Progressive Alteration of UCP and ANT in Skeletal Muscle of Fasted Chickens

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Avian uncoupling protein (avUCP), sharing 71–73% amino acid homology with both UCP2 and UCP3, is one of the mitochondrial anion carrier proteins. Its precise physiological roles in the cell remain elusive. A confusing aspect of these UCP variants (namely, UCP2, UCP3 and avUCP), is that their expression is enhanced in response to fasting; that is, in response to basal metabolic state in which such energy expenditure would be expected to be depressed.

In this study, we examined progressive alterations in the expression of genes encoding for mitochondrial uncoupling proteins, not only UCP but also avian adenine nucleotide translocator (avANT), in the skeletal muscle tissue of fasted chickens. The expression of avUCP gene was markedly enhanced after 8 h of fasting and then diminished slightly but remained elevated after 96 h of fasting compared to time 0 levels. In contrast, avANT was up-regulated only after 24 h of fasting but continued to be further increased after 96 h.

Taken together, these results demonstrate that transcription of each of the mitochondrial anion carriers, avUCP and avANT, is independently up-regulated during fasting periods, implying different control mechanisms and consequences of each in metabolic adaptations involved in prolonged fasting.

Key words: ANT, fasting, mitochondrial anion carrier, skeletal muscle, UCP

Introduction

Uncoupling proteins (UCPs) belong to a transporter family present in the mitochondrial inner membrane that, by dissipating the mitochondrial proton gradient, uncouples respiration from ATP synthesis (Palmieri, 1994). UCP1 is present exclusively in brown adipose tissue, which is the major site of regulatory thermogenesis in small rodents (Himms-Hagen, 1985). Additional uncoupling protein homologs have been identified. UCP2 is ubiquitously expressed (Fleury et al., 1997), while UCP3 gene expression has been demonstrated in skeletal muscle, adipose tissue and heart (Boss et al., 1997; Acin et al., 1999). It is now accepted that UCP1 is a key molecule involved in thermogenesis, in events such as cold- and diet-induced heat production (Champigny and Ricquier, 1990; Enerback et al. 1997). In contrast, little is known about the physiological roles of both mammalian UCP2 and UCP3. It has been proposed that they might play a role in the mediation of thermogenesis, in the regulation of lipids as fuel substrates, in the control of insulin secretion, and/or in controlling the production of reactive oxygen species (ROS) (Dulloo and Samec, 2001). UCP2 and UCP3 mRNA levels have been shown to be modulated by fasting (Samec et al., 1998; Spurlock et al., 2001).

Although bird species have no distinct stores of brown adipose tissue (BAT) or a related type of

Abbreviations: UCP : uncoupling protein, ANT : adenine nucleotide translocator
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thermogenic tissue (Johnston, 1971, Saarela et al., 1991), a new protein, named avian UCP (avUCP), which shares 71–73% amino acid homology with both UCP2 and UCP3, was identified in chicken skeletal muscles. Increased expression of avUCP was demonstrated in cold-acclimated ducklings (Raimbault et al., 2001) and chickens (Toyomizu et al., 2002). It was also reported that, as in mammals, avUCP is up-regulated during feed deprivation (Evoick-Clover, 2002). However, aspects concerning progressive alterations to avUCP during fasting, and the precise functions of avUCP, have not been clarified. Studies of avUCP with regard to the mitochondrial functions of chicken skeletal muscle during fasting is of particular interest because skeletal muscle tissue, not only in birds but also in large mammals whose BAT decreases with development, may play a role in controlling metabolic flux under conditions of nutritional stress (Rolfe and Brand, 1996).

As for the other mitochondrial anion carriers, roles for ANT (also known as ATP/ADP carrier) have been proposed in uncoupling by fatty acids in mitochondria (Skulachev, 1991). Recently, results from mitochondrial membrane potential measurements have shown that ANT is responsible for the major portion of thermoregulatory uncoupling in heart muscle mitochondria from cold-exposed (6°C, 48 h) rats (Simonyan and Skulachev, 1998). Indeed, previous studies have demonstrated increased mRNA for both avian ANT (avANT) and avUCP in cold-acclimated birds (Toyomizu et al., 2002; Talbot et al., 2004; Ueda et al., 2005). Moreover, Echtay et al. (2003) found that hydroxynonenal, one of the most reactive and important mediators of free-radical damage, induced uncoupling of mitochondria through ANT, as well as through UCPs. Thus, interrelations between UCP and ANT in metabolic regulatory uncoupling during fasting are of interest.

We describe here progressive alterations in the expression of genes encoding the mitochondrial uncoupling proteins, UCP and ANT, and discuss their functions in the skeletal muscle of fasted chickens.

Materials and Methods

Animals and Experimental Design

Male, White Leghorn chicks (Jula) were obtained from a commercial hatchery (Koiwai Farm, Ltd., Iwate, Japan) at 1 day of age. The chicks were housed in electrically heated batteries and provided with water and commercial starter diet ad libitum for 4 weeks. Birds (ca. 390 g) were selected from a 2-fold larger population in order to obtain uniform body weights; these birds were kept in wire-bottomed cages under conditions of controlled temperature (25±1°C) and continuous light. Twenty chickens were assigned to 5 groups of 4 animals each for 0, 12, 24, 48, or 96 h from the beginning of fasting. All birds were provided with free access to water. Their pectoralis muscles were excised quickly, frozen, powdered in liquid nitrogen, and stored at −80°C until required for total RNA extraction. All experiments were performed in accordance with institutional guidelines concerning the care and use of animals.

Northern Blotting

Basically, the analysis was performed as shown previously (Toyomizu et al., 2002).

General methods: Standard molecular biological techniques were carried out, essentially as described by Sambrook et al. (1989). Tissues used were homogenized in Trizol-Reagent (Invitrogen Gibco-BRL, Bethesda, MD, USA) and total RNA isolated according to the manufacturer’s protocol.

Northern analysis: To examine changes in the levels of avUCP and avANT mRNAs in the skeletal muscle tissue of each fasted chicken, total RNA was electrophoresed in a 1.0% agarose gel containing formaldehyde, as described by Lehrach et al. (1977), and transferred to a Zeta-Probe Membrane (Bio-Rad Laboratories, Hercules, CA, USA) for hybridization. Probes were labeled with ORFs of avUCP (921bp) and avANT (894bp) by random priming with [α-32P]dCTP (3000 Ci mmol−1) (Takara BcaBEST™ Labeling Kit). Hybridized RNA blots were washed in a solution of 4xSSC (1xSSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0)/0.1% sodium dodecyl sulfate (SDS) at room temperature for 5 min, in 1xSSC/0.1% SDS at 55°C for 20 min, in 1xSSC/0.1% SDS at 58°C for 20 min, and in 1xSSC/0.1% SDS at 60°C for 20 min. The signals for avUCP and avANT mRNAs were detected and quantified using a Molecular Imager FX (Bio-Rad), which allows direct counting of emitted β-radiation by the 32P-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.

Statistical Analyses

Data were analyzed using the Statistical Analysis System (SAS, 1985). Means within a group were compared using Duncan’s least significance multiple-range test for the results from the mRNA expression analyses. All data are expressed in the form of mean ± standard error (S.E.). Differences were considered significant for values of P < 0.05.

Results and Discussion

Little is known about the precise physiological roles of the UCP1-homologues (UCP2, UCP3). Avian UCP is one of the mitochondrial anion carrier proteins, whose roles still also remain elusive. The confusing aspect regarding the physiological characterization of these UCP variants (that is, UCP2, UCP3 and avUCP) is that their expression was enhanced in response to fasting (Boss et al., 1998; Cardenas et al., 1999; Evock-Clover et al., 2002), a peculiar result considering that the enhanced expression of these variants occurred in muscle in the fasted state, a basal metabolic state in which energy expenditure would be expected, instead, to be depressed. More recently, Dulloo’s group demonstrated a close association between fasting-induced changes in UCP2 and UCP3 gene expression with those of key regulators of lipid oxidation, being consistent with the hypothesis that these UCP homologs may be involved in the regulation of lipid metabolism (Samec et al., 2002). However, information regarding how UCP variants progressively alter during fasting is scarce.

Figures 1 and 2 show fasting-induced alteration in avUCP and avANT mRNA levels in the pectoralis muscle, as analyzed by Northern blot. avUCP transcripts produced much stronger signals in skeletal muscle for the 12 and 24 h fasted groups compared to time 0 (Fig. 1a). The level of avUCP mRNA, which was quantified by scanning photodensitometry and normalized using GAPDH mRNA expression, was significantly increased up to 2-fold after only 12 h of fasting. The levels then diminished to about 1.5-fold at 24, 48 and 96 h (Fig. 1b). In contrast, avANT transcripts produced much stronger signals in skeletal muscle only after 48 and 96 h of fasting (Fig. 2a). Quantitative analysis showed that avANT mRNA levels were not significantly changed during the first 24 h of fasting, but the avANT mRNA levels did increase up to about 2-fold and 3-fold after 48 and 96 h respectively (Fig. 2b). This is accordance with our result normalized to even 18s rRNA level, analyzed by real-time RT-PCR (Abe et al., unpublished data). Thus, the current results exhibited different patterns of alterations to gene transcripts for these uncoupling proteins, avUCP and avANT: expression of avUCP gene was markedly enhanced after only 12 h of fasting and then reduced slightly but remained elevated even after 96 h of fasting, whereas those of avANT were only up-regulated after fasting for 24 h and continued to be further increased at 96 h. The former results are in agreement with previous reports indicating that mRNA levels of UCP2 and UCP3 (Samec et al., 2002), and avUCP (Evock-Clover et al., 2002) are markedly
Increased in the gastrocremius and tibialis anterior (fast-twitch muscles) in 48 h fasted mice and in pectoralis major and iliotibialis muscle in 24 and 48 h fasted broiler chickens, respectively. We, however, further extend the findings to report a decline in expression of UCP transcripts after reaching a plateau. In this regard, one could speculate that the utilization of fatty acid and uncoupling in the mitochondria from 12 h fasted chickens may be simultaneously upregulated if plasma FFA is enhanced during fasting as occurs in the case of rodents (Menahan and Sobocinski, 1983). This speculation does lead us to put forward the idea that co-upregulated UCP alleviates mitochondrial ROS production from β-oxidation in the skeletal muscle of fasted animals: fatty acid-mediated mild uncoupling via UCP would decrease mitochondrial production of ROS (Papa and Skulachev, 1997; Rolfe and Brand, 1997).

Explanation of increasing ANT transcription during the later period of fasting, requires some discussion. It is known that not only UCP but also ANT can mediate uncoupling by free fatty acids (Andreyev et al., 1989; Skulachev, 1991; Talbot et al., 2004). It has also been shown that knocking out of one of the two ANT isoenzymes (muscle-specific ANT1) results in a strong increase in ROS production by muscle mitochondria (Esposito et al., 1999). These reports allow us to postulate that ANT possibly suppresses mitochondrial ROS formation: ROS may be increasingly generated along with increased β-oxidation after fasting. We are currently examining this possibility. Alternatively, the increased ANT observed in the present study may be related, in part, to the increased exchange of ATP/ADP, namely via mitochondrial ATP export and ADP import, as the oxidation capacity of the fatty acid steadily increases. It does not, however, seem that ADP levels following a fast remain high in the cell (state 4: respiratory status when ADP is absent), or that the requirement for ANT in exchanging ATP/ADP may be high, as judged by Jucker et al. (2000) who found no differences in ATP synthesis flux in fasted versus control groups. In any event, as ROS, the main intracellular producers of which are mitochondria, can damage DNA, phospholipids and alter proteins such as transcriptional factors (Lee et al., 1999), uncoupling the respiratory chain from oxidative phosphorylation, via UCP or ANT, may play an important role in the regulation of ROS production.

In conclusion, the results presented here demonstrate that transcription of the mitochondrial anion carriers, avUCP and avANT is independently upregulated during fasting, implying different control mechanisms and consequences of each in the metabolic adaptations required during prolonged fasting. Elucidation of the physiological mechanisms involved in the regulation of UCP/ANT expression and the metabolic changes to β-oxidation in skeletal muscle will help us further understand biochemical adaptation in fasted animals. These issues will need to be addressed in future studies.

Fig. 2. Progressive alteration in the expression of avANT in chicken skeletal muscle during fasting. A, Northern blot analyses of RNA (30 μg/lane) from pectoralis superficialis muscle were performed using avANT cDNA cloned in our laboratory. Figures shown are representative of 2–3 independent analyses. Blots were subsequently hybridized with a GAPDH cDNA probe to correct for differences in the amounts of RNA loaded onto the gel. B, Results were normalized to GAPDH mRNA levels. Values are means ± SE (represented by vertical bars) for 4 chickens per treatment. *P < 0.05 compared within treatments.
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