presence (○) of 10 μM PA. Endogenous proton leak is labeled as (.....), PA- induced leak as (●●●●●). Data deal with 8 (heart) and 5 (skeletal muscle) mitochondrial preparations

Endogenous proton leak, the non-specific membrane pathway (Porter et al. 1996), determined in the absence of PA did not contribute significantly in state 3 respiration in canary's skeletal muscle and heart mitochondria. Moreover, we observed that the endogenous proton leak in canary birds (6 and 12 nmoles of oxygen/min/mg of protein for the skeletal muscle and heart, respectively) was much lower compared to mammals with similar body size such as mice (endogenous proton leak in heart mitochondria equals 20 nmoles of oxygen/min/mg of protein, unpublished data). One possible explanation of different proton leak in canary and mice might be due to the degree of mitochondrial fatty acid unsaturation. It has been reported that long-lived animals have a lower degree of mitochondrial fatty acid unsaturation than short-lived ones (Pamplona et al. 1999).

Based on the uncoupling activity we observed in canary UCP (cUCP), we propose that cUCP might have implication in adaptative thermogenesis in canary birds just like UCP1. Canary birds have a high metabolic rate, thus may require abundant food and oxygen for energy supply, especially under the stress of food deprivation or exposure to cold. To date, the studies on birds have not shown any

distinct BAT or a related thermogenic tissue like mammals (Gimeno et al. 1997). In the absence of BAT and UCP1, skeletal muscle and heart of birds have been suggested to be the main site for non-shivering thermogenesis (Duchamp et al. 1991). It has been reported that avUCP mRNA is primarily expressed in skeletal muscle and heart, and increases significantly during the period of food deprivation (Vianna et al. 2001, Raimbault et al. 2001; Bicudo et al. 2002). Our unpublished data on the starved canary birds confirm the above observations. We observed the increase of inhibitory efficiency of GTP in state 4 respiration and augmented drop in ADP/O ratio in state 3 respiration. These results indicate an increase in UCP activity and overexpression of cUCP protein in canary's heart mitochondria upon fasting. Thus, upregulation of UCPs during diet-induced thermogenesis suggests a plausible role of avian UCP in energy expenditure.

Another function of cUCP may not be related to thermogenesis. There is 70% sequence homology between avUCP and mammalian UCP2 and UCP3, suggesting they might have similar physiological relevance. Although the role of UCP2 and UCP3 is still controversial, they have been proposed to play an important role in regulation of lipids as fuel substrate and in controlling production of reactive oxygen species (ROS) (Cortez-Pinto et al. 1999; Lee et al. 1999). The activation of UCPs allows an increase in electron flux, and it has been shown to lower ROS production *in vitro* and *in vivo* by decreasing the reduced state of ubiquinone (Skulachev 1996; Maxwell et al. 1999). Diminishing ROS production is important to minimize oxidative damage to the cell or mitochondrial lipids, proteins and DNA (Davies 1987; Du et al. 1998) and in consequence may help in slowing down the aging process. Thus, the higher cUCP activity and the higher cUCP content in canary heart and skeletal muscle mitochondria may contribute to the significantly higher maximum lifespan of canaries than mice. Therefore, the hypothesis that mitochondrial UCPs may play a significant role in longevity by the reduction of ROS production seems to be quite tempting.

Acknowledgments We thank Olivia Li Ling Tan for important technical assistance. This work is supported in part by research grants: National Medical Research Council (NMRC), Singapore (R-185-000-058-213), and Biomedical Research Council (BMRC), Singapore (R-185-000-065-305) to Y. Deng.

References

Altman P, Dittmer D (1972) Biol Data Book Fed Am Soc Exp Biol, Betsheda, 229–235
Andreyew AY, Bondareva TO, Dedukhova VI, Mokhova EN, Skulachev VP, Tsofina LM, Volkov NI, Vygodina TV (1989) Eur J Biochem 182:585–592
Barja B, Cadenas S, Rojas C, Perez-Campo R, Lopez-Torres M (1994) Free Rad Res 21:317–328
Bicudo JE, Bianco AC, Vianna CR (2002) J Exp Biol 205:2267–2273
Boss O, Samec S, Paoloni-Giacobino A, Rossier C, Dulloo A, Seydoux J, Muzzin P, Giacobino JP (1997) FEBS Lett 408:39–42
Boss O, Hagen T, Lowell B (2000) Diabetes 49:143–156
Bradford M (1976) Anal Chem 72:248–254
Cannon B, Nedergaard J (1985) J Physiol 20:110–164
Cortez-Pinto H, Zhi Lin H, Qi Yang S, Odwin Da Costa S, Diehl AM (1999) Gastroenterology 116:1184–1193
Davies KJ (1987) J Biol Chem 262:9895–9901
Deng Y, Mieczkowski M (1998) Protoplasma 203:16–25
Deng Y, Kohlwein SD, Mannella CA (2002) Protoplasma 219:160–167
Du G, Mouithys-Mickaland A, Sluse FE (1998) Free Radical Biol Med 25:1066–1074
Duchamp C, Barre H, Rouanet JL, Lanni A, Cohen-Adad F, Berne G, Brebion PI (1991) Role of skeletal muscle. Am J Physiol 261: R1438–R1445
Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, Brand MD (2002) Nature 415:96–99
Essop M, Razeghi P, McLeod C, Young ME, Taegtmeyer H, Sack MN (2004) Biochem Biophys Res Comm 314:561–564
Fleury C, Neverowa M, Collins S, Raimbault S, Champigny O, Levi-Meyrueis C, Bouillaud F, Melchior JC, Seldin M, Surwitt R, Ricquier D, Warden C (1997) Nat Gen 15:269–272
Fridell YC, Sánchez-Blanco A, Silvia BA, Helfand SL (2004) J Bioenerg Biomembr 36:219–228
Giacobino JP (2001) Biochem Soc Trans 29:774–777
Gimeno RE, Dembski M, Weng X, Shyjan AW, Gimeno CJ, Iris F, Ellis SJ, Deng N, Woolf EA, Tartaglia LA (1997) Diabetes 46:900–906
Himms-Hagen J, Harper ME (2001) Exp Biol Med 226:78–84
Iser WB, Kim D, Bachman E, Wolkow C (2005) Mech Ageing Dev 126(10):1090–1096
Jaburek M, Varecha M, Gimeno RE, Dembski M, Jezek P, Zhang M, Burn P, Tartaglia LA, Garlid KD (1999) J Biol Chem 274:26003–26007
Jarmuszkiewicz W, Sluse-Goffart CM, Hryniewiecka L, Michejda J, Sluse FE (1998) J Biol Chem 273:10174–10180
Jarmuszkiewicz W, Sluse-Goffart CM, Hryniewiecka L, Sluse FE (1999) J Biol Chem 274:23198–23202
Jarmuszkiewicz W, Almeida AM, Vercesi AE, Sluse FE, Sluse-Goffart CM (2000a) J Biol Chem 275:13315–13320
Jarmuszkiewicz W, Milani G, Fortes F, Schreiber A, Sluse FE, Vercesi AE (2000b) FEBS Lett 467:145–149
Jarmuszkiewicz W, Navet R, Alberici LC, Douette P, Sluse CM, Sluse-Goffart FE, Vercesi AE (2004) J Bioenerg Biomembr 36(5):1–10
Ježek P, Žačkova M, Růžička M, Škobisová M, Jabůrek M (2004) Physiol Res 53:S119–S211
Johnston DW (1971) Comp Biochem Physiol A Physiol 40:1107–1108
Klingenberg M (1990) Trends Biochem Sci 15:108–112
Klingenberg M, Winkler E, Echtay K (2001) Biochem Soc Trans 29:806–811
Kowaltowski AJ, Costa AD, Vercesi AE (1998) FEBS Lett 425:213–216
Lee FY, Le Y, Zhu H, Yang S, Lin HZ, Trush M, Diehl AM (1999) Hepatology 29:677–687
Maia IG, Benedetti CE, Leite A, Turcinelli SR, Vercesi AE, Arruda P (1998) FEBS Lett 429:403–406
Mao W, Yu XX, Zhong A, Li W, Brush J, Sherwood SW, Adams SH, Pan G (1999) FEBS Lett 443(3):326–330
Maxwell DP, Wang Y, McIntosh L (1999) Proc Natl Acad Sci USA 96:8271–8276
Mitchell P (1979) Science 206:1148–1159
Navet R, Douette P, Puttine-Marique F, Sluse-Goffart CM, Jarmuszkiewicz W, Sluse FE (2005) FEBS Lett 579(20):437–442
Nicholls DG, Locke RM (1984) Physiolol Rev 64:1–64

Pamplona R, Portero-Otin M, Riba D, Ledo F, Gredilla R, Herrero A, Barja G (1999) Aging Clin Exp Res 11:44–49
Porter RK, Hulbert AJ, Brand MD (1996) Am J Physiol 271: R1550–R1560
Raimbault S, Dridi S, Denjean F, Lachuer J, Couplan E, Bouillaud F, Bordas A, Duchamp C, Taouis M, Ricquier D (2001) Biochem J 353:441–444
Ricquier D, Bouilland F (2000) Biochem J 345:161–179
Samec S, Seydoux J, Dulloo AG (1998a) Diabetes 47:1693–1698
Samec S, Seydoux J, Dulloo AG (1998b) FASEB J 12:715–724
Sanchis D, Fleury C, Chomiki N, Goubern M, Huang Q, Neverova M, Grégoire F, Easlick J, Raimbault S, Lévi-Meyrueis C, Miroux B, Collins S, Seldin M, Richard D, Warden C, Bouillaud F, Ricquier D (1998) J Biol Chem 273(51):34611–34615
Skulachev VP (1996) Q Rev Biophys 29:169–202
Skulachev VP (1998) Biochim Biophys Acta 1363:100–124
Slautterback DB (1965) J Cell Biol 24:1–21
Stuart JA, Harper JA, Brindle KM, Brand MD (1999) Biochim Biophys Acta 1413:50–54
Talbot DA, Duchamp C, Rey B, Hanuise N, Rouanet JL, Sibille B, Brand MD (2004) J Physiol 558:123–135
Vercesi AE, Martins IS, Silva MAP, Leite HMF, Cuccovia IM, Chaimovich H (1995) Nature 375:24
Vianna CR, Hagen T, Zhang CY, Bachman E, Boss O, Gereben B, Moriscot AS, Lowell BB, Eduardo J, Bicudo PW, Bianco AC (2001) Genomics 5:137–145
Wieckowski MR, Wojtczak L (1997) Biochem Biophys Res Commun 234:414–417
Žačkova M, Kramer R, Ježek P (2000) Int J Biochem Cell Biol 32:499–508
Žačkova M, Škobisová M, Urbánková E, Ježek P (2003) J Biol Chem 278:20761–20769