Cold-induced mitochondrial uncoupling and expression of chicken UCP and ANT mRNA in chicken skeletal muscle

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Abstract **Although bird species studied thus far have no distinct brown adipose tissue (BAT) or a related thermogenic tissue, there is now strong evidence that non-shivering mechanisms in birds may play an important role during cold exposure. Recently, increased expression of the duckling homolog of the avian uncoupling protein (*av*UCP) was demonstrated in cold-acclimated ducklings [Raimbault et al., Biochem. J. 353 (2001) 441–444]. Among the mitochondrial anion carriers, roles for the ATP/ADP antiporter (ANT) as well as UCP variants in thermogenesis are proposed. The present experiments were conducted (i) to examine the effects of cold acclimation on the fatty acid-induced uncoupling of oxidative phosphorylation in skeletal muscle mitochondria and (ii) to clone the cDNA of UCP and ANT homologs from chicken skeletal muscle and study differences compared to controls in expression levels of their mRNAs in the skeletal muscle of cold-acclimated chickens. The results obtained here show that suppression of palmitate-induced uncoupling by carboxyatractylate was greater in the subsarcolemmal skeletal muscle mitochondria from cold-acclimated chickens than that for control birds. An increase in mRNA levels of *av*ANT and, to lesser degree, of *av*UCP in the skeletal muscle of cold-acclimated chickens was also found. Taken together, the present studies on cold-acclimated chickens suggest that the simultaneous increments in levels of *av*ANT and *av*UCP mRNA expression may be involved in the regulation of thermogenesis in skeletal muscle.**

Key words: Uncoupling; Thermogenesis; *av*ANT; *av*UCP; Skeletal muscle; Cold-acclimated chicken

1. Introduction

It has been proposed that the mechanism of fatty acid-induced uncoupling of oxidative phosphorylation in skeletal muscle mitochondria is composed of (i) the influx of protonated fatty acids into mitochondria via phospholipid regions of the inner mitochondrial membrane and (ii) the efflux of protonated fatty acid anions mediated by any one of a number of anion carriers [1], such as brown fat uncoupling protein (UCP1) [2,3], the adenine nucleotide translocator (ANT) [4], the aspartate/glutamate antiporter [5], and the dicarboxylate carrier [6]. Recently, two molecular homologs of the brown-fat UCP1, called UCP2 and UCP3, were identified in mammals [7,8] and biochemically characterized [9].

It is well-established that in rodents, brown adipose tissue (BAT) plays a major role in thermogenesis in response to exposure to cold via the action of the BAT-specific mitochondrial uncoupling protein (UCP1) [10]. The gene expression of UCP2 and UCP3 in BAT have also been observed to be up-regulated in rats exposed to 5°C for 2 d [11]. Additionally, it was reported that UCP2 and UCP3 expression in skeletal muscle of rats were enhanced after 48 h [12] and 24 h [13] of cold exposure, respectively.

Although bird species studied thus far have no distinct stores of BAT or a related thermogenic tissue [14,15], the cold-induced uncoupling in skeletal muscles of a bird (pigeon) had already been described by Skulachev's group [16,17], and it was found to be mediated by fatty acid [18]. There is also evidence that non-shivering mechanisms associated with the non-phosphorylating oxidation might play an important role in birds during cold exposure, as in the case of cold-acclimated ducklings [19]. Recently, a new protein, named *av*UCP, which shares a 70% amino acid identity with both UCP2 and UCP3, was identified in chicken skeletal muscles, while the relatively increased expression of the duckling homolog of the *av*UCP in cold-acclimated ducklings was also demonstrated [20].

Among the mitochondrial anion carriers mentioned above, roles for the ANT (also known as ATP/ADP carrier) as well as the above UCP variants in thermogenesis are proposed [2]. Increases in ANT protein and the carboxyatractylate (cAtr: specific inhibitor of ANT)-sensitive portion of myristate-stimulated respiration were observed in the liver mitochondria of hyperthyroid rats [21]. More recently, results from mitochondrial membrane potential measurements have shown ANT is responsible for the major portion of thermoregulatory uncoupling in heart muscle mitochondria from cold-exposed (6°C, 48 h) rats [22].

Thus, skeletal muscle thermogenesis could be mediated not only by *av*UCP but also by ANT in cold-acclimated chickens. Studies of the expression levels of these carriers are therefore of particular interest because skeletal muscle tissue in chickens is an important source of cold-induced thermogenesis for the whole body of the bird.

The aims of this work were: (i) to examine the effects of cold acclimation on the fatty acid-induced uncoupling of oxidative phosphorylation in skeletal muscle mitochondria and (ii) to clone the cDNA of homologs of UCP and ANT from

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Abbreviations: UCP, uncoupling protein; ANT, adenine nucleotide translocator; ADP/O ratio, adenosine diphosphate to oxygen ratio; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ΔΨ, transmembrane electric potential difference

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chicken skeletal muscle and then study changes in mRNA expression in the skeletal muscle of cold-acclimated chickens compared to controls. The results obtained show that the suppression of palmitate-induced uncoupling by cAtr was greater in subsarcolemmal skeletal muscle mitochondria from cold-acclimated chickens than that seen for control birds. It was also found that cold acclimation induced an increase in mRNA levels of skeletal muscle *av*ANT and, to a lesser degree, of *av*UCP.

2. Materials and methods

2.1. Animals

Two sets of animal experiments were similarly conducted: twelve 3-week-old White Leghorn male chicks were used (Iwashima Sangyou, Miyagi, Japan). Six chicks were transferred to 4–6°C for 10–12 d while the remainder were maintained at 24°C. Chicks were housed in a wire-bottomed cage under conditions of continuous light, and provided with free access to the experimental diet used in this laboratory and to water. They were killed by decapitation, and *pectoralis superficialis* muscles were quickly removed. For the first set of experiments the muscles were placed in ice-cold isolation buffer A (see below) for the subsequent isolation of mitochondria, and in the second set of experiments the muscles were frozen, powdered in liquid nitrogen, and stored at −80°C until Northern blot analysis for examination of the expression levels of *av*UCP and *av*ANT transcripts. All experiments were performed in accordance with institutional guidelines concerning animal use.

2.2. Isolation of mitochondria and measurement of respiration

Muscle subsarcolemmal and intermyofibrillar mitochondria were isolated from the muscle tissue according to the methods of Barre et al. [23]. The muscles were trimmed of fat and connective tissue, blotted dry, weighed and then minced with scissors. The mince, suspended in ice-cold buffer A containing 100 mM sucrose, 50 mM tris(hydroxymethyl)aminomethane (Tris) base, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 100 mM KCl, 1 mM ATP, pH 7.4, was homogenized with a Potter–Elvehjem homogenizer (5 passages).

The homogenate was then centrifuged at 800×*g* for 10 min and the resulting precipitate was subsequently used for the preparation of the intermyofibrillar mitochondria. The supernatant was centrifuged at 1000×*g* for 10 min and then at 8700×*g* for 10 min. The resulting pellet, containing subsarcolemmal mitochondria, was suspended in buffer A and recentrifuged at 8700×*g* for 10 min. The resulting pellet was resuspended in buffer B containing 250 mM sucrose, 20 mM Tris base, 1 mM EGTA, pH 7.4, and then washed by centrifugation at 8700×*g* for 10 min. The final subsarcolemmal mitochondrial pellet was suspended in a minimal volume of buffer B, and kept on ice. The pellet from the 800×*g* centrifugation was resuspended in isolation buffer A and treated with the protease nagarse (2 mg/g muscle wet wt) for 5 min in an ice bath. The mixture was diluted with an equivalent volume of buffer A (without nagarse), homogenized and centrifuged at 1000×*g* for 10 min to pelletize the myofibrils. The resulting supernatant was filtered through sterile gauze and centrifuged at 8700×*g* for 10 min. The resulting pellet was suspended in buffer A and recentrifuged at 8700×*g* for 10 min. The pellet from this stage was resuspended in buffer B and washed by centrifugation at 8700×*g* for 10 min. The intermyofibrillar mitochondrial pellet was resuspended in a minimal volume of buffer B and kept on ice. All procedures were carried out at 4°C.

The reaction medium was composed of 110 mM mannitol, 60 mM KCl, 60 mM Tris–HCl, 10 mM potassium phosphate and 1 mM EGTA, pH 7.4. Mitochondrial protein used was 250 μg/ml and oxidation rate, expressed in ng atom of O/mg mitochondrial protein/min, was measured polarographically in a total reaction volume of 2 ml at 37°C using an O_2 monitor [24]. For calculation of respiration rate, an oxygen concentration of 390 ng atoms O/ml was adopted. Partly uncoupled states were adjusted by addition of micromolar concentrations of a 7.5 mM ethanolic solution of palmitate [21]. To determine the cAtr-sensitive portion of this uncoupled respiration, 5 μM cAtr (f.c.) was added to the mitochondria suspension.

2.3. cDNA cloning and Northern blotting

2.3.1. General methods. Standard molecular biology techniques were carried out essentially as described by Sambrook et al. [25]. Tissues used were homogenized in Trizol-Reagent (Gibco BRL, Bethesda, MD, USA), and total RNA was isolated by the method of Chomczynski and Sacci [26].

2.3.2. Isolation of UCP and ANT cDNA expressed in chicken skeletal muscle. For the cloning of chicken cDNA homologous with mammalian UCP3, the cDNA library was constructed in pUC118 vector (Takara Shuzo, Kyoto, Japan) with poly(A)$^+$-RNA, which originated from skeletal muscle of cold-acclimated chicken. We obtained a cDNA library with 250 000 independent clones. The human UCP3 cDNA probe of 772 bp was obtained by PCR from a human skeletal muscle cDNA library (Takara Shuzo, #9514), using sense and antisense primers (sense: 5′-TGACCTCGTTACCTTTCCACTG-3′; antisense: 5′-TCCCAAACGCAAAAAGGAGGGT-3′) corresponding to nucleotides 179–200 and 929–950 on human UCP3 cDNA (GenBank Accession AF011449). This cDNA was used as a probe to screen a chicken skeletal muscle cDNA library. For the cloning of chicken cDNA homologous with mammalian ANT, sense and antisense primers (sense: 5′-TGTCTCTTTCCCGGCAGCTGC-3′; antisense: 5′-CTACAAGTATCAGCTGGCTCCG-3′) were prepared on the basis of nucleotides 11–31 (BI395064) and 448–469 (BI066017) of chicken EST (expressed sequence tag) clones, which are significantly homologous to the human ANT1 cDNA (GenBank Accession J02966). Reverse transcription-PCR (RT-PCR) was primed using Superscript II reverse transcriptase (Gibco BRL) and oligo-dT12–17 primers with total RNA from chicken skeletal muscle. The PCR products were subcloned into the pCR2.1 (Invitrogen, San Diego, CA, USA).

2.3.3. Clone sequencing. cDNA clones were purified using a Quiagen plasmid kit according to the manufacturer's instructions (Quiagen, Santa Clarita, CA, USA) and sequenced using standard protocols for the LI-COR Model 4000LS DNA sequencer with the M13 forward and reverse primers for pUC118 and M13 reverse and T7 promoter primers for pCR2.1 until the sequence was determined on both strands.

2.3.4. Northern analysis. To examine changes in levels of expression of *av*UCP and *av*ANT mRNAs in the skeletal muscle tissue of cold-acclimated chickens, total RNA was electrophoresed in a 1.0% agarose gel containing formaldehyde, as described by Lehrach et al. [27], and transferred to Zeta-Probe Membrane (Bio-Rad Laboratories, Hercules, CA, USA) for hybridization. Probes were labeled by random priming with [α-^{32}P]dCTP (3000 Ci mmol^{-1}) (Takara Bca-BEST™ Labeling Kit). Hybridized RNA blots were washed in a solution of 4×SSC (1×SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate (SDS) at RT for 5 min, in 1×SSC/0.1% SDS at 55°C for 20 min, in 1×SSC/0.1% SDS at 58°C for 20 min, and in 1×SSC/0.1% SDS at 60°C for 20 min. The signals for *av*UCP and ANT mRNA were detected and quantified using a Molecular Imager FX (Bio-Rad), which allows a direct counting of emitted β-radiation by the ^{32}P-labeled cDNA probes hybridized to the dotted target DNA. The blots were subsequently hybridized with GAPDH cDNA probe to correct for differences in the amounts of RNA loaded onto the gel.

2.4. Statistics

Statistical analysis was performed using a Student's *t*-test ($P < 0.05$) for comparison of results from cold and control groups [28].

3. Results

Body weight gain was lower in the cold-acclimated animals compared to controls (control chickens, 174 ± 2 g; cold-acclimated chickens, 114 ± 3 g; 34% decrease: $P < 0.001$), even though the cold-acclimated chickens ate more feed than the controls (540 ± 20 g and 590 ± 8 g; 9% increase: $P < 0.05$). As a result, and as expected, the feed efficiency decreased in the cold-acclimated animals (0.325 ± 0.017 and 0.192 ± 0.004; 41% decrease: $P < 0.001$), implying that cold acclimation induced more heat production.

Table 1 shows the results for state 3 and state 4 respiration,

Table 1
Oxidative metabolism with succinate used as a substrate in subsarcolemmal and intermyofibrillar mitochondria isolated from *pectoralis superficialis* muscle of control and cold-acclimated chickens[a]

Muscle mitochondrial populations	Animal	Oxygen consumption State 3	Oxygen consumption State 4	Respiratory control ratio[b]	ADP/O ratio	ATP synthesized (nmol min^{-1} mg^{-1})
		(ng atoms O min^{-1} mg^{-1})				
Subsarcolemmal mitochondria	control	129 ± 19	38 ± 8	3.67 ± 0.30	2.13 ± 0.08	277 ± 44
	cold-acclimated	212 ± 24*	60 ± 6*	3.53 ± 0.19	2.15 ± 0.11	448 ± 36*
Intermyofibrillar mitochondria						
	control	370 ± 38	64 ± 7	5.85 ± 0.18	2.37 ± 0.07	872 ± 78
	cold-acclimated	359 ± 28	69 ± 7	5.35 ± 0.35	2.48 ± 0.10	889 ± 70

[a]Data are expressed in means and standard errors for six chickens in each group.
[b]Respiratory control ratio was calculated as state 3:state 4 oxidation rates.
*$P < 0.05$ for cold-acclimated group vs. control group.

as well as of RCR and the adenosine diphosphate to oxygen (ADP/O) ratio of the subsarcolemmal and intermyofibrillar mitochondria of skeletal muscle. For subsarcolemmal mitochondria, the rates of both state 3 and state 4 respiration significantly increased in the cold-acclimated chicken, while no differences in RCR or ADP/O ratio were observed. Intermyofibrillar mitochondria, by contrast, did not exhibit any differences in state 3 or state 4 respiration, RCR or ADP/O ratio between groups.

Fig. 1 clearly shows that the state 4 respiration rate increased with stepwise increases in concentrations of palmitate in the subsarcolemmal and intermyofibrillar skeletal muscle mitochondria from control and cold-acclimated groups. Palmitate-stimulated increments in respiration were significantly greater for subsarcolemmal mitochondria (Fig. 1A) from the cold-acclimated group compared with the controls at the cumulative concentrations of palmitate above 11.25 μM, while no differences were observed between groups for intermyofibrillar mitochondria (Fig. 1B). Thus, the palmitate-induced uncoupling effect on subsarcolemmal mitochondria was significantly enhanced by cold treatment. Moreover, Fig. 2 shows that subsarcolemmal mitochondria from the cold-acclimated group also resulted in a greater cAtr-sensitive portion of the fatty acid-stimulated respiration, as measured at the two palmitate concentrations of 3.75 and 15 μM, though the magnitude of cAtr sensitivity was dependent on the concentration of palmitate.

After screening approximately 25 000 clones for cloning of chicken UCP cDNA homologous with mammalian UCP3, we isolated one positive clone, and predicted a polypeptide con-

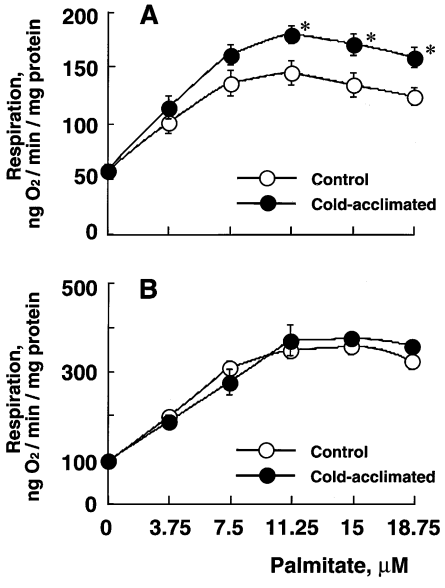

Fig. 1. Concentration dependence of palmitate-stimulated resting state respiration in subsarcolemmal mitochondria (A) and intermyofibrillar mitochondria (B) from *pectoralis superficialis* muscle of control (open symbols) and cold-acclimated (filled symbols) chickens. For experimental conditions, see Section 2. Values are means ± S.E.M. for four chickens in each group. *$P < 0.05$ for cold-acclimated group vs. control group.

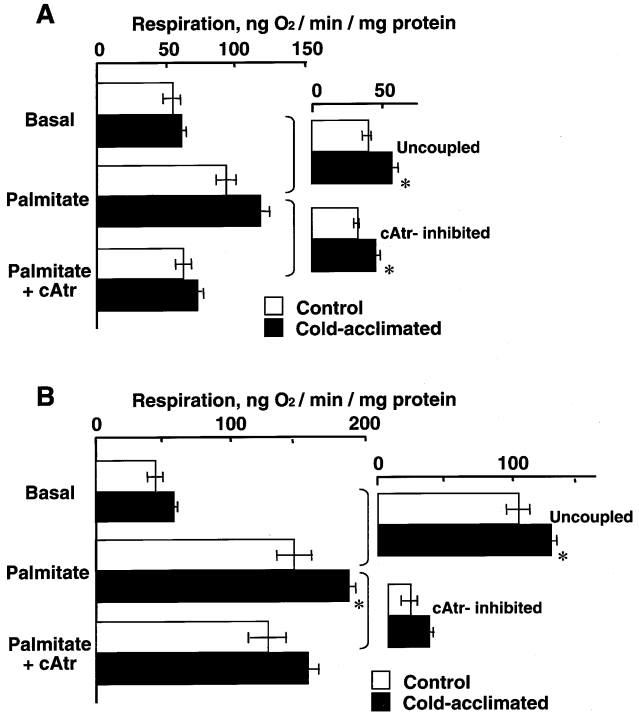

Fig. 2. Effect of cold acclimation on palmitate-stimulated resting state respiration of the subsarcolemmal mitochondria of chicken skeletal muscle in the presence and absence of 5 μM carboxyatractyloside (cAtr; f.c.). Respiration was stimulated by the addition of palmitate to a final concentration of 3.75 μM (A) or 15 μM (B). See Section 2 for experimental conditions. Values are means ± S.E.M. for four chickens in each group. *$P < 0.05$ for cold-acclimated group vs. control group. The right parts of the figure show (i) values of the additional oxygen consumption initiated by palmitate and (ii) a decrease in the oxygen consumption when cAtr was added after palmitate.

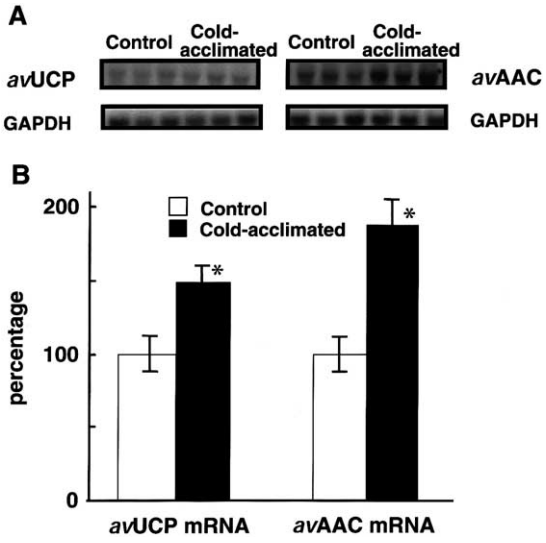

Fig. 3. Effect of cold acclimation on the expression of the mRNAs for *av*UCP and *av*ANT in chicken skeletal muscle. A: Northern blot analyses of RNA (30 μg/lane) from *pectoralis superficialis* muscle were performed using *av*UCP cDNA and *av*ANT cDNA cloned here. Figures shown are representative of 2–3 independent analyses. B: Blots were subsequently hybridized with a GAPDH cDNA probe to correct for differences in the amounts of RNA loaded onto the gel. The results are shown as percentage of control group. Values are means ± S.E.M. for 5–6 chickens in each group. $*P < 0.05$ for cold-acclimated group vs. control group.

taining 307 amino acids. During the preparation of this manuscript, Raimbault et al. [20] recently reported the cloning of *av*UCP cDNA. The 307-amino-acid protein obtained in our study is identical to their *av*UCP, except for differences in two codons (GenBank Accession AB088685). A comparison of the amino-acid sequences of *av*UCP and the human UCPs showed the *av*UCP has amino acid identities of 55, 71, and 73% to UCP1, UCP2 and UCP3 (GenBank Accession U28480, U76367 and AF011449), respectively. Furthermore, the three energy-transfer protein signature domains [29] are well-conserved in *av*UCP. On the other hand, *av*ANT cDNA cloned in the present study encodes a 298 amino-acid protein (GenBank Accession AB088686) showing 89%, 93%, and 93% identity with human ANT1 [30], ANT2 [31] and ANT3 [32], respectively. The identity of the *av*ANT to human ANT1 is the lowest among human ANTs, and the abundant gene expression of the *av*ANT in skeletal muscle showed similar major bands to human ANT1 mRNA [33]. Like other mitochondrial carriers, *av*UCP and *av*ANT possess six putative transmembrane domains, and conserves the 14-amino-acid residues [29].

We then moved on to examine the modulation of *av*UCP and *av*ANT transcripts in skeletal muscle of cold-acclimated chickens. Fig. 3a shows that both *av*UCP and *av*ANT transcripts produced much stronger signals in skeletal muscle for the cold-acclimated group compared to the control group. The levels of *av*UCP and *av*ANT mRNAs, which were quantified by scanning photodensitometry and normalized using corresponding GAPDH mRNA expression was increased 1.5 and 2.0-fold by cold acclimation, respectively (Fig. 3b). Therefore, the increased uncoupling action observed in the mitochondria of cold-acclimated chickens might be involved in the expression of both *av*UCP and *av*ANT mRNAs.

4. Discussion

Early studies by Halawani et al. [34] demonstrated an increased capacity for non-shivering thermogenesis (NST) in cold-acclimated birds. Similarly, Barre et al. [35] demonstrated a large increase in the metabolic rate of cold-acclimated Muscovy ducklings that could not be attributed to shivering activity. Though these two studies demonstrating a non-shivering thermogenic component to cold adaptation in birds did not identify the site of this response, Duchamp and Barre [36] suggested that the NST mainly originates from skeletal muscles. The present results showed that cold acclimation increased the rates of state 3 respiration and ATP synthesis in the subsarcolemmal mitochondria of skeletal muscle. These increases allow us to suggest a potentially greater ATP production of muscle in response to cold stress, thereby eventually dissipating heat via an increased metabolic flux and/or futile cycle. It is very likely that cold acclimation increases long-chain acyl ester levels in skeletal muscle [37] as a result of fat deposit mobilization [35]. These related metabolites may contribute to muscle NST in birds by enhancing the ATP-consuming process of sarcoplasmic reticulum (SR) Ca^{2+} cycling (i.e. Ca^{2+} cycling across SR membranes) and its associated heat production [37]. Moreover, an increase in fatty acid supply to skeletal muscles by cold acclimation [36,38] may also contribute to NST by uncoupling mitochondrial oxidative phosphorylation processes [19,39–41]. Indeed, the present experiments showed that the state 4 respiration rate was increased to a greater extent in cold-acclimated chickens than in controls following the addition of palmitate to the subsarcolemmal mitochondria suspension, and that the cAtr-sensitive portion of the palmitate-stimulated respiration was considerably higher for the cold-acclimated chicken, even though the magnitude of cAtr sensitivity was dependent on the concentration of palmitate used. These results therefore suggest that ANT and other mitochondrial anion carriers in cold-acclimated chickens may dominate in mediating fatty acid-induced uncoupling of mitochondrial oxidative phosphorylation processes. The dependency of the cAtr sensitivity on palmitate concentration also implies that other uncoupling mechanisms might also be involved [1], although the possibility of fatty acid-induced opening of the permeability transition pore can be excluded in our interpretation of these results since EGTA was present in the reaction medium.

The results of the Northern blots hybridized as probes using *av*UCP and ANT cDNAs cloned here revealed that a 10–12 d period of acclimation of chickens to 5°C resulted in 1.5-fold and 2.0-fold increases in the UCP and ANT expressions (see Fig. 3) in skeletal (*pectoralis superficialis*) muscle, respectively. The latter observation is supported by our result (Fig. 2) as well as the observation of Roussel et al. [42], indicating that cold acclimation resulted in a 1.7-fold increase in the subsarcolemmal mitochondrial ANT content of duckling gastrocnemius muscle, which was determined by the amount of CAT required to reduce state 3 respiration to state 4 respiration using cAtr. Such an increase is presumably accompanied by a decrease in $\Delta\Psi$ in muscle mitochondria, thereby making them more sensitive to fatty acid-induced uncoupling. The cold acclimation-induced increase in chicken UCP mRNA expression observed here is also in good agreement with data of Raimbault et al. [20], who showed that *av*UCP mRNA expression was increased 2.6-fold in cold-acclimated

ducklings developing muscle NST. One could postulate that elevation of the UCP transcript, in addition to the ANT transcript, may be responsible for some of the fatty acid-induced uncoupling in skeletal muscle mitochondria in cold-acclimated chickens.

This postulation does, however, require some discussion. In rats acclimated to cold, a condition known to strongly increase UCP1 in BAT [43], 20 days of the adaptation had no effect on UCP3 expression in the tibialis anterior muscle [29], suggesting that changes in BAT UCP1 but not muscle UCP3 levels might play an important role in the maintenance of body temperature. It is conceivable that, because BAT thermogenesis is increased during cold acclimation, an excessive increase in body temperature could be prevented by no change in muscle UCP3 expression. In birds, UCP1 is not expressed and neither BAT nor a related thermogenic tissue has been discovered [14,15]. Therefore, it seems likely that *av*UCP in skeletal muscle, in contrast to UCP3 in rat skeletal muscle, may contribute to the heat production by fatty acid-induced uncoupling of oxidative phosphorylation in skeletal muscle mitochondria in cold-acclimated chickens. This difference in skeletal muscle function between birds and mammals is not suprising if we take into account the complexity of the organization of higher organisms.

It is noteworthy that, in heart tissue from the cold-exposed animals (6°C, 48 h), ANT seems to dominate compared with UCP in mediating uncoupling by both endogenous and exogenous fatty acids [22]. This conclusion is evidenced by the fact that in the heart mitochondria recoupling effects of cAtr are always stronger than those of GDP, specific inhibitor of UCP. The present studies also showed that cold acclimation induced an increase in the level of *av*ANT mRNA and, to a lesser degree, of *av*UCP in skeletal muscle. At this point in time, however, a mechanism of regulation of chicken UCP by nucleotides has not been identified, as was previously shown by Echtay et al. [9], for mammalian UCP2 and UCP3. Therefore, further biochemical studies are required to elucidate the mediating part of the fatty acid-induced uncoupling activity of ANT and UCP using proteoliposomes reconstituted with the recombinant proteins as previously described [9,44], thereby assessing how the ANT and UCP contribute to uncoupling activities for heat production in the skeletal muscle of cold-acclimated chickens.

It should be emphasized that subsarcolemmal mitochondria exhibited higher rates of state 3 and state 4 respiration and larger palmitate-stimulated increments in respiration than intermyofibrillar mitochondria. The latter observation supports the data of Roussel et al. [41], indicating that the sensitivity of ATP synthesis to fatty acid inhibition was much more affected by cold acclimation in the subsarcolemmal compared to intermyofibrillar mitochondria. These different responses to cold acclimation in the two mitochondria could be explained on the basis of their localization. Intermyofibrillar mitochondria could satisfy contractile element ATP requirements [45,46], which would be required for shivering thermogenesis but not for NST, while subsarcolemmal mitochondria adjacent to the SR and capillary vessels might contribute to the ATP-consuming process of SR Ca^{2+} cycling and the uncoupling of oxidative phosphorylation, both of which are upregulated by cold acclimation [19,37,39].

In conclusion, from our results it appears that the decreased feed efficiency by cold-acclimated chickens is partly involved in the expression of mRNAs for two mitochondrial anion carriers, *av*UCP and ANT, which leads to an increase in the fatty acid-stimulated uncoupling action of subsarcolemmal mitochondria that mediates skeletal muscle thermogenesis. Initial evidence for the simultaneous expression of both genes strongly supports previous findings that cold acclimation resulted in a 1.7-fold increase in the subsarcolemmal mitochondrial ANT content of duckling gastrocnemius muscle [42] and that *av*UCP mRNA expression was increased 2.6-fold in cold-acclimated ducklings developing muscle NST [20]. Elucidation of the physiological mechanism involved in the UCP/ANT expression and molecular mechanisms involved in fatty acid-stimulated UCP/ANT-mediated uncoupling would help us understand the regulation of thermogenesis in cold-acclimated chickens. These issues will need to be addressed in future studies.

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