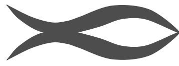

www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 16: 839-845 (2016) DOI: 10.4194/1303-2712-v16_4_10

RESEARCH PAPER

Cloning of PGC-1α from Grass Carp and Its Response to Cadmium-Induced Stress

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Received 02 March 2016
Accepted 12 June 2016

Abstract

Cadmium (Cd) is a demonstrated pollutant in the aquatic environment. The bioaccumulation of Cd in fish affects the mitochondrial viability such as free radicals generation and lipid peroxidation. Peroxisome proliferator-activated receptor (PPAR) γ co-activator α (PGC-1α) is crucial for mitochondrial biogenesis and metabolism. However, only a few PGC-1α s have been identified in fish, and their functions remain obscure. In the present study, PGC-1α gene was cloned from *Ctenopharyngodon idellus* (grass carp). Sequencing analysis revealed that grass carp PGC-1α also contained a PPARγ binding site, a RNA recognition motif and several serine-arginine repeats like other PGC-1α s, suggesting a similar function of PGC-1α in grass carp compared with that in mammals. PGC-1α expression was mainly observed in tissues with high content of mitochondria such as kidney and red muscle, then heart>brain> white muscle> liver > adipose. When the fish were exposed to cadmium for 24h, 5 μM cadmium promoted mitochondrial succinate dehydrogenase activity and PGC-1α expression enhancement. While 20 μM cadmium slightly raised the enzyme activity, which was consistent with PGC-1α expression increment. These results suggested that grass carp PGC-1α was involved in cadmium induced stress and it may be associated with mitochondrial function.

Keywords: PGC-1α; grass carp; cadmium; succinate dehydrogenase; mitochondria.

Introduction

Over the past decades, the ever increasing release of agricultural, industrial and domestic waste led to a significant contamination of the environment and particularly of the aquatic compartment (Akaishi *et al*. 2007; Clarke *et al*. 2009). Cadmium (Cd) is a demonstrated pollutant in the aquatic environment (Burger 2008). Due to little excretion rate of the metal, it is retained within the body and thus, even at very low concentration, the metal can be readily accumulated within the vital organs, causing hepatotoxicity, nephrotoxicity, reproductive system toxicity (Mitsumori *et al*. 1998; Toman *et al*. 2005; Reynders *et al*. 2006; Kumar *et al*. 2007; Roodbergen *et al*. 2008). Liver is one of the target organs in which cadmium primarily accumulates and exerts its deleterious effects, including histopathological and cellular changes, enhancement of lipid peroxidation, influence on mitochondrial function and DNA chain damage in mammals (Tzirogiannis *et al*. 2003; Koyu *et al*. 2006). Some aquatic vertebrates like fish are more vulnerable to cadmium (Burger 2008), but the molecular mechanism of cadmium stress response in fish still hasn't been clearly elucidated.

In order to respond to cadmium stress, fish cells must be able to perceive the signal and convert them into appropriate responses, which in turn confer on fish the ability to tolerate the unfavorable conditions. Mitochondria are the main organelle responsible for cell metabolism. The number, structure and functions of mitochondria differ in animal cells and tissues in response to physiological or environmental alterations (Enriquez *et al*. 1999; Moraes 2001). Mitochondria function is tightly regulated by peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α). Activation of PGC-1α not only stimulates mitochondrial DNA replication but also regulates other vital cellular events such as mitochondrial fusion, fission and antioxidant defense (Ventura-Clapier *et al*. 2008). PGC-1α interacts with nuclear receptors and transcription factors to activate specific target genes upon response to multiple stimuli including calcium ion, reactive oxygen species (ROS), insulin, exercise, and cytokines (Kang and Ji 2012). However, whether PGC-1α is involved in regulating cell response under cadmium stress or not hasn't been explored. In the present study, we

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characterized PGC-1α in grass carp and detected the effect of cadmium on PGC-1α expression.

Materials and Methods

Experimental Fish

Healthy grass carps (300±50 g) were purchased from a local dealer (Chengdu, China). These fish were transferred into automatic-cycle, glass aquaria, fed with a commercial fish diet and kept for 7 days at 18 ± 4 °C before experiments. Animal studies were approved by the Southwest University for Nationalities Institutional Committee for the Care and Use of Animals.

Cloning of PGC-1α Gene in Grass Carps

Ten healthy grass carps were anesthetized, slaughtered and liver samples were harvested. Total RNA was extracted from grass carp with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. cDNA was synthesized by reverse transcription from 2 µg RNA as described in the product protocol (Fermentas Life Science, Hanover, MD, US). The primers (Table 1) were designed to amplify the entire open reading frame of PGC-1α cDNA in grass carp using Primer Premier 5 software based on the sequences of PGC-1α from Danio rerio (GenBank accession No. XM_002667531, AY998087, FJ710604, and DQ017637). PCR amplification was performed with the following program: 1 cycle at 95 °C for 5 min; 38 cycles at 94 °C for 45 s, 62 °C for 60 s, and 72 °C for 90 s; followed by 1 cycle at 72 °C for 10 min. The PCR fragments were purified by 1% agarose gel electrophoresis and cloned into pMD19-T vector (TaKaRa, Dalian, China), transformed into *E.coli* DH5α, and sequenced.

Bioinformatics Sequence Analysis

The sequence, isoelectric point and molecular weight of the deduced PGC-1α protein were analyzed using ExPASy-Tools (http://www.expasy.org/tools). Signal peptide was identified by SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) and amino acid sequence homology of PGC-1α proteins from related species was established with CustalW2. The phylogenetic tree was constructed using neighbour-joining (NJ) methods based on PGC-1α amino acid sequences using MEGA software version 3.1 (Kumar *et al*. 2004).

Waterborne Cadmium Exposure Experiment

Healthy grass carps were randomly distributed into three groups (306±17 g, n=10; 298±15 g, n=10; 312±23 g, n=10), and exposed to 0, 5, or 20 µM $CdCl_2$ for 24 h, respectively. Then these fish were anesthetized, sacrificed and liver samples were collected for further processing.

Succinate Dehydrogenase Activity

About 200 mg liver sample from each fish was collected and triturated in liquid nitrogen, stirred and dissolved in 2 ml PBS, then centrifuged at 10,000 g for 30 min. The total protein concentration in supernatant was analyzed with BCA kit (Bio-Rad, Hercules, CA, USA). Activity of mitochondrial succinate dehydrogenase (SDH) activity was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. 100 µL solution of each sample was transformed into 96-well plate, then 0.5 mg mL^{-1} of MTT (Sigma-Aldrich, St. Louis, MO) dissolved in 20 µL PBS was added. Plate was incubated at 37 °C for 3 h and centrifuged at 2500 g for 10min, 150 µL isopropanol was added into each well to solubilize the resulting formazan crystals. The optical density was read on a Microplate Reader (Bio-Rad, Hercules, CA, USA) with a test wavelength of 570 nm. SDH activity was normalized to the control group, which was considered to be 100%.

Real Time Quantitative RT-PCR

Heart, liver, spleen, kidney, brain, gill, fin, adipose, red muscle and white muscle were collected from ten healthy grass carps. Liver samples from cadmium treated fish and controls were triturated in liquid nitrogen. Total RNA and cDNA were prepared as described above, real-time quantitative PCR was carried out to analyze the level of PGC-1α expression in liver by a fluorescence temperature icycler (Bio-Rad, Hercules, CA, USA). The gene specific primers

Table 1. Primers for specific target genes

Gene	Primer	Sequence(5'-3')	Annealing temperature(°C)
PGC-1α	S1	GGATGGCGTGGGACAGGTGTAATC	62
(clone)	A1	GCTGG GGTGGTGCTGTCTCGTT	
	S2	CTGAGCAAGGCGTCCTCCACTATG	62
	A2	TTACCTT CTCAG GCTGTACTGGG	
PGC-1α	S	CCAGTCAAGAGAACCCTTTCAA	58
(analyze)	A	CCCTTCCGAATAGAACCGC	
β -Actin	S	ATCCTCCGTCTGGACTTGG	58
	A	TCCGTCAGGCAGCTCATAG	

for PGC-1α were presented in table 1. Conditions for amplification were 90 s at 95 °C, followed by 45 cycles of 5 s at 95 °C, and 20 s at 58 °C for primer annealing, then 40 s at 72 °C for elongation. Relative mRNA expression level was analyzed using the comparative Ct method (Livak and Schmittgen, 2001), with β-actin as a loading control for normalization.

Statistical Analyses

Data were showed as mean±SEM and the differences between $CdCl_2$ treatments and controls were analyzed using SPSS13.0 for Windows Software (SPSS, Chicago, IL, USA). Differences were considered as significant at $P<0.05$.

Results

Characterization of PGC-1α in Grass Carp

Two fragments were amplified by RT-PCR and a total 2,640 bp cDNA of grass carp PGC-1α was spliced (GenBank accession number JN195739.1). The obtained nucleotide sequence covered an entire ORF of 2,638 bp encoding 878 amino acids (Figure 1). Molecular weight of the predicted PGC-1α protein was 97220.7 Da and the isoelectric point was 5.83.

Figure 1. The cDNA and deduced amino acid sequence of grass carp PGC-1α. The ORF encodes an 878 amino acid polypeptide containing a LXXLL motif, a PPARγ binding site, a RNA recognition sequence and several serine-arginine repeats as indicated in the figure. The asterisk represents termination codon. This sequence was submitted to the NCBI GenBank with accession No. JN195739.1.

No signal peptide was found in the deduced PGC-1α protein with SignalP 4.0 analysis. Amino acid sequence analysis revealed that the PGC-1α protein contained a canonical LXXLL (a.a. 142-146) motif, a PPARγ binding site (a.a. 335-384), a RNA recognition motif (RRM) (a.a. 757-824) and several serine-arginine repeats (a.a. 648-679), which were conserved among PGC-1α s from different species (Figure 1). In addition, fish specific serine and glutamine rich sequences were found in grass carp PGC-1α.

The deduced amino acid sequence of grass carp PGC-1α was 93.74%, 90.92%, 55.52%, 55.06%, 54.94%, 54.62%, 54.50%, 54.11%, 54.05%, 54.05% and 53.54% identical to schizothorax prenanti, zebrafish, chicken, human, monkey, pig, chiru, cattle, rat, goat, mouse PGC-1α, respectively. Thus, grass carp PGC-1α protein exhibited high degree of sequence identities with schizothorax prenanti and zebrafish, but relatively low identities with mammals and birds (Figure 2). The phylogenetic tree was constructed according to the deduced grass carp PGC-1α and the PGC-1α amino acid sequences from other species (Figure 3).

Tissue Distribution of PGC-1α in Grass Carp

Real time quantitative RT-PCR was employed to detect the relatively levels of PGC-1α mRNA in ten tissues of grass carp. High expression levels of PGC-1α were observed in kidney and red muscle, then heart, brain and white muscle, low expression levels were detected in liver and adipose. A relatively low level of PGC-1α expression was found in spleen, gill and fin (Figure 4).

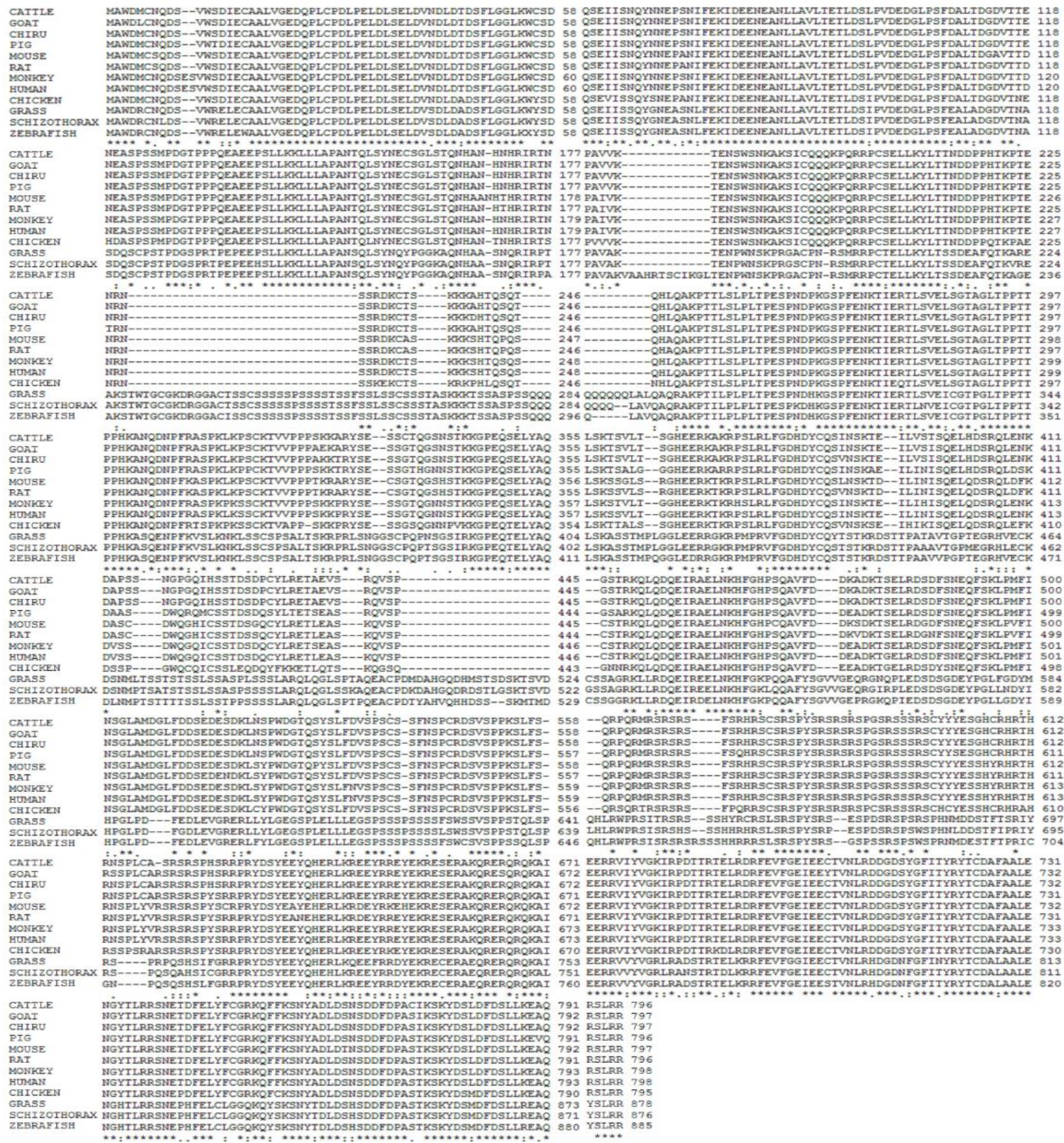

Figure 2. Multiple alignment of the PGC-1α amino acid sequences from twelve species. The GenBank accession number of the PGC-1α s used in this analysis are indicated in the following phylogenetic tree. The sequence alignments were performed using Clustal W2.

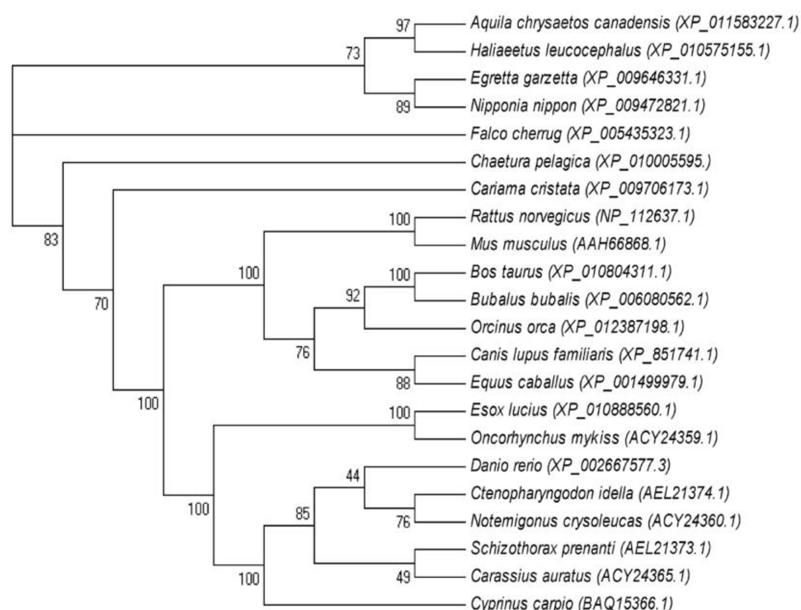

Figure 3. Phylogenetic analysis of PGC-1α amino acid sequences. The phylogenetic tree was generated using neighbour-joining (NJ) methods (Kimura two-parameter model, 10,000 replicates, boostrap phylogeny test) based on PGC-1α amino acid sequences using MEGA software version 3.1. Bootstrap values and genetic distance are also indicated.

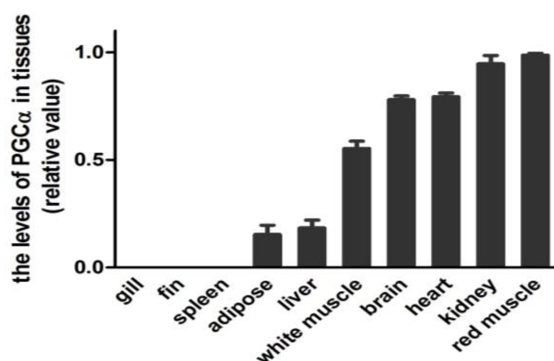

Figure 4. Relative levels of grass carp PGC-1α mRNA in different tissues. The mRNA was detected by RT-PCR. (n=10).

Effect of Cadmium on Liver Mitochondrial Succinate Dehydrogenase Activity

MTT assay was used to test succinate dehydrogenase activity after 24 h cadmium exposure. A stimulatory effect of $CdCl_2$ on succinate dehydrogenase activity was found at 5 µM (149.4±21.8% vs 100.0±24.1%, P=0.12), while a relatively slightly enhanced effect at 20 µM (137.8±29.8% vs 100.0±24.1%, P =0.43) (Figure 5). The result indicated that cadmium activated mitochondrial succinate dehydrogenase after short-term (24 h) exposure at low concentration (5 µM), and the stimulatory effect decreased at high concentration (20 µM).

Influence of Cadmium on PGC-1α Expression

Furthermore, to explore the mechanism underlying the effect of $CdCl_2$ on liver mitochondrial function, the expression of the mitochondrial function regulator PGC-1α was examined. As summarized in Figure 6, the expression level of PGC-1α was 18.4±4.2 fold with 5 µM $CdCl_2$ administration (P=0.0018) and 3.2±0.7 with 20 µM $CdCl_2$ administration (P=0.006) compared to controls. Therefore, cadmium activated PGC-1α expression in liver with a more notable enhancement at 5 µM than at 20 µM. The increment was consistent with the influence of cadmium on mitochondrial succinate dehydrogenase activity.

Discussion

Water pollution by heavy metals is a serious environmental problem. Among heavy metal pollutants, cadmium is considered to be one of the most toxicants. Because of its high solubility in water,

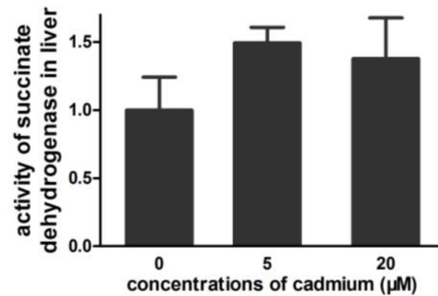

Figure 5. Effect of cadmium on liver mitochondrial succinate dehydrogenase activity. Grass carps were exposed to 0, 5 and 20 μM $CdCl_2$ for 24 h. Succinate dehydrogenase activity was detected by MTT. The enzyme activity was normalized to controls, which were considered to be 100%. Data represent the mean ± SEM, n=10.

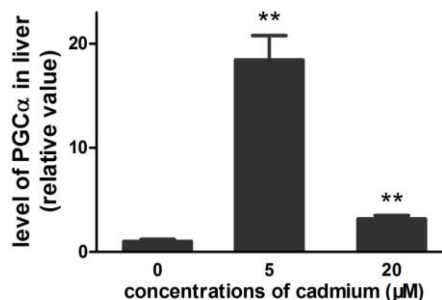

Figure 6. Influence of cadmium on expression of PGC-1α. Grass carps were exposed to 0, 5 and 20 μM $CdCl_2$ for 24 h. The levels of PGC-1α mRNA were detected by real time fluorescent RT-PCR. PGC-1α expression levels were normalized to controls, which were considered to be 100%. Data represent the mean ± SEM, n=10. (**P<0. 01).

it is promptly taken up by fish. There is growing evidence that oxidative stress via reactive oxygen species (ROS) generation is one of the fundamental molecular mechanisms of cadmium toxicity (Espinoza *et al.* 2012; Williams and Gallagher 2013). Mitochondria are an important source of ROS in eukaryotic cells (Turrens 2003; Andreyev *et al*. 2005). PGC-1α, a master regulator of mitochondria function, modulates oxidative phosphorylation and fatty acid oxidation gene expression and has been reported coordinated balancing of muscle oxidative metabolism (Summermatte *et al*. 2010). Thus PGC-1α could be involved in cadmium induced stress.

In the present study, grass carp PGC-1α was identified and found that it also contained a canonical LXXLL pattern, a PPARγ binding site, a RNA recognition motif and several serine-arginine repeats, suggesting that PGC-1α in grass carp could also be a regulator of mitochondria function.

In humans, PGC-1α is mainly expressed in tissues with high content of mitochondria, such as heart, skeletal muscle, and kidney *et al* (Larrouy *et al*. 1999). Consistent with this, high expression levels of PGC-1α were observed in kidney and red muscle, then heart, brain and white muscle in our study, indicating that PGC-1α expression is related with mitochondrial biogenesis and functions.

Furthermore, the effect of cadmium on liver mitochondrial succinate dehydrogenase activity was explored. According to the report of Velasquez-Vottelerd *et al*, Cd exposure effect on mitochondrial viability varied across fish tissues and was related to the exposure duration (Velasquez *et al*. 2015). In our study, 5 μM cadmium in water promoted grass carp liver SDH activity after short-term (24 h) exposure, and 20 μM cadmium in water slightly increased SDH activity compared to control after short-term (24 h) exposure. The effects of cadmium on SDH activity might also be associated to the level of cadmium and exposure duration.

Mitochondrial biogenesis and function are regulated by master transcriptional coactivators (Ventura-Clapier *et al*. 2008). Undoubtedly, PGC-1α is pivotal for mitochondrial function. Furthermore, the influence of cadmium on PGC-1α was detected and found that 5 μM cadmium significantly enhanced PGC-1α expression with short-term (24 h) exposure, and 20 μM cadmium slightly increased PGC-1α expression compared to control, which was consistent with SDH activity improvement. The results suggest that enhanced SDH activity could be partial consequence of PGC-1α up-regulation.

Taken together, PGC-1α gene was characterized in grass carp and detected relative levels of PGC-1α mRNA in different tissues, demonstrated it is involved in cadmium induced stress and may be associated with mitochondrial function.

Acknowledgments

This work was supported by the Natural Science Foundation of China (No. 31201990, 31400794);

Applied Basic Research Program of Sichuan Province, China (No.2014JY0088).

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