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Molecular characterization and expression of a novel homolog of uncoupling protein 5 (UCP5) from the eastern oyster *Crassostrea virginica* (Bivalvia: Ostreidae)

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ABSTRACT

Uncoupling proteins (UCPs) belong to the mitochondrial anion carrier gene family which has been implicated in diverse physiological functions ranging from thermoregulation to antioxidant defense. In mammals, the UCP family is well characterized and contains five members (UCP1-5). In contrast, invertebrate homologues of uncoupling proteins are much less studied both from the viewpoints of structure and function. In this study we report nucleotide and predicted protein structure of an important member of UCP family, UCP5 from eastern oysters *Crassostrea virginica*. UCP5 from oysters appears to be a close homolog of the mammalian brain mitochondrial carrier protein (BMCP1, or UCP5) and is the first full-length UCP described from a Lophotrochozoan invertebrate. Evolutionary analysis of UCP sequences indicates at least three monophyletic UCP branches (UCP1-3, UCP4 and UCP5) that have diverged early in the evolution, prior to the divergence of vertebrates and invertebrates. In oysters, two forms of UCP5 transcript are found (UCP5S and UCP5L) that differ by 152 bp in length due to the presence of an intron in UCP5L. UCP5 was expressed in all studied oyster tissues, unlike mammals, where UCP5 is predominantly expressed in brains and male gonads. Hypoxia-reoxygenation stress, sublethal Cd exposure (50 μg L^{-1} Cd for 56 days) and acclimation to different temperatures (12 and 20 °C) had no significant effect on UCP5 mRNA expression in oysters indicative of its relative unimportance in antioxidant defense and temperature adaptation of oyster mitochondria. These data suggest that despite the relatively high degree of evolutionary conservation of the UCP5 amino acid sequence, its functional significance in mitochondria changed in the course of evolution of mollusks and vertebrates.

1. Introduction

Uncoupling proteins (UCPs) consist of group of genes in the mitochondrial anion carrier gene family which have been implicated in a variety of physiological functions such as regulation of mitochondrial membrane potential, antioxidant defense, apoptosis and thermoregulation (review in: Adams, 2000; Erlanson-Albertsson, 2003). In mammals, five members of this group have been described, including UCP1, 2, 3, 4 and 5 with partially overlapping expression in various organs. As their name suggests, UCPs can uncouple ATP production from mitochondrial respiration by causing proton leakage, although the degree of contribution to physiological proton leak varies between different UCP isoforms. UCP5 is an important mitochondrial protein that has been linked to regulation of metabolism and mitochondrial function in mammals and insects. It has also been implicated in antioxidant protection through the control of generation of the reactive oxygen species (ROS) in the mitochondria due to the mild mitochondrial uncoupling (Sanchis et al., 1998; Yu et al., 2000). Consistent with its putative role in antioxidant protection, UCP5 mRNA was overexpressed in response to hyperoxia and transient global ischemia (during the induced cardiac arrest and resuscitation), and downregulated during hypoxia in neuronal cell lines and in rat brain tissue (Pichiule et al., 2004). UCP5 also appears to be involved in the control of metabolic rate and calorie use, although physiological and molecular mechanisms for this involvement are less clear. In *Drosophila*, UCP5 affects sensitivity to starvation and low-calorie diets (Sánchez-Blanco et al., 2006). In obesity-resistant mice, UCP5 was upregulated in response to a high-fat diet and downregulated during fasting in the liver (Yu et al., 2000). It was also upregulated in mice in response to the cold exposure and endotoxin exposure, going hand-in-hand with elevated metabolic rates (Yu et al., 2000).

In mice and humans, three UCP5 isoforms with different tissue-specific expression patterns have been described (Yu et al., 2000). The UCP5L, a long form, is the less common form. In humans, it is the only form found in the brain, and composes only about 25% of the UCP5 transcripts in other tissues. In contrast to humans, mice have

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substantially lower levels of UCP5L and it is found in the brain and white adipose tissue only. The short form, UCP5S, is lacking three amino acids (Val-Ser-Gly) at position 23–25 compared to UCP5L and is the most common UCP5 transcript in mammals. It is found in all tissues in mice and all but brain tissues of humans (Yu et al., 2000). The human-specific UCP5SI has an additional 31 amino acids inserted between transmembrane domains III and IV that adds a second hydrophobic segment not found in other isoforms. In mammals, UCP5L and UCP5SI are more potent with respect to their ability to reduce the mitochondrial membrane potential than the short form UCP5S (Yu et al., 2000). UCP5 homologs have also been isolated from insects and nematodes, but their expression profiles (including tissue-specific expression patterns and presence of different isoforms) have not been studied in invertebrates. Moreover, no UCPs have been so far characterized in the majority of other invertebrate groups, including mollusks and other Lophotrochozoa.

In this study, we have isolated and characterized full-length transcripts of two forms of UCP5 from a marine bivalve, the eastern oyster *Crassostrea virginica*, and studied their expression profiles in different tissues of oysters. Analysis of the relationships of a molluscan UCP5 with other vertebrate and invertebrate UCPs was conducted in order to shed further light on evolution of this important gene family. We have also analyzed the effect of conditions known to elicit oxidative stress and/or to affect mitochondrial proton leak and coupling in oysters (including hypoxia-reoxygenation stress, Cd exposure and acclimation at different temperatures) on expression of UCP5. This study will for the first time test whether pro-oxidant stressors and/or elevated temperature results in up-regulation of UCP5 transcription in a marine mollusk consistent with the putative role of UCP5 in mitochondrial uncoupling and antioxidant defense and would be important for future functional studies (outside the scope of this paper) that will pinpoint the physiological role of this protein in invertebrates.

2. Materials and methods

2.1. Oyster and tissue collection

Adult oysters *C. virginica* (Gmelin) (Bivalvia: Ostreidae; 70–120 mm shell length) were collected in winter 2005 from Stump Sound, North Carolina, transported to the University of North Carolina at Charlotte within 8 h and placed in tanks with artificial sea water (ASW) (Instant Ocean®, Kent Marine, Acworth, GA, USA) at 12 ± 1 °C and 30 ± 0.5‰. Oysters were allowed to recover for 3–5 days, and temperature in the tanks was then gradually changed to reach 20 °C. The rate of the temperature change was <2 °C d^{-1}. Oysters were acclimated for at least 3 weeks at 12 or 20 °C prior to the experiments. Oysters were fed *ad libitum* on alternate days with a commercial algal blend (2 mL/oyster) containing *Nannochloropsis*, *Tetraselmis*, and *Isochrysis* spp. with a cell size of 2–15 μm (PhytoPlex; Kent Marine) or *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* with a cell size of 2–20 μm (DT's Live Marine Phytoplankton, Premium Reef Blend Sycamore, IL, USA).

In order to determine the effects of temperature acclimation and Cd exposure on UCP5 expression, half of the tanks were randomly selected, and Cd (as $CdCl_2$) was added to the nominal concentration of 50 μg L^{-1}. The remaining tanks were used as controls. Oysters were exposed to Cd (50 μg L^{-1}) or clean ASW (controls) for 56 days at 12 °C or 20 °C. To avoid pseudoreplication, at least two replicate tanks were set up for each treatment. Water was changed every other day. In order to avoid Cd depletion in Cd-exposed tanks, a static-renewal design was used, with cadmium supplementation to the nominal concentration of 50 μg L^{-1} during each water change.

For hypoxia-reoxygenation experiments, oysters were maintained in normoxia (100% air saturation, controls) or subjected to a gradual hypoxia with subsequent hyperoxic reoxygenation (hypoxia-reoxygenation stress) at 20 °C. Experimental oysters were randomly placed into one of the two air-tight Plexiglas chambers and allowed to acclimate for 8 h under normoxic conditions. The chambers were then closed and oysters allowed to become gradually hypoxic over the period of 19 h. Our pilot experiments indicate that oxygen concentrations drop below 5% of air saturation within the first 4 h of exposure under these conditions (data not shown). After hypoxia exposure, water was changed without disturbing oysters and bubbled with 100% O_2 for additional 6 h to achieve oxidative stress. Control oysters were handled in the same way but maintained in normoxic, well-aerated water throughout the experiment.

After the experiments, oysters were dissected, and their tissues collected and shock-frozen in liquid nitrogen until further use. For RNA isolation, equal amounts of specific tissues from 3–4 individuals were pooled in order to reduce variation in mRNA expression due to the individual differences between oysters. For ganglia, tissues of 5–6 oysters were pooled to obtain sufficient amounts of tissue for RNA extraction. All subsequent experiments used these pooled samples; 3 to 6 of such pooled samples were used for each experimental group.

2.2. RNA isolation

Total RNA was extracted from the gill tissue of control and cadmium-exposed oysters using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. Tissue to TRI reagent ratio was kept below 1:10 (weight:volume). This method yielded high purity total RNA with 280/260 absorbance ratio ≥1.9. For RLM-RACE PCR (see below), mRNA was extracted from 150–200 μg of total RNA using Oligotex mRNA Mini Kit (QIAGEN, Valencia CA, USA). RNA samples were stored at −80 °C until further analysis. cDNA was obtained from 1–5 μg total RNA using SuperScriptTM III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction.

2.3. Isolation and characterization of the full-length UCP5 transcript

Full-length UCP5 transcripts were isolated using FirstChoice® RLM-RACE kit (Ambion, USA) according to the manufacturer's instructions. Specific primers for RLM-RACE were designed using a fragment of UCP5, which was earlier isolated in this laboratory using degenerate primers (Sokolova and Sokolov, 2005) and a partial UCP5 sequence obtained from the Marine Genomic Database (*C. virginica*, clone 16491, http://www.marinegenomics.org/). For 5′-RLM-RACE, primers UCP5-r-R534 and UCP5-r-R326 were used (Table 1). Amplified fragments were gel-purified using QIAquick Gel Extraction Kit

Table 1
Primer sequences used for UCP5 and β-actin amplification in RLM-RACE PCR and qPCR.

Primers #	Primer names	Primer sequence (5′ to 3′)	T_{ann}, °C
1	UCP5-r-R534	GCC ATG AAA TGA GTG GAC ATG CTG	55
2	UCP5-r-R326	TTT GAG CTT GCA TGC GCA CCT TGA	58
3†	FW: UCP5-2F117	AGA CTT GTA GAT GGG TGC AGC CTC	58
	RV: UCP5-2R453	GCA AGC TCA AAG GGA GAA TGG A	
4	FW: UCP5-2F5	CAG GAT CGT GTT AAC GTC TAC AAG GGA	56
	RV: UCP5-2R692	TGT CGT AGG CGG GCA GAA TGA	
5	FW: UCP5-3F631	TGT CAG TCC TAC TGC CCA GCG	56
	RV: UCP5-3R1084	AGA GGT CTG CCT GGA GCG TTG	
6	FW: UCP5-4F1051	CAT CTA CAC ACA CCA ACG CTC C	56
	RV: UCP5-4R1552	GAT GGC CTA TAT AAT GCA CGC A	
7*	FW:UCP 5-F217	TGC ATC TGT TGC TGC TGA AAG TGG	55
	R:UCP5-R377	ATG CTC GCA CTC CTT CTT CTG CAT	
8*	FW: Act-Cv-F437	CAC AGC CGC TTC CTC ATC CTC C	55
	RV: Act-Cv-R571	CCG GCG GAT TCC ATA CCA AGG	
9†	FW: Act-Cv-F437	CAC AGC CGC TTC CTC ATC CTC C	55
	RV: Act-Cv-R571	CCG GCG GAT TCC ATA CCA AGG	

T_{ann} — annealing temperature used in PCR. *Primer pairs used in qPCR. †Primer pairs used in semi-quantitative PCR. 1–7 — primers for UCP5, 8 — primers for β-actin.