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Are the mammalian-like uncoupling proteins 1 and 2 expressed in cold-acclimated Muscovy ducklings?

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Despite their lack of brown adipose tissue (BAT), birds can exhibit nonshivering thermogenesis (NST) in the cold. Avian NST mainly originates from skeletal muscle and may involve fatty acid-induced uncoupling of mitochondrial respiration. In mammals, uncoupling of oxidative phosphorylation is based on the presence of uncoupling proteins (UCPs) in the inner mitochondrial membrane. Because both the BAT-specific UCP1 and the ubiquitous UCP2 are up-regulated in cold-acclimated (CA) rodents, the question arises as to whether CA birds exhibiting NST express mammalian-like UCPs. Tissue expression of UCP1 and UCP2 mRNA was therefore investigated in CA Muscovy ducklings (Cairina moschata, 5-wk old), reared at 4°C from the age of 1-wk and exhibiting muscle NST. Rat fragments of UCP1 or UCP2 cDNA were used as probes in Northern blots. UCP1 mRNA was detected only in rat BAT while UCP2 mRNA was detected in rat BAT, liver and soleus muscle. Conversely, no UCP1- or UCP2-like mRNA were detected in duckling skeletal muscle, white adipose tissue and liver. It is concluded that CA ducklings do not express a mRNA with sufficient homology to hybridise with rat UCP1 or UCP2 probes.

1. Introduction

Being endotherms, birds must increase their heat production to survive in the cold. It has long been thought that birds rely exclusively on shivering thermogenesis for regulatory thermogenesis (Chaffee & Roberts 1971) because of the importance of shivering in most avian species and the lack of brown adipose tissue (BAT), the main site of NST in small rodents. However, studies in cold-acclimated (CA) young birds including ducklings and penguin chicks (Barré et al. 1986a, Duchamp et al. 1989) or long-term CA adult chickens (El Halawani et al. 1971) have demonstrated the occurrence of NST despite the lack of BAT.

Because the sites of cold-induced NST are different in mammals and birds, the molecular mechanisms involved may also differ. In mammals, BAT NST is mainly based on the presence of a mitochondrial protein known as uncoupling protein (UCP1) which acts as a proton ionophore in the inner mitochondrial membrane and thus uncouples respiration from ATP synthesis (Nicholls et al. 1986). It is now clear that the BAT specific

UCP1 is not the only uncoupling protein expressed in mammals, following recent discoveries of other members of the UCP family (UCP2 and UCP3). Contrary to UCP1, UCP2 is ubiquitously expressed in rat, mouse and human tissue (Fleury et al. 1997, Gimeno et al. 1997). Similarly, UCP3 is principally expressed in skeletal muscle and BAT of rodents (Vidal-Puig et al. 1997). These proteins show 57% homology with UCP1. Furthermore, when transfected in yeast, these proteins partially uncouple mitochondrial respiration, suggesting a role of UCP2 and UCP3 in the control of tissue energy expenditure. Interestingly, the ubiquitous UCP2, like the BAT-specific UCP1, is upregulated in CA rodents (Boss et al. 1997, Denjean et al. unpub. data) suggesting the possible involvement of tissues other than BAT in mammalian NST.

In the absence of BAT, proposed mechanisms for avian NST include those based (1) on an alteration of the mitochondrial energetic efficiency and (2) on an increased ATP hydrolysis by futile cycles. The former mechanism is supported by results obtained in vitro supports from experiments using isolated mitochondria which showed an enhanced fatty acid-induced loose-coupling of respiration in muscle mitochondria from CA ducklings (Barré et al. 1986b). These in vitro data have recently been supported by in vivo data demonstrating a lower energetic efficiency of locomotor activity in CA ducklings (Denjean et al. unpub. data). The molecular mechanisms of such uncoupling are still unclear, and the involvement of mammalian-like UCPs is a distinct possibility. The presence of UCP1 in birds has been investigated using Western blots and specific antibodies against mammalian UCP1 (Saarela et al. 1991). No immunoreactivity could be found despite the use of large amounts of mitochondrial proteins from various tissues of winter-acclimatized birds including pheasants (Phasianus colchicus), Japanese quails (Coturnix coturnix japonica), pigeons (Columba livia), house sparrows (Passer domesticus), and great tits (Parus major) (Saarela et al 1991). However, there was no indication that these species exhibited NST even after cold-acclimatization. Thus it remains unknown whether mammalian-like UCP1 is expressed in avian models in which muscle NST has been demonstrated. To our knowledge, no experiment has been carried out to investigate the expression of other members of the UCP family in bird species.

The aim of the present work was therefore to investigate the expression of both mammalian-like UCP1 and UCP2 in several tissues (liver, pink adipose tissue (PAT) and gastrocnemius muscle) of cold-acclimated ducklings known to possess the capacity for muscle NST. Expression of mammalian-like UCP2 was investigated at the mRNA level using Northern blots given that there is as yet no antibody capable of discriminating between the various UCPs. Rat tissues were used as positive controls.

2. Materials and methods

2.1. Animals

Male Muscovy ducklings (Cairina moschata L, pedigree R31, Institut National de la Recherche Agronomique) were obtained from a commercial stock breeder (Ets Grimaud). They were fed a commercial mash (Aliment Genthon Démarrage) ad libitum and had free access to water. Ducklings were kept under a constant photoperiod (8:16-h light–dark cycle). The cold-acclimation schedule described by Barré et al. (1986a) was used: from the age of 1 wk, ducklings were caged in groups of six for a period of 4 wk at 4°C ambient temperature (Ta).

Male Wistar rats weighing 200–220 g were obtained from a commercial stock-breeder (Iffa Credo, France) and fed a commercial mash (UAR 105) ad libitum. Animals were housed in individual cages and reared for 1 week at 4°C Ta under a constant photoperiod (8:16 light-dark).

At the time of experiment, animals were killed rapidly by decapitation. Interscapular brown adipose tissue (BAT), white adipose tissue (WAT), liver and soleus muscle were sampled in rats. Pink adipose tissue, liver and gastrocnemius muscle were sampled in CA ducklings. Tissues were rapidly dissected, weighed and frozen in liquid nitrogen. Samples were then stored at – 70°C until analysis. Animals were cared for under the French Code of Practice for the Care and Use of Animals for Scientific purposes, and the experimental protocols were approved by the French Ministry of Agriculture Ethics Committee (Animals).

2.2. Total RNA extraction

Total RNA was isolated from 0.2–0.4 g portions of frozen tissue (about 1 g for WAT) using a simplified version of the guanidium thiocyanate method (Chomczynski et al. 1987). Briefly, tissues were homogenised with a polytron (Konematica) in a solution containing guanidium isothiocyanate 2M, sodium citrate 12.5 mM, β-mercaptoethanol 0.4%, sarcosyl 0.25% and sodium acetate 0.125M, pH 4. Total RNA was then extracted by phenol-chloroform, precipitated by isopropanol and washed by ethanol. Total RNA was stored in water and quantified by absorbance at 260 nm (1optical density unity representing 40 μg RNA/ml). The 260/280 ratio of the RNA sample was between 1.8 and 2. The integrity of total RNA extracted was routinely checked by gel electrophoresis. The 18S and 28S RNA bands always indicated excellent integrity of the preparation.

2.3. Northern blot

10μg (rat) or 20μg (duckling) of total RNA was denatured, electrophoresed on 1% formaldehyde-agarose gel and transferred to Nylon membranes (Amersham). Prehybridization (4h) and hybridization (18h) were carried out at 42°C. Two different probes were used. Firstly, a portion of the rat UCP1 complementary DNA (cDNA) was amplified by reverse transcription and polymerase chain reaction (RT-PCR) on rat BAT total RNA using a pair of specific oligonucleotide primers (forward corresponding to nucleotides 409–428 and reverse to nucleotides 605–586) selected according to the published rat UCP1 cDNA sequence (RNUCPG.PE1, EMBL). Secondly, a portion of the rat UCP2 cDNA was amplified by RT-PCR on rat BAT total RNA using a pair of oligonucleotide primers (forward corresponding to nucleotides 488–507 and reverse to nucleotides 780–761) chosen according to the rat UCP2 cDNA sequence (U69135, GENBANK). Probes were labelled by random priming with [α-^{32}P]deoxyCytidine 5′-triphosphate (NEN) and washed at 56°C in 1× standard sodium citrate with 0.1% sodium dodecyl sulfate. Blots were exposed to Hyperfilm XAR5-Kodak at – 70°C for 4 days.

2.4. Statistics

Statistical differences between means were assessed by Student's t-test and recognized at $P < 0.05$. Values presented are means (SE).

3. Results

The yield of total RNA (per g wet weight) from several tissues of cold-exposed rats or cold-acclimated ducklings is shown in Table 1. As expected, the yield was the highest from liver and was similar between the two species. Similarly, no difference in yield was observed between rat and duckling oxidative muscles (soleus or internal gastrocnemius). Conversely, the yield of total RNA was higher (more than × 7, $P < 0.05$) from the pink adipose tissue of CA ducklings than in the WAT of CA rats. Interestingly, the yield was considerably increased by cold-acclimation, being nearly 20-fold greater from the pink adipose tissue of CA ducklings with respect to the WAT of thermoneutral control ducklings. It was however lower than that obtained from rat BAT.

Amplified DNA fragments obtained after RT-PCR of BAT total RNA (1μg) are shown in Fig.1. Using UCP1 primers, a single band of 197pb was obtained after 21 cycles of PCR (Fig 1A). Using UCP2 primers, a single band of 293 pb was obtained after 27 cycles of PCR (Fig. 1B). These bands were sampled and sequenced to confirm

Table 1: Total RNA yield expressed in mg/g from several tissues of 1-wk cold-exposed rats (n = 8) and cold-acclimated ducklings (n = 2–4). The value for duckling WAT is for animals reared at thermoneutrality (25°C) for 5 wks (n = 4). Total RNA was then used in Northern blots. BAT = brown adipose tissue; WAT = white adipose tissue; PAT = pink adipose tissue; Means (SE).

	Rats	Ducklings
BAT	1.05 (0.07)	
WAT	0.038 (0.006)	0.015 (0.003)
PAT		0.29 (0.12)
Liver	6.37 (0.19)	6.51 (0.10)
Soleus muscle	1.18 (0.05)	
Gastrocnemius muscle		1.15 (0.09)

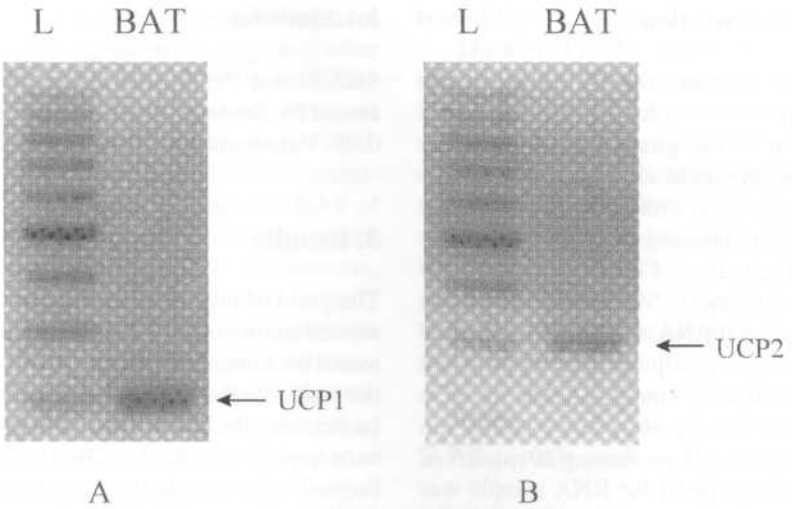

Fig. 1: Amplified products obtained by RT-PCR using rat UCP1 (A) or UCP2 (B) specific primers. 1 μg total RNA of rat interscapular brown adipose tissue was used in the reverse reaction. 21 cycles of PCR were used for UCP1 and 27 cycles for UCP2 (denaturation at 94°C for 45s, annealing at 60°C (UCP1) or 61°C (UCP2) for 60s, extension at 72°C for 60s). Sizes of products were 197 pb for UCP1 and 293 pb for UCP2. Amplified products were then purified and used as probes for Northern blot analysis. BAT: interscapular brown adipose tissue; L: ladder of DNA fragments ranging from 200 to 1000 pb in 100 pb increments (Promega).

the specificity of the amplification. The sequences obtained perfectly matched those published for UCP1 and UCP2 cDNA. These fragments were used as probes in Northern blots.

In an initial experiment, the distribution of UCP1 mRNA was investigated in several tissues of cold-exposed rats and CA ducklings. A representative autoradiogram obtained with the UCP1 probe is shown in Fig. 2. One large and one faint signal were detected with rat BAT RNA. These results indicate that a predominant transcript of approximately 1.6–1.7 kb and a relatively minor transcript of 1.9–2.0 kb are expressed in rat BAT. Similar sizes of UCP1 mRNA have been reported by others (Ricquier et al. 1986). It should be noted that UCP1 transcripts could be detected in rat BAT after only a few hours of film exposure. As expected, no signal was observed in rat liver nor soleus muscle. In duckling tissues, no signal was detected in pink adipose tissue, liver, nor gastrocnemius muscle despite prolonged exposure of the autoradiograms and the use of twice as much total RNA.

In a second experiment, distribution of UCP2 mRNA was investigated in rat and duckling tissues. A typical example of a Northern blot performed with the UCP2 probe is presented in Fig. 3. The presence of UCP2 transcripts approximately 1.9 kb in size was detected in the three rat tissues examined (BAT, liver and soleus muscle). Interestingly, the band intensity was highest in soleus muscle and lowest in liver, suggesting that the relative expression of UCP2 transcripts in cold-exposed rats would be the highest in oxidative skeletal muscles. By contrast, no signal was obtained with the total RNA from pink adipose tissue, liver and gastrocnemius muscle of CA ducklings.

4. Discussion

Present results therefore showed that, contrary to rat tissues, no mammalian-like UCP1 and UCP2 transcripts could be detected in various tissues of CA ducklings in which NST has been demonstrated.

Validation of the Northern blot analysis was an important prerequisite of the study. It was performed by firstly sequencing the probes developed for rat UCP1 and UCP2, and secondly by using rat tissues as positive controls for UCP1 and

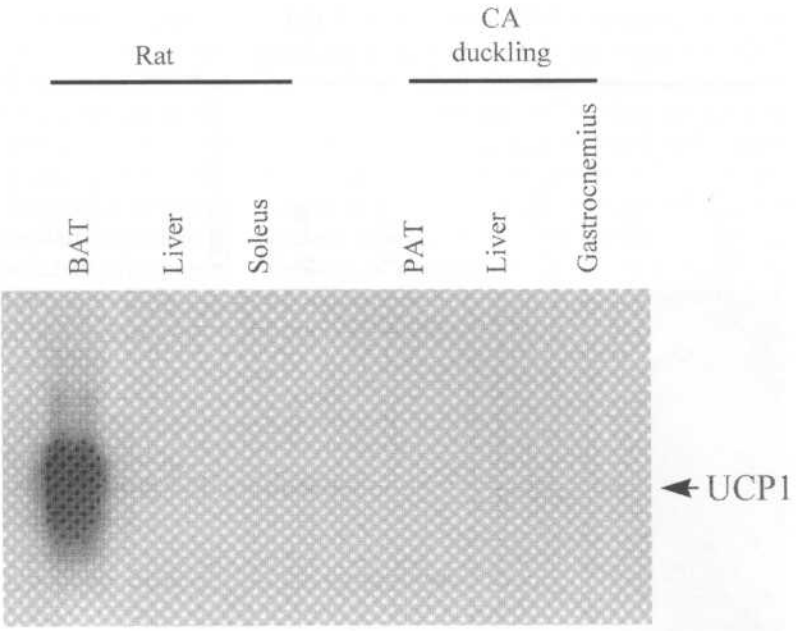

Fig. 2: Northern blots of UCP1 mRNA in tissues of a cold-exposed rat (interscapular brown adipose tissue (BAT), liver and soleus skeletal muscle (Sk. muscle)) or of a cold-acclimated duckling (liver, pink adipose tissue (PAT) and gastrocnemius skeletal muscle (Sk. muscle)). The film was exposed for 4 days at – 70°C.

UCP2 expression. Sequences obtained for the amplified fragment perfectly matched published sequences for UCP1 and UCP2 cDNA, confirming the specificity of amplification. Further, the detection of UCP1 transcripts (1) with a size similar to that described elsewhere (Ricquier et al. 1986) and (2) only in rat BAT indicates that the probe specifically targeted UCP1 mRNAs. Similarly, both the size of the detected transcripts and their tissue distribution obtained with the UCP2 probe agree well with other studies (Fleury et al. 1997), indicating that the UCP2 probe specifically targeted UCP2 mRNA. Contrary to UCP1, UCP2 transcripts were detected in rat BAT, liver and soleus muscle confirming the relative ubiquitous expression of the UCP2 gene as opposed to the UCP1 gene (Fleury et al. 1997). Interestingly, hybridization with the UCP2 probe, which had nearly twice the specific activity of the UCP1 probe, gave a much fainter signal after 4 days exposure of the films, suggesting that the relative abundance of UCP2 transcripts in BAT should be much lower than that of UCP1 mRNA. Nevertheless, the results obtained in rats validate our use of the Northern blot technique to specifically detect mammalian-like UCP1 and UCP2 transcripts.

The present results indicate that no transcript with a sufficient homology to hybridise with rat UCP1 probe could be detected in various tissues of CA ducklings. This was despite the increase in the duration of autoradiogram exposure and the use of a high amount of total RNA. These results therefore confirm and extend previous studies aimed at detecting mammalian-like UCP1 expression in avian tissues by Western blots (Saarela et al. 1991). This study could not detect any immunoreactive mammalian-like UCP1 in various species of winter-acclimatized birds. Present results show that even in a species in which NST has been demonstrated, no UCP1-like mRNA could be detected. Thus avian muscle NST is unlikely to be based on the expression of a UCP1-like protein in skeletal muscle. Birds therefore differ from mammals in which UCP1 mRNA can be detected in skeletal muscle under some circumstances such as a treatment with β3 adrenergic agonists (Nagase et al. 1996).

Interestingly, no UCP1-like transcripts were detected in the pink adipose tissue of CA ducklings. Indeed, in response to cold acclimation, this tissue undergoes differentiation resulting in an intensification of the pink coloration and the appearance of multilocular adipocytes with more mitochondria and surrounded by more capillaries (Barré et al. 1986a). This altered tissue is perfused by a higher blood flow in response to either cold

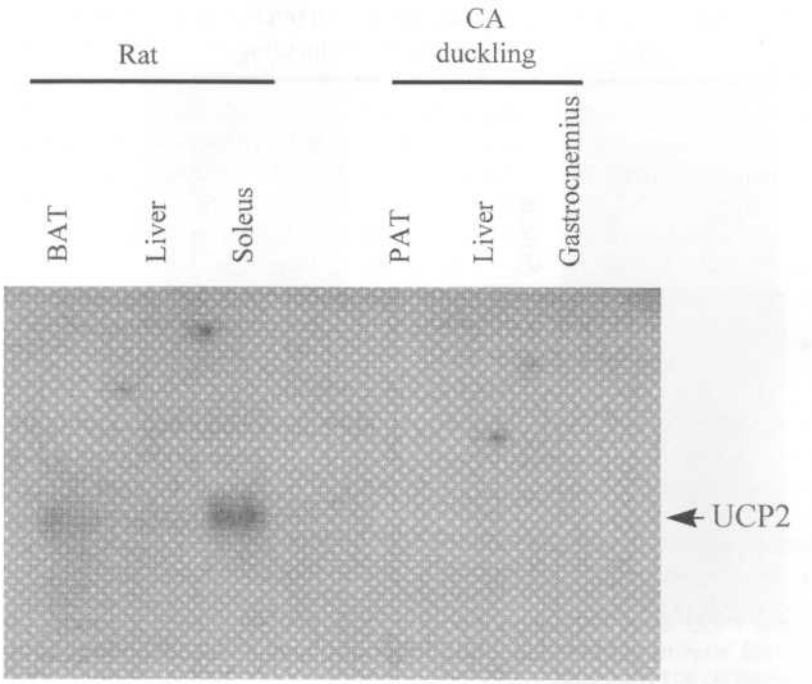

Fig. 3: Northern blots of UCP2 mRNA in tissues of a cold-exposed rat (interscapular brown adipose tissue BAT, liver and soleus skeletal muscle (Sk. muscle)) or of a cold-acclimated duckling (liver, pink adipose tissue (PAT) and gastrocnemius skeletal muscle (Sk. muscle)). The film was exposed for 4 days at – 70°C.

or glucagon stimulation (Duchamp & Barré, 1993, Duchamp et al. 1993). Present results are therefore in keeping with biochemical studies showing that PAT mitochondria are functionally different from those of rat BAT (Barré et al. 1986a). Nevertheless, the oxidative capacity of PAT was higher than that of the WAT of thermoneutral control ducklings (Barré et al. 1987). It should be noted that this augmentation only resulted in an oxidative capacity similar to that of rat WAT. Present results indicate that the total RNA yield is also tremendously increased in PAT as compared with WAT (x20). Part of this effect may however be related to the fact that multilocular adipocytes of PAT are smaller than unilocular adipocytes of WAT. Barré et al. (1986a) reported a reduction of 50% in diameter in PAT adipocytes which would induce a larger reduction in adipocyte volume (× 8). In the absence of mammalian-like UCP1 both at the mRNA (this study) and protein (Saarela et al. 1991) levels, multilocular adipose tissue of CA birds appears to be functionally similar to the white adipose tissue of rats. Multilocularity may actually signify an intense lipolytic activity of the tissue in the cold in order to provide fuels to other thermogenic organs, this function being assisted by the higher perfusion of the tissue in the cold (Duchamp & Barré 1993). In support of this hypothesis, both an increased lipolytic responsiveness and a higher sensitivity to glucagon have recently been reported in PAT of CA ducklings (Bénistant et al. 1998). Overall, these results are consistent with the view that thermogenic brown adipose tissue is absent from avian species (Barré et al. 1986a, Olson et al. 1988, Saarela et al. 1989, 1991).

Present results show for the first time that CA ducklings do not express mRNAs with sufficient homology with mammalian UCP2 to be detected in Northern blots. This situation therefore differs from mammals in which UCP2 mRNA could be detected in several species including rats, mice and humans. In mammals, cold exposure has been shown to increase UCP2 mRNA levels in most tissues (Boss et al. 1997, Denjean et al. unpub. data) as it does for UCP1 mRNA in BAT. However, no up-regulation of UCP3, the third member of the UCP family, was observed in skeletal muscles of cold-exposed rats (Carmona et al. 1998). This explains why we investigated the potential presence of mammalian-like UCP2 instead of UCP3 in birds. Because UCP2 does actu-

ally alter the energetic efficiency when expressed in yeasts (Fleury et al. 1997), it was hypothesised that it may act as an uncoupling protein and thus play a role in the thermogenic activity of tissues. This has renewed interest in the potential role of tissues other than BAT in facultative energy dissipation and possibly NST. It should however be noted that UCP2 is also up-regulated in situations where increased thermogenic activity is doubtful such as fasting (Fleury et al. 1997, Boss et al. 1997, Millet et al. 1997). Although these results were not confirmed by Gong et al. (1997), they suggest that UCP2, and potentially UCP3, could play a role in the regulation of lipids as fuel substrate rather than as mediators of regulatory thermogenesis (Samec et al. 1998). Alternatively, UCPs may play a role in inhibiting reactive oxygen species generation by the respiratory chain (Kowaltowski et al. 1998). Whatever the physiological role of these proteins, we could not detect their expression in avian tissues. It therefore appears that the proposed mechanism for avian NST based on a loose-coupling of mitochondrial respiration does not involve mammalian-like UCP2.

We cannot however refute the argument that putative avian UCPs, if they exist, are so different from those in mammals that they cannot be detected with the probes used. This argument is reinforced by the revelation that UCPs are in fact present in vegetables (Laloi et al. 1997). The deduced amino acid sequence of these plant UCPs are only 44% and 47% identical to human UCP1 and UCP2, respectively. It is therefore puzzling that these proteins are expressed in potatoes but not in endothermic birds, especially if they play a role as mediators of regulatory thermogenesis, modulators of lipid catabolism or as protectors against reactive oxygen. It should be noted that within mammals, cross hybridisation generally exists across widely diverse species hence there is a high homology between the published sequences of rat, mouse, hamster, bovine and human UCPs. To avoid the non-detection of partial hybrids, membranes were only lightly washed with × 1 SSC solution before exposure to films but this did not lead to positive detection.

In conclusion, the present work has failed to find evidence for the presence of mammalian-like UCP1 and UCP2 mRNA in cold-acclimated Muscovy ducklings. No mRNA with a sufficient homology to hybridise with rat UCP1 or UCP2 probes was indeed detected in pink adipose tissue, liver and skeletal muscles. Although the possibility cannot be entirely excluded that a putative avian UCP might be too dissimilar to be detected by Northern blots with mammalian UCP probes, these results are not in favour of the expression of mammalian-like UCPs in birds. The molecular mechanisms of the mitochondrial loose-coupling observed in skeletal muscles of birds exhibiting nonshivering thermogenesis may not be based on the existence of such proteins.

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Selostus: **Esiintyykö nisäkästyyppisiä uncoupling 1 ja 2 -proteiineja kylmäakklimoidulla myskisorsalla (*Cairina moschata* L.)?**

Vaikka linnuilla ei olekaan ruskeaa rasvakudosta, voivat ne poikasvaiheessa tuottaa lämpöä ns. nonshivering termogeneesin (NST, ei-lihasvärinäperäinen lämmöntuotto) avulla luurankolihaksissa. Lintujen lihassolujen mitokondrioissa, aivan samoin kuin nisäkkäiden ruskeassa rasvakudoksessa, lämpöä syntyy rasvahappojen indusoimassa tapahtumassa, missä hengitysketjun protoninsiirto irtikytkeytyy oksidatiivisesta fosforylaatiosta ja protonigradientin purkautumisesta syntyvä energia ei sitoudukaan ATP-molekyyleihin, vaan vapautuu lämpönä. Nisäkkäillä uncoupling-tapahtuma (irtikytkeytyminen) perustuu mitokondrion sisäkalvolla oleviin uncoupling-proteiineihin (UCP), joiden kautta protonivuoto ja gradientin purkautuminen tapahtuvat. Jyrsijöillä on havaittu ruskean rasvakudoksen spesifisen UCP1 ja muissa kudoksissa olevan UCP2 määrän lisääntyvän kylmäakklimaatiossa. Kirjoittajat tutkivat molekyylibiologisin menetelmin, esiintyykö linnuilla nisäkkäiden kaltaisia uncoupling-proteiineja, jotka ovat yhteydessä kylmäaltistukseen. Koe-eläimenä käytettiin viiden viikon ikäisiä myskisorsia. Kun myskisorsia kasvatetaan + 4°C:een lämpötilassa,

niiden lihakset alkavat tuottaa lämpöä NST:n avulla jo viikon vanhana. Rotan UCP1:n ja UCP2:n cDNA-jaksoja käytettiin Northern-blottauksen koettimena. Menetelmällä voidaan tutkia kyseisen proteiinin geeniekspressiota. UCP1:n lähetti-RNA:ta (mRNA) löydettiin vain rotan ruskeasta rasvakudoksesta, kun taas UCP2:n lähetti-RNA:ta löydettiin rotan ruskeasta rasvakudoksesta, maksasta ja pohjelihaksesta. Kummankaan uncoupling-proteiinin lähetti-RNA:ta ei löydetty sorsan lihaksesta, rasvasta tai maksasta.

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