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Comparative Biochemistry and Physiology Part A 136 (2003) 655–661

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Abundant and constant expression of uncoupling protein 2 in the liver of red sea bream *Pagrus major*

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Received 16 August 2002; received in revised form 18 July 2003; accepted 19 July 2003

Abstract

Four overlapping cDNA fragments encoding a partial sequence for uncoupling protein 2 (UCP2) were amplified by PCR using degenerate primers from the liver of a marine teleost fish, red sea bream (*Pagrus major*). The partial sequence was 674 bp long, encoding 224 amino acids. The deduced amino acid sequence from the cDNA partial sequence contained the signature motifs for mitochondrial transporter protein and revealed positional identity higher than 72.8% with UCP2 from mammals. The fish UCP2 gene was highly expressed in the liver but almost undetectable in the visceral mesenteric adipose tissue. Using beta-actin as control, the UCP2 mRNA level was determined to be at least 20-fold higher in the liver than in the visceral mesenteric adipose tissues. Neither 48 h starvation nor high lipid diet had any significant effect on liver UCP2 gene expression, indicating that the abundant UCP2 gene expression was stable and might have some basic function in a fish liver that always contains high lipid content. The striking contrast of UCP2 gene expression in the two fish fat-depot organs is consistent with their large differences in oxidative capacity. We suggest that the fish liver may adapt to a constantly high fat deposit by maintaining high UCP2 expression to constrain reactive oxygen species (ROS) production and protect hepatocytes from apoptosis.

Keywords: Uncoupling protein 2; cDNA sequence; Gene expression; Liver; Adipose; Feeding; Lipid; Reactive oxygen species; Fish; *Pagrus major*

1. Introduction

Uncoupling protein (now UCP1) is a demonstrated uncoupler of oxidative phosphorylation (Nicholls and Locke, 1984). UCP1 expression is restricted to mammalian brown adipose tissue, where it provides a pathway for protons pumped out of the mitochondrial matrix by the electron transport chain to pass back in. The futile cycling of proton pumping and leaking allows an augmentation of heat production in this tissue. Since this sort of net proton conductance is found not only in the mitochondria of mammalian brown adipose tissue, but also in almost all other mitochondria of animals, plants and yeasts (Brand et al., 1999), the recently discovered UCP1 homologues, uncoupling protein 2 (UCP2) (Fleury et al., 1997; Gimeno et al., 1997) and uncoupling protein 3 (UCP3) (Boss et al., 1997; Vidal-Puig et al., 1997) have been considered candidates for the catalysis of this basal level of uncoupling oxidative phosphorylation in mammals. In part because of the high sequence similarity of the UCP homologues

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1095-6433/03/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/S1095-6433(03)00218-6

to UCP1, early hypotheses for their physiological role(s) have focused on thermogenesis. However, Stuart et al. (1999) have identified uncoupling protein 2 (UCP2) from a carp (*Cyprinus carpio*) peritoneal exudates cell cDNA library and from a zebrafish (*Danio rerio*) day 0 fin regeneration cDNA library. Heat is conducted, and thus dissipated, in water at a rate some 25 times that in air. In fish, such as carp, where specific anatomical adaptations to retain metabolic heat (e.g. epidermal fat layers) are absent, a strategy of active thermogenesis is untenable. The presence of UCP2 in fish (ectothermic vertebrate), strongly suggests that UCP2 may have function(s) not related to thermogenesis (Stuart et al., 1999).

Whereas nothing is known at present about the tissue expression and physiological regulation of fish UCP2 gene in vivo, the work on mammalian UCP2 gene expression has revealed a tissue distribution pattern related to lipid metabolism, especially fatty acid oxidation (Fleury et al., 1997; Samec et al., 1998; Ricquier and Bouillaud, 2000). A recent study by Echtay et al. (2002) indicates that the interaction of superoxide with UCPs may be a mechanism for decreasing the concentrations of reactive oxygen species inside mitochondria.

Liver is the major site in mammals for the increase of beta-oxidation induced by fish oil feeding (Lock et al., 1989). Although mammalian hepatocytes do not normally express uncoupling proteins, UCP2 is expressed in hepatocytes of genetically obese mice with fatty livers, or induced by dietary fish oil and fibrates (Chavin et al., 1999; Cortez-Pinto et al., 1999; Rashid et al., 1999; Tsuboyama-Kasaoka et al., 1999; Murase et al., 2001).

In fish with a discrete visceral mesenteric adipose tissue, such as red sea bream (*Pagrus major*), liver also functions partially as a fat depot organ (Ando and Mori, 1993). In consistent to the high lipid content of fish liver, our previous work showed a substantial expression of lipoprotein lipase (LPL) mRNA in the liver of red sea bream and revealed a reciprocal regulation of LPL mRNA abundance by dietary fatty acids in the liver and visceral mesenteric adipose, indicating that the fish will partition excessive supply of lipid substrates to the liver in response to over feeding (Liang et al., 2002). The purpose of this study was to compare the UCP2 gene expression of the two fat depot organs with distinctive oxidative capacity in the fish (ectothermic vertebrate), which would test the new idea about the physiological function of vertebrate UCP2.

2. Materials and methods

2.1. Fish, feed, feeding and sampling

Juvenile red sea bream *P. major* (average mass 46 g) were used in this study. At the beginning of the experiment the fish were randomly distributed (45 fish/tank) into two circular 500 l indoor tanks. The fish were reared under natural photoperiod (February–June). The water temperature rose from 15.0 to 21.0 °C over the course of the feeding experiment.

The experimental feeds consisted of a low lipid diet (control diet) that was a standard commercial feed (C-3000, Kyowa Hakko, Tokyo, Japan), and a high lipid diet that was a batch of the low lipid diet, top-dressed with 10% (w/w) pollack viscera oil (Riken Vitamin, Tokyo, Japan). The low lipid diet contained 60.6% protein and 15.3% lipid and the high lipid diet 54.9% protein and 24.6% lipid, expressed on a dry basis. The fish were hand fed to satiation once a day for 14 weeks.

Following the feed conditioning, fed and starved fish were sampled at 5 and 48 h post-feeding, respectively. Ten fish were sampled at each time from each tank. They were anaesthetized (0.01% ethyl 3-aminobenzoate methanesulfonic acid salt solution, Aldrich Chemical Company, WI, USA), immediately frozen in liquid nitrogen and stored at −85 °C until use.

2.2. RNA isolation and reverse transcription

The liver and visceral mesenteric adipose tissue were dissected from the partially frozen fish and weighed quickly. Total RNA was isolated using S N A P. Total RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with oligo$(dT)_{18}$ primer using 1st-strand cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA).

2.3. PCR cloning and sequencing of liver UCP2 cDNA

The following four degenerate primers were synthesized to perform PCR: 5‘TTTCCA-CTGGACACCGCAAAA(G)GT5’ (designated as UCP01F), 5‘GTGGCGGGACTGCAGCGT-

CAA(G)ATG3' (designated as UCP02F), 5'GCGGAGTTCATGTATCTTGTT(C)TT3' (designated as UCP03R), and 5'GTGACAAACA-TAACCACA(G)TTCCA3' (designated as UCP04R). The Takara PCR Kit (Takara, Otsu, Shiga, Japan) was used for PCR amplification of the four overlapped liver UCP2 cDNA fragments. The following cycle was used for PCR: denaturation for 1 min at 94 °C, annealing for 30 s at 50 °C, and extension for 2 min at 72 °C. These steps were repeated for 30 cycles with an additional initial 1-min denaturation at 94 °C and a 3-min final extension at 72 °C.

The PCR product was gel purified and recovered using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA, USA), and cloned into TA (pCRII) vector (Invitrogen). Inserts were sequenced using ABI prism dRhodamine terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA). Nucleotide sequence was obtained and confirmed from at least five clones of each of the four overlapped fragments.

2.4. Quantitation of relative UCP2 mRNA abundance

The relative UCP2 mRNA abundance was determined by PCR amplification of tissue cDNA samples within the exponential phase, using beta-actin as an external control. Two gene-specific PCR primers were used to amplify a 650 bp fragment of UCP2 cDNA: 5'TCGGGACGATCA-GCACCATGATCAAAAC3' (designated as UCP05F), and 5'TCAGAAACGAGGGCACAA-ATCCTTTGTA3' (designated as UCP06R). A 554 bp fragment of beta-actin cDNA was amplified by PCR using following two primers: 5'TG-GCAGGCAGAGACCTGACGGACTATCT-GATGAA3' (designated as ACT01F), and 5'CTCATCATATTCCTGCTTGCTGATCCA-CAT3' (designated as ACT02R). The PCR products were separated on a 2% agarose gel and stained with ethidium bromide. The gels were then photographed and analysed by densitometric scanning (Densitograph Version 2.5, ATTO Corporation, Tokyo, Japan). The relative UCP2 cDNA level in the fish tissue was expressed as the ratio UCP2/beta-actin cDNA (%).

2.5. Statistical analysis

Statistical analyses of differences among treatment means were carried out by ANOVA and Fisher's protected least significant difference (StatView 4.51, Abacus Concepts, Berkeley, CA, USA). Differences were considered significant if $P < 0.05$.

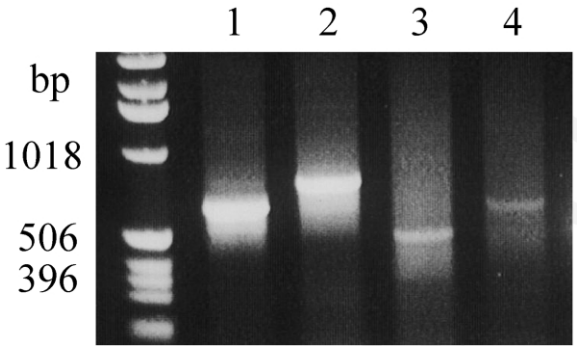

Fig. 1. UCP2 cDNA fragments generated by PCR from the liver of red sea bream (*Pagrus major*) using degenerate primers. 1, UCP01F and UCP03R; 2, UCP01F and UCP04R; 3, UCP02F and UCP03R; 4, UCP02F and UCP04R.

3. Results

PCR using the four pairs of degenerate primers (UCP01F and UCP03R, UCP01F and UCP04R, UCP02F and UCP03R and UCP02F and UCP04R), designed to amplify four overlapped UCP2 cDNA fragments (647 bp, 779 bp, 491 bp and 623 bp, respectively), yielded four products of expected length from the liver cDNA of red sea bream (Fig. 1). Nested PCR using primers UCP02F and UCP03R and the PCR product from primers UCP01F and UCP04R as template, also yielded a 491 bp fragment (figure not shown), indicating that the four fragments were overlapped as designed.

The partial liver UCP2 cDNA of red sea bream was 674 bp long, encoding 224 amino acids (Fig. 2). It seems that the incomplete amino acid sequence of the fish UCP2 lacked the first 59 and the last 28 amino acid residues. The deduced amino acid sequence contained the signature motifs (PTDVVKVR, 78–85; PVDVVKTR, 177–184) for mitochondrial transporter protein (Walker, 1992; Stuart et al., 1999). Like other UCP2, the fish UCP2 did not possess the two histidines that are believed to be essential for UCP1 function (Klingenberg, 1990) and on the identical position were leucine and glycine instead (L91 and G93 in red sea bream numbering). The similarity of amino acid sequences between red sea bream (perciform)

and carp or zebrafish (cypriniform) is 73.2–75.9%, between red sea bream and mammals (mouse, hamster, rat, dog, pig, cow and human) is 72.8–74.1%, and between carp or zebrafish and mammals (mouse, hamster, rat, dog, pig, cow and human) is 82%. This multiple alignment result shows nearly the same pattern of sequence homology as that for the growth hormones of these vertebrates (Chang et al., 1992; Zhu et al., 1992).

The fish UCP2 gene was highly expressed in the liver but was almost undetectable in the visceral mesenteric adipose tissue of this fish (Fig. 3). Using beta-actin as control, the UCP2 mRNA level was determined to be at least 20-fold higher in the liver than in the visceral mesenteric adipose tissues under the four nutritional conditions in this study ($P<0.0001$) (Table 1). Neither starvation nor high lipid diet had any significant effect on the liver UCP2 mRNA abundance ($P>0.05$) (Table 1), indicating that the UCP2 gene expression was not only extremely high but also much stable in the fish liver that has a constant high lipid content.

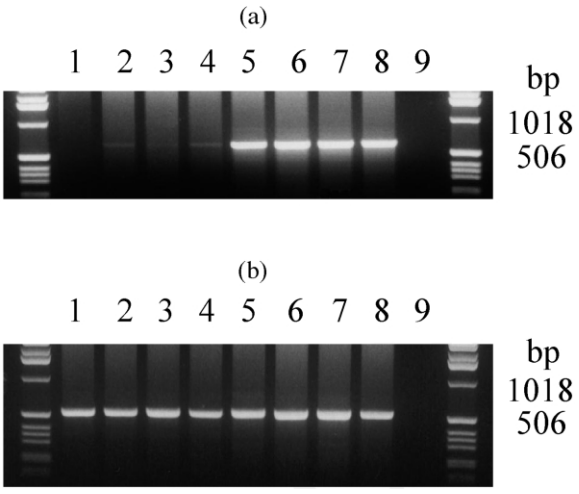

Fig. 3. Analysis of UCP2 (a) and beta-actin (b) mRNA expression in the liver (5–8) and visceral mesenteric adipose tissue (1–4) of red sea bream (*Pagrus major*) in response to nutritional treatments by RT-PCR. 1 and 5, control diet under fed condition; 2 and 6, control diet under fasted condition; 3 and 7, high-fat diet under fed condition; 4 and 8, high-fat diet under fasted condition; 9, negative control without template.

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TACAGAGGAGTGTTCGGGACGATCAGCACCATGATCAAAACAGAGGGACCCAGGTCTCTG      60
 Y  R  G  V  F  G  T  I  S  T  M  I  K  T  E  G  P  R  S  L       20
TACAACGGGCTGGTGGCAGGGCTGCAGAGACAGATGTGCTTCGCCTCCATCAGAATCGGC     120
 Y  N  G  L  V  A  G  L  Q  R  Q  M  C  F  A  S  I  R  I  G       40
CTCTACGACAACGTCAAAAACTTCTACACTGGGGGCAAAGACAACCCTAATGTGCTGATA     180
 L  Y  D  N  V  K  N  F  Y  T  G  G  K  D  N  P  N  V  L  I       60
CGTATCCTGGCCGGCTGTACTACAGGCGCCATGGCAGTCTCGTTTGCACAACCCACAGAT     240
 R  I  L  A  G  C  T  T  G  A  M  A  V  S  F  A  Q  P  T  D       80
GTGGTCAAGGTTCGATTTCAGGCCCAGAGCAACCTGGACGGCGTGGCCCGTCGCTACACT     300
 V  V  K  V  R  F  Q  A  Q  S  N  L  D  G  V  A  R  R  Y  T      100
GGCACCATGCAGGCCTACAAACACATCTTCCAGAATGAGGGCATGCGTGGACTCTGGAAA     360
 G  T  M  Q  A  Y  K  H  I  F  Q  N  E  G  M  R  G  L  W  K      120
GGCACACTACCCAACATCACTAGAAATGCGCTTGTCAACTGCACAGAACTGGTTACATAC     420
 G  T  L  P  N  I  T  R  N  A  L  V  N  C  T  E  L  V  T  Y      140
GACCTGATCAAGGAGGCCATCCTCAAACACAACCTGTTGTCAGACAACCTGCCCTGCCAC     480
 D  L  I  K  E  A  I  L  K  H  N  L  L  S  D  N  L  P  C  H      160
TTTGTTTCTGCGTTTGGTGCAGGCTTTGTTACCACAGTGATAGCTTCCCCAGTAGATGTG     540
 F  V  S  A  F  G  A  G  F  V  T  T  V  I  A  S  P  V  D  V      180
GTGAAAACTAGATACATGAACTCCCCACCAGGCCAGTACAAGAGTGCTATCAACTGTGCC     600
 V  K  T  R  Y  M  N  S  P  P  G  Q  Y  K  S  A  I  N  C  A      200
TGGACCATGATGACAAAAGAGGGGCCGACTGCTTTCTACAAAGGATTTGTGCCCTCGTTT     660
 W  T  M  M  T  K  E  G  P  T  A  F  Y  K  G  F  V  P  S  F      220
CTGAGGTTGGGATC     674
 L  R  L  G         224
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Fig. 2. Partial nucleotide sequence of cDNA and deduced amino acid sequence for UCP2 from the liver of red sea bream (*Pagrus major*).

Table 1
Nutritional regulation of UCP2 gene expression in the liver and visceral mesenteric adipose tissue of red sea bream (*Pagrus major*)

Tissue	Ratio UCP2/beta-actin mRNA (%)			
	CF	CS	HF	HS
Liver	43 ± 14^a	55 ± 7^a	62 ± 4^a	60 ± 5^a
Visceral adipose	0.5 ± 0.1^b	2.1 ± 0.9^b	1.3 ± 0.6^b	3.1 ± 2.2^b

A value followed by a superscript differs significantly ($P < 0.05$) from all other values not followed by the same superscript. CF, control diet under fed condition; CS, control diet under starved condition; HF, high-fat diet under fed condition; HS, high-fat diet under starved condition.

4. Discussion

Some studies on mammalian UCP2 gene expression has revealed a tissue distribution pattern related to lipid metabolism, especially fatty acid oxidation (Fleury et al., 1997; Ricquier and Bouillaud, 2000). In the gastrocnemius muscle (a mixed fiber type muscle with a high capacity to shift between glucose and lipids as fuel substrate), the UCP2 mRNA expression was found to be markedly up-regulated during food deprivation and the expressions was subsequently found to be markedly down-regulated upon transition to refeeding. The differential mRNA expression of muscle UCP2 during food deprivation and refeeding, are much more consistent with a role for UCP2 in the regulation of lipids as fuel substrate rather than as mediators of regulatory thermogenesis (Samec et al., 1998). Our study has demonstrated that the UCP2 gene was highly expressed in the liver but was almost undetectable in the visceral mesenteric adipose tissue of red sea bream. It would be very interesting and important to further identify the type of liver cells expressing UCP2 and also to conduct an expanded analysis of the pattern of UCP2 expression among tissues. The striking contrast of UCP2 gene expression in the two fish fat-depot organs is consistent to the great distinction of their oxidative capacity, suggesting the involvement of UCP2 expression in lipid oxidation instead of lipid deposition. As the abundant UCP2 gene expression in the liver was constant, it might be essential to the basic function of the fish liver that always contains high lipid content. We should also point out that translational regulation of the UCP2 mRNA has been shown for human UCP2 expression under stress conditions (Pecqueur et al., 2001). It still needs to be determined whether translational regulation may exist for the fish UCP2 expression under our experimental condition.

The physiological function of UCP2 remains to be elucidated. However, there is evidence showing that UCP2 modulates the mitochondrial generation of reactive oxygen species (ROS) in some cell types, with active ROS production (e.g. immune cells) or with excessive provision of fatty acids (e.g. intestinal epithelial cells) (Fleury et al., 1997; Negre-Salvayre et al., 1997; Murase et al., 2001). Because ROS are basically harmful to tissue cells, as indicated by our previous studies (Hwang and Liang, 1989, 1991; Chen et al., 1992), it is proposed that aerobic cells develop a special protective system against ROS toxicity: the existence of the mild uncoupling of respiration and phosphorylation by means of increased H^+ leak through the mitochondrial inner membrane (Skulachev, 1996; Ricquier and Bouillaud, 2000). In fact, uncouplers increase the rate of electron transfer and inhibit O_2^- radical formation by mitochondria. The explanation of this effect is that partial uncoupling stimulates O_2 consumption, shortens the lifetime of CoQ^- radical (which is an excellent one-electron O_2 reductant and an initiator of ROS formation), and inhibits ROS production. Therefore, respiration uncoupling represents a powerful system for the limitation of ROS production. This suggests that one of the functions of proteins that uncouple respiration might be the limitation of ROS production (Ricquier and Bouillaud, 2000).

That human UCP2 is expressed at high levels throughout the immune system, including spleen, thymus, leukocytes, macrophage, etc., strongly suggests a putative role for this protein in immunity (Fleury et al., 1997). Because fatty acids and their metabolites, especially ROS, are essential for immune function, including cytotoxic activity (Trinchieri, 1989; De Sanctis et al., 1994), it is reasonable to speculate that the high level of UCP2 expression in the immune system, might be a protective response to the active ROS production in the immune cells. This speculation is consistent to the work of Larrouy et al. (1997), who found that UCP2 is also expressed at a very high level in Kupffer cells.

Echtay et al. (2002) showed that superoxide increased mitochondrial proton conductance through effects on UCP1, UCP2 and UCP3. Superoxide-induced uncoupling requires fatty acids and is inhibited by purine nucleotides. It correlates

with the tissue expression of UCPs, appears in mitochondria from yeast expressing UCP1 and is absent in skeletal muscle mitochondria from UCP3 knockout mice. Their findings indicate that the interaction of superoxide with UCPs may be a mechanism for decreasing the concentrations of reactive oxygen species inside mitochondria.

Although lipids and their metabolites are potentially hepatotoxic, the absence of overt injury in fatty livers suggests that adaptive responses to lipid accumulation occur. Despite the up-regulation of factors that threaten cell viability, hepatocyte death was not increased in mice with fatty livers, confirming that the protective response of the liver were sufficient under the conditions (Rashid et al., 1999). This must be also true to some fish livers, for they function partially or even totally as fat depot organs and always keep high lipid content.

Murase et al. (2001) found that fish oil feeding up-regulated UCP2 expression in the small intestine as well as in the liver and suggest that intestine UCP2 is up-regulated through direct activation of peroxisome proliferator-activated receptor by dietary fatty acids, whereas liver UCP2 is up-regulated through some indirect mechanism involving lipid metabolism. Cortez-Pinto et al. (1999) found that lipid emulsions resulted in a dose- and time-dependent induction of UCP2 mRNA and protein in cultured hepatocytes. Consistent with the possibility that ROS generated intracellularly during lipid metabolism participates in UCP2 induction, addition of the cell-impermeable antioxidant did not alter lipid-related induction of UCP2. Moreover, tert-butyl hydroperoxide, which is known to increase hepatocyte mitochondrial ROS production, also increased UCP2 mRNA levels. In our study, it also seems unlikely that the liver UCP2 expression of the fish could be directly regulated by fatty acids, because neither 48 h starvation nor high lipid diet had any significant effect on the liver UCP2 mRNA abundance.

In conclusion, the fish UCP2 gene was found to be highly expressed in the liver but was almost undetectable in the visceral mesenteric adipose tissue. The striking contrast of UCP2 gene expression in the two fish fat-depot organs is consistent to the great distinction of their oxidative capacity. We suggest that high fat deposit in the fish liver, may keep a stable ROS generation under the oxidative condition. This might further induce a constant and abundant UCP2 expression in the fish liver to restrain over production of ROS and protect the fish hepatocytes from apoptosis.

Acknowledgments

We wish to express our thanks to Dr Jeffrey T. Silverstein and four anonymous referees for their helpful review of the manuscript. This work was financially supported by the STA Fellowship of Japan (Project No. 298157), the Guangdong Natural Science Foundation of China (Project No. 980702), and the National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences (Beijing, China).

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