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Expression of Avian UCP and ANT in Skeletal Muscle of Cold-exposed Laying and Meat-type Chickens

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Most bird species studied thus far have no distinct brown adipose tissue (BAT) or a related type of thermogenic tissue ; however, our recent studies on cold (4–6℃)-acclimatized chickens suggest that simultaneous increments in mRNA levels of the mitochondrial anion carriers, avian adenine nucleotide translocator (avANT) and avian uncoupling protein (avUCP), may be involved in the regulation of thermogenesis in avian skeletal muscle (FEBS Lett. 529 : 313–318, 2002). The present experiments were conducted to compare the responses of laying- and meat-type chickens in regard to the expression of avUCP and avANT when they undergo acute (24–48 h) mild cold (8℃) exposure. Twenty-four male laying- and twelve meat-type chickens (3-wk old) were used. Groups from each species were exposed to mild cold for 24 and 48 h, while the control group remained at 23℃. In laying-type chickens, weight gain and feed efficiency were both compensated in some degree after 48 h of cold exposure. In contrast, meat-type chickens exhibited a linear decrease in weight gain corresponding to the duration of cold exposure. There were differences in the expression of avANT mRNA in *Pectoralis superficialis* muscle, between laying- and meat-type chickens, i.e. expression of avANT mRNA was significantly increased in laying-type chickens exposed to cold for 48 h, but not in meat-type chickens. However, no differences were observed in the expression of avUCP mRNA in pectoral muscle between control and mild cold groups for both type of chickens, showing that acute mild cold exposure was not sufficient to induce increased expression of avUCP mRNA in skeletal muscle of either laying- or meat-type chickens. Up-regulation of avANT mRNA, but not avUCP mRNA, in laying-type chickens exposed to acute mild cold suggested that avANT might be involved in thermogenesis and adaptation to acute mild cold exposure. These findings will contribute to our understanding of potential differences in the responses of laying- and meat-type chickens to cold exposure at the molecular level.

Key words : UCP, ANT, cold exposure, chicken

Introduction

Poultry production faces serious problems when chickens are exposed to extreme thermal conditions. Heavy economic losses due to mortality, low production and

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immunosuppression (Regnier and Kelley, 1981) could impose serious threats to the poultry industry in response to such conditions of low thermal exposures. In future farming systems resembling more natural conditions, animals will face various kinds of environmental stressors, including cold stress (van Loon *et al.,* 2004). Growth rate and feed conversion efficiency decrease when the environmental temperature is lower than the thermoneutral zone (Ueda *et al.,* 2005). In order to overcome the negative effects of cold exposure on performance it is of utmost importance to know the mechanism of thermogenesis in chicken.

Most of the work on thermogenesis is being done on mammals and, therefore, may not be relevant to avian species. In mammals, brown adipose tissue (BAT) is specialized for adaptive, non-shivering thermogenesis via the activation of an uncoupling protein gene, UCP1, expressed in the BAT (Nicholls *et al.,* 1978). This uncoupling protein gene encodes for a mitochondrial protein carrier, which uncouples oxidative phosphorylation for ATP production and stimulates heat production (Ricquier *et al.,* 1991). UCP3 is uniquely expressed in skeletal muscle (Boss *et al.,* 1997 ; Vidal-Puig *et al.,* 1997) and is a likely candidate to be involved in energy metabolism. Birds lack BAT, yet show adaptive thermogenesis by activating non-shivering thermogenesis in skeletal muscles (Duchamp and Barre, 1993 ; Duchamp *et al.,* 1999). In fact, chickens (*Gallus gallus*) (Raimbault *et al.,* 2001 ; Toyomizu *et al.,* 2002) and humming birds (*Eupetomena macroura*) do possess an uncoupling protein (UCP) homologue, avian UCP (Vianna *et al.,* 2001). Our previous studies (Toyomizu *et al.,* 2002) showed an increase in mRNA levels of avian uncoupling protein (avUCP) and avian adenine nucleotide translocator (avANT) in the skeletal muscle of cold-acclimatized laying-type chickens and reported that these increases were involved in the adaptive response to cold. More recently, Ueda *et al.* (2005) reported that avUCP and avANT transcription levels concomitantly increased from days 1 to 2 of cold exposure and then decreased slightly over the next 5 days, but remained higher than control values.

Importantly, it was reported that meat-type chickens exhibit high sensitivity to heat treatment, as shown by a significant increase in body temperature (Altan *et al.,* 2003). Our recent results showed that superoxide production was enhanced in skeletal muscle mitochondria of hyperthermia-treated meat-type chickens, whereas no such increase was observed in laying-type chickens (Mujahid *et al.,* 2005). Thus, some type-dependent differences between laying- and meat-type chickens in terms of tolerance to environmental stress have been observed. Here, in order to discern whether the response to acute mild cold stress (8℃ for 24–48 h) is specific to particular chicken types, we investigated the influence of acute mild cold stress on the expression of avUCP and avANT in the skeletal muscle of laying- and meat-type chickens. Both of these genes encode for mitochondrial anion carriers and, as such, are candidates that may be involved in the control of adaptive thermogenesis in birds. To explore this possibility, the present experiments were carried out to compare laying- and meat-type chickens with regards to their expression of skeletal muscle avUCP and avANT when exposed to cold for 24 and 48 h, and to examine potential type-specific differences in tolerance to acute mild cold exposure on molecular basis.

Materials and Methods

Birds

Male laying-type (Julia) and meat-type chicks (Cobb) were obtained from a commercial hatchery (Economic Federation of Agricultural Cooperatives, Miyagi and Koiwai Farm, Ltd., Iwate Japan, respectively) at 1 d of age. Chicks were housed in electrically-heated batteries under continuous light for three weeks and provided with water and commercial starter layer (CP 20%, ME 2950 Kcal/kg) and meat-type diet (CP 22%, ME 3000 Kcal/kg) *ad libitum*. Temperature of batteries and cages were maintained according to chicken raising guide lines provided by the chick's suppliers and reduced gradually *as per* instructions on daily basis : 31, 29 and 23℃ for laying-type chickens and 32, 28 and 23℃ for meat-type chickens were set during the first, second and third week, respectively. During their second week of age, birds were shifted to individual cages to provide an adjustment period of one week. During the third week, sixteen laying-type chickens and eight meat-type chickens were transferred to cold environment (8℃) and kept for 24 or 48 h, while eight laying- and four meat-type chickens were maintained at 23℃ as controls. Birds were sacrificed by decapitation and *Pectoralis superficialis* muscles quickly removed. Muscle samples were snap frozen in liquid nitrogen and stored at −80℃ until Northern blot analysis for examination of the levels of avUCP and avANT transcripts. The experiment was performed in accordance with institutional guidelines concerning animal use.

Weight Gain and Feed Consumption

Bird weights at the start and end of the experiment were recorded and feed consumption was also measured. Body weight gain/loss and feed efficiency of each chicken were determined individually.

Northern Analysis

To examine changes in the levels of avUCP and avANT mRNAs in the skeletal muscle tissue of cold exposed chickens Northern blot analyses were performed as described previously (Toyomizu *et al.,* 2002). Briefly, muscle tissues were homogenized in Trizol-Reagent (Invitrogen Gibco-BRL, Bethesda, MD, USA) and total RNA isolated according to the manufacturer's protocol. The RNA was electrophoresed in a 1.0% agarose gel containing formaldehyde, as described by Lehrach *et al.* (1977), 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 (220 TBq/mmol) (Takara BcaBEST™ Labeling Kit). Hybridized RNA blots were washed in a solution of 4×SSC (1×SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min, in 1×SSC/0.1% SDS at 55℃ for 20 min, in 1×SSC/0.1% SDS at 58℃ for 20 min, and in 1×SSC/0.1% SDS at 60℃ for 20 min. The signals for avUCP and avANT mRNAs were detected and quantified using a Molecular Imager FX (Bio-Rad), which allowed 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.

Statistics

Statistical analysis of the data was performed using Student's *t*-tests for comparison of data from control and cold exposed groups within each chicken type, with regards to weight gain, feed consumption and feed efficiency. For mRNA expression results, effect of two factors, chicken type and duration of cold exposure was first analyzed by a two-way factorial design for general linear model analysis of variance procedure to separate the effects of cold exposure from those due to chicken type, and the means were compared using Duncan's least significance multiple-range test (SAS, 1985). All data are expressed in the form of mean ± standard deviation (S.D.). Differences were considered significant for values of $P < 0.05$.

Results

The weight gains of acute mild cold-exposed laying- and meat-type chickens were significantly decreased compared to control (Table 1). Cold-exposed laying- and meat-type chickens also showed significant decreases in their feed efficiency compared to control chickens. In the case of laying-type chickens, feed efficiency decreased sharply during the first 24 h of cold exposure and then increased, although it was still significantly less than that of control chickens after 48 h.

The effect of cold exposure on the expression of avUCP and avANT mRNAs in skeletal muscle (*Pectoralis superficialis*) of laying- and meat-type chickens, as determined by Northern blot analyses, is shown in Fig. 1 A. Two-way analysis of variance demonstrated no significant effect of chicken type and duration of cold exposure on avUCP mRNA expression : there was no difference observed in the expression of avUCP mRNA in skeletal muscles of laying- and meat-type chickens (Fig. 1 B). Non-significant differences in avUCP expression were observed between control chick-

Table 1. Effect of cold exposure on weight gain, feed consumption and feed efficiency of laying- and meat-type chickens

Treatments	Weight gain (g)		Feed consumption (g)		Feed efficiency (%)	
	Time after cold exposure (h)					
	0–24	24–48	0–24	24–48	0–24	24–48
Laying-type chickens (n=8)						
Control	9.64±3.42	8.18±5.30	31.59±6.84	31.93±4.90	27.01±7.68	24.03±12.28
8℃ for 24 h	0.48±2.19*	NA	27.63±2.96	NA	2.41±8.82*	NA
48 h	2.06±2.02*	5.48±3.12*	26.63±3.82	29.35±4.37	7.74±6.98*	18.67±6.08*
Meat-type chickens (n=4)						
Control	70.20±14.00	82.00±13.42	96.75±10.53	100.13±4.05	72.08±7.42	81.95±13.92
8℃ for 24 h	36.65±8.25*	NA	91.00±8.68	NA	40.27±9.46*	NA
48 h	29.27±4.61*	16.63±7.15*	93.20±8.15	95.40±5.03	31.57±5.49*	17.43±4.07*

Data expressed as means and standard deviations for four or eight chickens in each group.
*$P < 0.05$ for cold-exposed group vs. control group.
NA, not applicable.

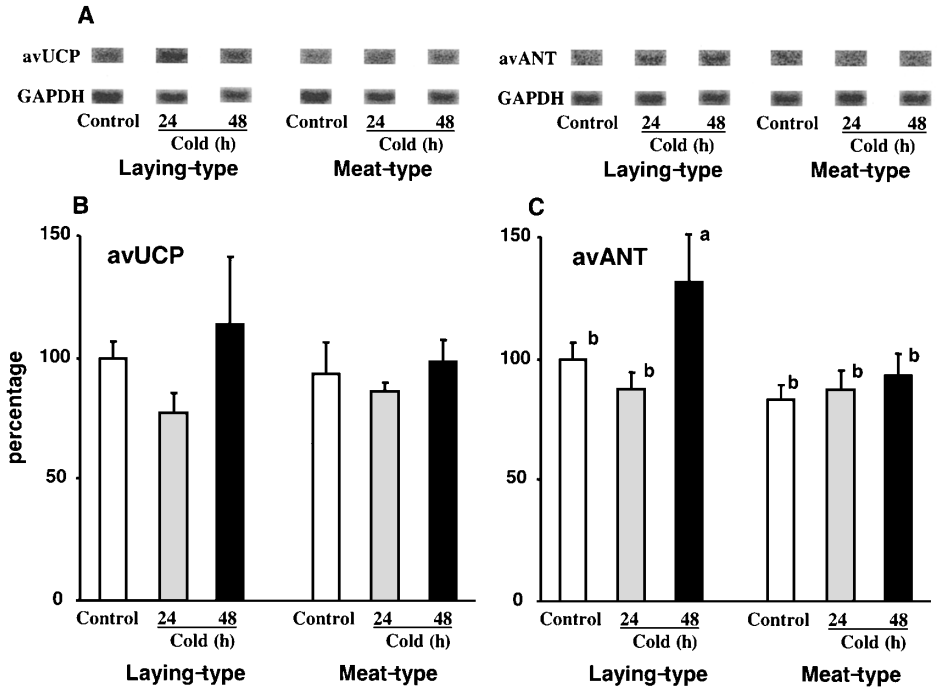

Fig. 1. Expression of avian UCP and ANT in skeletal muscle of cold-exposed laying and meat-type chickens. A : Northern blot analyses of RNA (25 ug/lane) from *Pectoralis superficialis* muscle were performed using avUCP and avANT cDNA cloned here. B and C : Blots were subsequently hybridized with a GAPDH cDNA probe to correct for differences in the amounts of RNA loaded onto the gel. The results are shown as percentage of control groups from laying-type chickens. Values are means±SD for 4 chickens in each group.
[a,b] Bars with different letters are significantly different ($P<0.05$).

ens and those exposed to cold either for 24 or 48 h, both in laying- and meat -type chickens. However, analysis of variance for avANT mRNA showed that laying-type chickens had significantly higher expression in skeletal muscle compared to meat-type chickens ($P<0.05$). Significant interaction between chicken type and duration of cold exposure was also observed : there was a significant increase in avANT mRNA in laying-type chickens exposed to cold for 48 h (Fig. 1 C), but 24 h cold exposure did not yet exhibit any increases. In contrast, there was no difference in avANT mRNA levels between control and cold-exposed meat-type chickens.

Discussion

Laying- and meat-type chickens exhibited different responses when exposed to cold. Weight gain and feed efficiency of laying-type chickens were significantly decreased during the first 24 h of cold exposure. There seemed to be some adaptation in weight gain and feeding efficiency of laying-type chickens when the duration of cold exposure was increased to 48 h, though no change was observed in feed consumption. In the case

of meat-type chickens, there was a linear decrease in weight gain and feed efficiency with increasing duration of cold exposure, thus showing no adaptation as was observed in laying-type chickens.

Recently, expression of an avUCP in skeletal muscle of chickens (Toyomizu *et al.,* 2002 ; Evock-Clover *et al.,* 2002), ducks (Raimbault *et al.,* 2001), and humming birds (Vianna *et al.,* 2001) were reported. In the present study, no difference in the expression of avUCP was observed in either the laying- or meat-type chickens when exposed to cold (8℃) for 24 or 48 h. Toyomizu *et al.* (2002) reported a 1.5-fold increase in mRNA for avUCP in laying-type chickens. However, the chickens were exposed to cold at 4–6℃ for 10–12 days. Collin *et al.* (2003) reported a slight, but significantly higher relative avUCP expression on day 14 in cold-exposed (20℃) meat-type chickens compared to control (29–31℃). It is likely that acute mild cold exposure of 8℃ for 24 or 48 h, used in present study was not sufficient to induce increased expression of avUCP in skeletal muscle of three week old chickens.

The significantly higher expression of avANT in laying-type chickens exposed to cold compared to control chickens may explain the adaptation of laying-type chickens to cold exposure. It has been reported that expression of ANT *in vivo* is regulated by external condition. Cai *et al.* (1997) reported ANT gene expression was inducible in different tissues of frogs by freezing exposure. Roussel *et al.* (2000) reported that cold acclimation (4℃ for 5 weeks) resulted in a 1.7-fold increase in the subsarcolemmal mitochondrial ANT content of duckling gastrocnemius muscle. Toyomizu *et al.* (2002) reported a two-fold increase in ANT mRNA in laying-type chickens when exposed to cold stress (4–6℃ for 10–12 days), and that cold adaptation increased the rates of state 3 respiration and ATP synthesis in the subsarcolemmal mitochondria of skeletal muscle. Ueda *et al.* (2005) reported that avANT transcript levels increased in the skeletal muscle of laying-type chickens from days 1 to 2 of cold exposure (4℃), and then decreased slightly over the next 5 days, but remained higher than control values. These increases suggested a potentially greater ATP production by the muscle in response to cold exposure, thereby eventually dissipating heat (thermogenesis) via an increased metabolic flux and/or futile cycle. Alternatively, avian ANT may also play an important role in thermogenesis via fatty acid-mediated uncoupling of mitochondrial oxidative phosphorylation (Toyomizu *et al.* 2002 ; Ueda *et al.* 2005).

The former postulation does, however, require some discussion. The known function of ANT is to catalyze the transmembrane exchange of cytosolic ADP with mitochondrial ATP, generated by oxidative phosphorylation (Vignais, 1976 ; Brandolin *et al.,* 1993). Thus, ANT is a key link between the mitochondrial and cytosolic compartments of cells. Elevated levels of ANT may contribute to minimize net ATP depletion during cold exposure. One possible function that it could play is in the regulation of the intra-mitochondrial adenine nucleotide pool size. ATP generated by re-phosphorylation of adenine nucleotide via oxidative phosphorylation could be transported to the cytosol in order to provide the energy for cells to survive under the cold-environmental condition. The elevated expression of avANT might be associated with the adaptive response of laying-type chickens.

More recently, Talbot *et al.* (2004) studied the mitochondrial basis of avian adaptive thermogenesis in penguins and found an increase in the amount of adenine nucleotide translocase on exposure to cold water. They reported that adaptive thermogenesis in juvenile king penguins was linked to uncoupling of oxidative phosphorylation in skeletal muscle mitochondria i.e. increased proton transport activity of the adenine nucleotide translocase. While repeated exposure to cold water leads to changes in the expression and activity of avANT, the changes could be for other reasons than thermogenesis, such as protection against oxidative stress, or to support increased ATP demand. These changes could be an essential prerequisite for the long-term survival of penguins in cold water. However, we are not sure whether this is also the case of laying-type chickens because we do not yet know even if oxidative stresses, such as reactive oxygen species, are enhanced in acute-cold exposed chickens.

In conclusion, in laying-type chickens there was no adaptive response during the first 24 h of acute mild cold exposure associated with non-significant expression of avANT mRNA. When cold exposure was increased to 48 h there was a significant increase in avANT mRNA that enabled the birds to adapt to the cold, as accompanied by concomitant increases in weight and feed efficiency during 24–48 h compared to the first 24 h of cold exposure. It is suggested that avANT could play a role in thermogenesis and adaptation to acute mild cold exposure. The differences in expression of avANT in skeletal muscles between laying- and meat-type chickens might be a possible explanation why they respond differently to acute-mild cold exposure.

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