FEBS Letters 580 (2006) 4815–4822

Possible role of avian uncoupling protein in down-regulating mitochondrial superoxide production in skeletal muscle of fasted chickens

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Received 6 June 2006; revised 25 July 2006; accepted 25 July 2006

Available online 4 August 2006

Edited by Vladimir Skulachev

Abstract **Little is known about the precise physiological roles of uncoupling protein 1 (UCP1) homologs (UCP2, UCP3, avian UCP) whose levels are up-regulated during fasting. UCPs in skeletal muscle are thought to play a role in the regulation of lipids as fuel substrates, and/or in controlling the production of reactive oxygen species (ROS). The aim of this investigation, using skeletal muscle from fasted chickens, was to examine alterations in the expression of genes encoding for avian UCP and key enzymes relevant to lipid flux across the mitochondrial β-oxidation pathway. We also clarified whether an increase in avUCP content could be associated with altered ROS production by mitochondria. Transcription levels of avUCP and CPT-I genes were increased 7.7- and 9.5-fold after a 24 h fast and slightly diminished but remained about 5.0- and 7.7-fold higher than baseline levels, respectively, after 48 h of fasting. In contrast, members of the β-oxidation pathway, LCAD and 3HADH, were gradually up-regulated from 12 to 48 h of fasting. This suggests that processes involved in the transfer and oxidation of fatty acids are up-regulated differently during the initial stage of fasting. Analysis of ROS production by lucigenin-derived chemiluminescence showed that the FFA-sensitive portion of carboxyatractyloside-upregulated ROS production was greater in skeletal muscle mitochondria from 24 h-fasted chickens compared with control, which leads us to postulate that ROS production is potentially down-regulated by UCP. The possible involvement of a backlog of fatty acid for oxidation, observed in chickens after a 24 h fast, in a transmembrane gradient of free non-oxidized fatty acids is also discussed.**

Keywords: UCP; Fasting; ROS; Fatty acid oxidation; Chicken

1. Introduction

Uncoupling proteins (UCPs) belong to a family of transporter proteins present in the mitochondrial inner membrane. By dissipating the mitochondrial proton gradient these proteins uncouple respiration from ATP synthesis [1]. UCP1 is present mainly in brown adipose tissue (BAT), which is the major site of regulatory thermogenesis in small rodents [2]. Five additional uncoupling protein homologs, UCP2-4, brain mitochondrial carrier protein type 1 (BMCP 1) and kidney mitochondrial carrier protein 1 (KMCP 1), have been identified to date. UCP2 is expressed ubiquitously [3], while UCP3 gene expression is seen in skeletal muscle, adipose tissue and heart [4,5]. UCP 4, BMCP 1 and KMCP 1, all of which have recently been identified [6–8], are expressed primarily in the brain and other neural tissues and within kidney cortex.

It is now accepted that UCP1 is a key molecule in thermogenesis, in particular cold- and diet-induced heat production [9,10]. In contrast, less is known of the physiological roles of mammalian UCP2 and UCP3. It is thought 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) (see for recent reviews [11–13]). UCP2 and UCP3 mRNA levels are modulated by fasting [14,15] and by re-feeding [16]. Feed deprivation increases skeletal muscle gene expression of UCP2 and UCP3 through the elevation of free fatty acid [FFA] concentrations in the serum [14]. A recent study [17] indicated a close association between fasting-induced changes in UCP2 and UCP3 gene expression with those of key regulators of lipid oxidation, which is consistent with the hypothesis that these UCP homologs may be involved in the regulation of lipid metabolism. On the other hand, considering the concept of mild uncoupling as a line of antioxidant defense [18], and that minor UCPs might mediate this uncoupling [19], one can speculate that enhanced UCP levels in fasted animals would provide an antioxidant function. More specifically, it was proposed that UCP3 functions as a regulator of mitochondrial ROS scavenging under fasting conditions, during which oxidative stress may be expected to be increased [20], i.e. the role of UCP3 may be to increase uncoupling and commensurate O_2 consumption, even during state 4 conditions, thereby minimizing ROS production. This is supported by a more recent hypothesis for the main, ancestral function of UCP2 and UCP3, that being to cause mild uncoupling and so diminish mitochondrial superoxide production [21].

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Abbreviations: UCP, uncoupling protein; ANT, adenine nucleotide translocator; CPT-I, carnitine-palmitoyl-transferase-I; 3HADH, 3-hydroxyacyl CoAdehydrogenase; LCAD, long-chain acyl CoA dehydrogenase; CS, citrate synthase; CAT, carboxyatractyloside; ROS, reactive oxygen species; LDCL, lucigenin-derived chemiluminescence; NEFA, non-esterified fatty acid; SS mitochondria, subsarcolemmal mitochondria

0014-5793/$32.00
doi:10.1016/j.febslet.2006.07.070

These findings suggest that the expression of genes encoding UCPs might progressively change, thus making UCPs key regulators of lipid oxidation and/or of ROS scavenging in animals during fasting.

Although bird species have no distinct stores of BAT or a related type of thermogenic tissue [22,23], a new protein, named avian UCP (avUCP), which shares a 71–73% amino acid identity with both UCP2 and UCP3, was identified in chicken skeletal muscle [24,25]. Increased expression of avUCP in cold-acclimated ducklings [24], chickens [25] and king penguins [26] has been demonstrated. Moreover, we also reported the negative correlation between avUCP content and mitochondrial superoxide production in chicken skeletal muscle during acute heat stress [27]. It was reported that, as in mammalian UCP, avUCP is up-regulated during feed deprivation [28,29]. Because skeletal muscle tissue, in birds and in large mammals whose BAT decreases with development, may play a significant common role in controlling metabolic flux under conditions of nutritional stress [30], studies of avUCP involvement in mitochondrial function in chicken skeletal muscle during fasting are of particular interest.

In order to provide further insight into the physiological role of enhanced avUCP in fasted chickens, we investigated the progressive alteration with fasting in the expression of genes encoding for mitochondrial protein UCPs along with transcripts for key enzymes relevant to lipid flux across the mitochondrial β-oxidation pathway. Further to this, a possible role of UCP in down-regulating mitochondrial superoxide production in the skeletal muscle of fasted chickens was also studied.

2. Materials and methods

2.1. Animals and experimental design

Male white leghorn chicks (Julia) 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 4–5 weeks. Birds used in experiments were selected from a 2-fold larger population in order to obtain uniform body weights; these experimental birds were kept in wire-bottomed cages under conditions of controlled temperature (25 ± 1 °C) and continuous light.

In the first series of experiments, six chickens (388 ± 12 g) were used for determination of the time course of plasma glucose and non-esterified fatty acid (NEFA) concentrations as a function of fasting time. Wing vein blood samples were taken at 0, 12, 24, and 48 h after the commencement of fasting. In a second series of experiments, to clarify the alteration of skeletal muscle target mRNA expression and mitochondrial enzyme activities, 20 randomly selected chickens (360 ± 14 g) were maintained in 5 groups of 4 animals, for 0, 12, 24, or 48 h, respectively, from the beginning of fasting. In a third series of experiments, for the estimation of mitochondrial superoxide production and the determination of avUCP protein content, 22 chickens (303 ± 20 g) were separated into two groups of equal number (0, 24 h of fasting). All birds were provided with free access to water. They were killed by decapitation and the *pectoralis* muscles rapidly excised. This method of killing was used in preference to overdose by general anesthesia, which is known to uncouple oxidative phosphorylation [31]. For mRNA analyses, muscles were frozen, powdered in liquid nitrogen, and stored at −80 °C until required for the extraction of total RNA. For isolation of mitochondria, muscles were placed in ice-cold isolation buffer A (see below). All experiments were performed in accordance with institutional guidelines concerning the care and use of animals.

2.2. Blood glucose and NEFA analysis

Blood samples were centrifuged at 700 × *g* for 10 min. Plasma glucose and NEFA concentrations were quantified using the Glucose CII-test and NEFA C-test (Wako Pure Chemical Industries, Osaka, Japan), respectively.

2.3. Quantitation of mRNA using real-time PCR

2.3.1. General methods. Standard molecular biological techniques were used, essentially as described by Sambrook et al. [32]. Tissues were homogenized in Trizol-Reagent (Invitrogen Gibco-BRL, Bethesda, MD, USA) and total RNA isolated according to the manufacturer's protocol.

To study progressive alterations in the expression of skeletal muscle target genes, that is, avUCP and fatty acid oxidation-related genes (CPT-I: carnitine-palmitoyl-transferase-I; 3HADH: 3-hydroxyacyl CoA dehydrogenase; LCAD: long-chain acyl CoA dehydrogenase; and CS: citrate synthase), real-time reverse transcription-polymerase chain reaction analysis was performed using the iCycler iQ Real Time Detection System (Bio-Rad Laboratories, Hercules, CA). Five micrograms of total RNA, prepared using Trizol-reagent (Invitrogen, San Diego, CA, USA), was reverse transcribed using a mixture of oligo(dT)$^{12-18}$ and random primers, and M-MLV reverse transcriptase (Invitrogen, San Diego, CA, USA). One microliter of each RT-reaction then served as a template in a 50 μl PCR reaction containing 2 mM $MgCl_2$, 0.5 μM of each primer and 0.5X SYBR green master mix (Bio Whittaker Molecular Applications). SYBR green fluorescence was detected at the end of each cycle to monitor the amount of PCR product formed during that cycle. At the end of each run, melting curve profiles were determined. Oligonucleotide sequences of sense and antisense primers and annealing temperatures are shown in Table 1. The specificity of the amplification product was verified by electrophoresis on a 0.8% agarose gel following a check of the DNA sequences. Results are presented as the ratio of each gene to 18s rRNA to correct for differences in the amounts of template DNA used.

2.4. Isolation of mitochondria and assay of 3HADH activity

Muscle subsarcolemmal (SS) mitochondria were isolated from muscle tissue according to our previously reported methods [25]. Muscles were trimmed of fat and connective tissue, blotted dry, weighed and then minced with scissors. The minced tissue was suspended in ice-cold buffer A (containing 100 mM sucrose, 50 mM tris (hydroxymethyl) aminomethane (Tris) base, 5 mM MgCl2, 5 mM ethylene glycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetra acetic acid (EGTA), 100 mM KCl, pH 7.4) and homogenized with a Potter-Elvehjem homogenizer (5 passages). The homogenate was then centrifuged at 800 × *g* for 10 min. The supernatant was centrifuged at 1000 × *g* for 10 min and

Table 1
Sequences for real-time PCR primers

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)	Fragment size (bp)	Annealing (°C)	GenBank® Accession Number
*av*UCP	ACTCTgTgAAgCAgCTCTACACC	ATgTACCgCgTCTTCACCACATC	433	65	AB088685
CPT-I	AAgggTACAgCAAAgAAgATCCA	CCACAggTgTCCAACAATAggAg	284	63	AY675193
3HADH	CTTggTgCCATATATggAAg	CTTCTACCAgTTTATCCAggAg	230	65	CD215336
LCAD	CAgTgACTTgCAAggAgTACggA	AgCCTCTCggTTTgTAACCgTAA	136	65	XM_421861
CS	AgggATTTCATCTggAACACACT	CACCgTgTAgTACTTCATCTCCCT	282	64	BG712956
18s rRNA	TAgATAACCTCgAgCCgATCg	gACTTgCCCTCCAATggATCC	312	63	AF173612

avUCP: avian uncoupling protein, CPT-I: carnitine-palmitoyl-transferase-I, 3HADH: 3-hydroxyacyl CoA dehydrogenase, LCAD: long-chain acyl CoA dehydrogenase, CS: citrate synthase.