Cadmium triggers kidney cell apoptosis of purse red common carp (Cyprinus carpio) without caspase-8 activation

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Abstract

Caspase-8, the essential initiator caspase, is believed to play a pivotal role in death receptor-mediated apoptotic pathway. It also participates in mitochondria-mediated apoptosis via cleavage of proapoptotic Bid in mammals. However, its role in fish remains elusive in Cadmium-induced apoptotic pathway. In this study, we isolated the caspase-8 gene from common carp, one of the most important industrial aquatic animals in China using rapid amplification of cDNA ends (RACE). The deduced amino acid sequence of caspase-8 comprised 475 amino acids, which showed approximately 64.1% identity and 79.8% similarity to zebrafish (Danio rerio) caspase-8, possessed two conserved death effector domains, a large subunit and a small subunit. Phylogenetic analysis demonstrated that caspase-8 formed a clade with zebrafish caspase-8. In kidney, cadmium (Cd) exposure triggered apoptosis and increased caspase-3 and -9 activities, whereas it did not affect caspase-8 activity. Real-time quantitative PCR analysis revealed that caspase-8 transcriptional level was not significantly increased in kidney after exposure to Cd. Using Western blot analysis, no caspase-8 cleaved fragment was detected and no significant alteration of procaspase-8 level was found with the same Cd-treated condition. Moreover, the immunopositive staining was predominantly limited to the cytoplasm of renal tubular epithelial cells and no remarkable changes of immunoreactivities were observed using immunohistochemical detection after Cd treatment. The results reveal that Cd can trigger apoptosis, while it cannot activate caspase-8 in purse red common carp. Crown Copyright © 2013 Published by Elsevier Ltd. All rights reserved.

1. Introduction

Apoptosis, or programmed cell death, is an important regulator of growth, development, defense and homeostasis in multicellular organisms (Elmore, 2007; Ulukaya et al., 2011). In mammals, cells undergo apoptosis through two major pathways, namely the intrinsic pathway (mitochondrial pathway) and the extrinsic pathway (death receptor pathway) (Jin and El-Deiry, 2005; Portt et al., 2011). The intrinsic pathway is initiated by the stress-mediated release of cytochrome c from the mitochondria, which promotes downstream effector caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex then leading to the characteristic phenotype of apoptosis (Fulda and Debatin, 2006). In the extrinsic pathway, stimulation of Fas, tumor necrosis factor receptor (TNFR) or TNF-related apoptosis-inducing ligand receptors (TRAILR) results in activation of the initiator caspase-8 (Fulda, 2009). Activated caspase-8 then triggers apoptosis via the activation of caspase-3 (Zhao et al., 2010).

In addition, caspase-8 may also cleavage Bid, a Bcl-2 family protein with a BH3 domain, enabling a crossstalk to the intrinsic apoptotic pathway (Indran et al., 2011; Kaufmann et al., 2012). Therefore, caspase-8 plays a critical role in caspase-dependent apoptotic pathway.

Apoptosis can be triggered by a variety of cellular stress stimuli, including chemotherapeutic drugs, irradiation, ethanol, p-dioxin, oxidative stress, and endoplasmic reticulum stress (Fulda et al., 2010). Besides these stimulating factors, growing evidence indicates that heavy metals such as cadmium (Cd) are also crucial ones in inducing apoptosis (Kim et al., 2008). Cd is one of the most toxic environmental and industrial pollutants (Templeton and Liu, 2010). In experimental animals and humans, kidney is a major target organ of Cd toxicity (Bernard, 2008). Long-term exposure to Cd causes renal tubular dysfunction as well as histopathological changes (Satarug et al., 2010). A large number of studies show that Cd induces different apoptotic pathways in various cell types depending on exposure conditions. In some cases, Cd triggers apoptosis through a mitochondrial pathway (Hossain et al., 2009; Lasfer et al., 2008; Li and Lim, 2007) or through a death receptor pathway (Song and Koh, 2012); while some other studies show that Cd induces apoptosis through a caspase-independent pathway...
Moreover, some studies indicate that Cd may induce apoptosis simultaneously via caspase-dependent and caspase-independent pathways (Coutant et al., 2006; Lee et al., 2006; Pathak et al., 2013). Overall, it is conceivable that Cd can induce apoptosis, and caspase family proteases are commonly involved in the regulation of apoptotic processes in most cases.

Caspase-8 has been shown to be involved in the regulation of apoptosis in fish. For example, previous studies indicate that fish caspase-8 exhibits structure and function similar to mammalian caspase-8 orthologs. However, to date only a few caspase-8 genes of fish have been characterized (Reis et al., 2010; Sakamaki et al., 2007; Sakata et al., 2007). In this study, to determine whether Cd induces apoptosis through the extrinsic pathway in common carp, caspase-8 was used as a marker. Apoptosis was detected using the fluorescence-activated cell sorter (FACS) and the MEGABASIC software package (version 5.1), and the reliability of the results was assessed by repeated measurements.

2. Materials and methods

2.1. Cloning of caspase-8 from common carp

Total liver RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. The cDNA sequence was amplified using a SMART PCR CDNA Synthesis Kit (Clontech, USA) according to the supplier’s protocol. The cDNA sequence was then subjected to a PCR amplification using primers specific for the conserved region of caspase-8 sequences in zebrafish (GenBank accession No. NM_001007404.2) and sea bass (Dicer2narchus labrax, GenBank accession No. FJ225665) degenerate primers (Csp8F, Csp8R) were designed to obtain partial sequence of caspase-8 with the following PCR cycling conditions: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 60 °C for 60 s, 72 °C for 60 s, with a final cycle of 10 min at 72 °C. To obtain the full cDNA sequence, the RACE PCR was performed using the gene-specific primers (Csp8-5′-1, Csp8-5′-2, Csp8-5′-3, Csp8-3′-1 and Csp8-3′-2) and adaptor primers (UPM) with the following PCR program: 1 cycle at 94 °C for 5 min; 10 cycles at 94 °C for 30 s, 60 °C for 60 s, and 72 °C for 60 s; 25 cycles at 94 °C for 30 s, 57 °C for 60 s, and 72 °C for 60 s; followed by 1 cycle at 72 °C for 10 min. The RT-PCR product was gel purified, cloned into pMD18-T vector (Takara, Japan) and sequenced. The primers used in this study are listed in Table 1.

2.2. Sequence and phylogenetic analysis

Protein prediction was conducted using the software at the Expasy Molecular Biology Server (http://expasy.org/). Sequence alignment was performed using the Clustal W 1.83 program. The protein sequences of some vertebrate and molluscan caspase-8 and the other known sequences were retrieved from the GenBank database for use in the alignment. Homology analysis, including identities and similarities between deduced amino acid sequences of common carp caspase-8 and the other known sequences was carried out using MatGat 2.0 (Campanella et al., 2003). Finally, phylogenetic tree construction was carried out using the neighbor joining method within the MEGA software package (version 5.1), and the reliability of the tree was based on 1000 bootstrap. The percentage of the bootstrap values was recorded.

2.3. Waterborne Cd exposure experiment

Healthy purse red common carp weighing 300 ± 50 g were obtained from a fish hatchery at the Institute of Purse Red Common Carp, Wuyuan County, China. Twenty-four carp were randomly placed into 6 tanks (4 fish per group) and were kept for 7 days at 25 ± 2 °C before experiments. Then fish were exposed to 2.5 μM Cd2+ [3(CdSO4)2]⋅8H2O, groups A and B] and 10 μM Cd2+ [groups C and D] and 0 μM Cd2+ [groups E and F, as controls]. One-third of the water in the tank was replaced every day through adding fresh water containing the same concentrations.

After the 96 h experiment, the fish groups A, C and E were anesthetized by MS-222 (Sandoz, Switzerland) and sacrificed. The kidney tissues were sampled. Then the samples were divided into 3 parts, weighed, immediately frozen in liquid nitrogen, and stored at −80 °C for further processing. Groups B, D and F were treated as groups A, C and E after 168 h Cd exposure.

2.4. Detection of DNA fragmentation by TUNEL assay

Kidney tissues from all the groups were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4 μm-thick sections. TUNEL assay was conducted to detect DNA fragmentation using Promega DeadEnd™ Colorimetric TUNEL System (Promega Corp, USA) according to the protocol. Briefly, tissue sections were deparaffinized and rehydrated and then were incubated with proteinase K (20 μg/ml) for 20 min at 37 °C. After further washes with PBS, the tissues were fixed in 4% paraformaldehyde in PBS for 10 min. After equilibration, 100 μl of TdT reaction mix was added to each slide and incubated at 37 °C for 1 h in the dark. The TUNEL-positive nuclei were visualized with HRP-conjugated antibody and 3,3′ diaminobenzidine (DAB) substrate reaction. The results were observed in an Olympus BX51 light microscope (Olympus Corp., Japan). At least 1000 cells were counted in 5 random fields, and the percentage of TUNEL-positive cells was then calculated.

2.5. Determination of caspase-3, -8 and -9 activities

Caspase-3, -8 and -9 activities were measured as previously described (Kemp et al., 2005) using Apo-One Homogenous Caspase-3/7, Caspase-Glo 8 Assay and Caspase-Glo 9 Assay (Promega Corp, USA), respectively. Briefly, kidney samples were homogenized in lysis buffer, and the homogenates were centrifuged at 15,000g
for 20 min at 4 °C. Supernatants were collected, and subsequently protein concentrations were determined with the Bradford Protein Assay Kit (Beyotime Biotechnology, China). Activities of caspase-3, -8, and -9 were determined using substrate peptides Ac-DEVD-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA, respectively. The endpoint fluorescence (RFU) values of untreated controls were subtracted from the reading of all the samples. Increase in caspase activities was then determined by comparing the reading from the Cd-treated samples with the level of the untreated controls.

2.6. Expression analysis of caspase-8 mRNA from kidney by real-time quantitative PCR

Total RNA from kidney was extracted using TRIzol reagent (Invitrogen, USA) according to manufacturer’s instructions. RNA samples were treated with RNase-free DNase (Fermentas, Lithuania) and then 2 μg of treated RNA was reverse-transcribed with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania). The caspase-8 and β-actin cDNA fragments were generated with RT-PCR. Amplicons were gel purified, and serial 10-fold dilution was used as a standard curve in each PCR. Real-time quantitative PCR was conducted on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Amplifications were carried out at a final volume of 20 μl containing 5 μl of 50-fold diluted cDNA template, 10 μl SYBR Green Real-time PCR Master Mix (Takara, Japan), 0.5 μl of each primer (Table 1), and 4 μl ddH2O in accordance with the manufacturer’s instructions. PCR amplification was performed in triplicate using the following procedures: Initial denaturation at 3 min at 94 °C, followed by 45 cycles of amplification (94 °C for 10 s, 53 °C for 15 s and 72 °C for 20 s). The reaction carried out without cDNA sample was used as a negative control. The results were expressed as the relative fold of the expression of the β-actin gene with the 2−ΔΔCT method (Livak and Schmittgen, 2001).

2.7. Production of fusion proteins and polyclonal antibodies

The expression primers were designed to include an EcoRI site within the sense primer and an XhoI site within the antisense primer and used to amplify all the 475 amino acids. The primers are listed in Table 1. PCR amplifications were performed using 1 cycle at 94 °C for 5 min; 10 cycles at 94 °C for 45 s, 53 °C for 45 s, and 72 °C for 45 s; 25 cycles at 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 50 s; followed by 1 cycle at 72 °C for 10 min. The purified products were digested and ligated into the EcoRI and XhoI sites of pET-32a expression vector (Novagen, USA), and then were transformed into DH5α competent cells. After sequencing the positive clones to ensure in frame insertion, the pET-32a-CSP8 construct was transformed into Escherichia coli BL21 (DE3) strain for protein expression. The fusion proteins were induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) and analyzed with 10% SDS-polyacrylamide gel (SDS–PAGE). To prepare the polyclonal antibodies, IPTG was added in a final concentration of 4 mM when the culture reached OD600 = 0.6. After 6 h culture at 37 °C, the cells were harvested by centrifugation and disrupted by sonication. The recombinant fusion proteins were purified by affinity chromatography in a column of Ni2+ charged resin (Novagen, Germany). Recombinant proteins were eluted from the resin with 6 M urea and 20 mM Tris–HCl pH 7.9, 50 mM imidazole and 0.5 M NaCl. The purity of the recombinant proteins were assessed on a 10% SDS–PAGE gel. Total 8 mg recombinant proteins were used to immunize 2 rabbits to generate polyclonal antibodies. The obtained polyclonal antibodies were purified by affinity chromatographic column matrix coupled with antigen. The specificity of the polyclonal antibodies was evaluated by Western blot analysis and immunohistochemical detection.

2.8. Western blot analysis

For identifying the protein expression of caspase-8 after exposure to Cd, 100 mg tissue samples from the kidney tissues of purse red common carp were washed with phosphate-buffered saline (PBS) and then homogenized with ice-cold extraction buffer [MEB, 100 mM sodium β-glycerophosphate, 15 mM MgCl2, 20 mM EGTA, 1 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM HEPES, 2.5 μg/ml aprotinin, and 2.5 μg/ml leupeptin; pH 7.5]. The homogenate was centrifuged at 120,000g for 30 min, and the supernatant containing the proteins was retained. Protein concentrations were determined using the BCA Protein Assay Kit (Beyotime Biotechnology, China). Each sample, equivalent to 30 μg of total protein, was run on 12% SDS–PAGE gel electrophoresis and subsequently transferred to a 0.45 μM PVDF membrane (Millipore, USA). After transfer, the blotting membrane was blocked with 5% dry milk for 1 h and then incubated overnight at 4 °C in blocking with primary antibody diluted 1000-fold. Mouse anti-GAPDH monoclonal antibody (Proteintech Group Inc., USA) diluted 1:10,000 was used as a loading control. After washing 3 times for 10 min, the membrane was further incubated for 2 h with 1:2000 diluted goat anti-rabbit IgG or goat anti-mouse IgG conjugated with peroxidase (Beyotime Biotechnology, China). After washing 3 times for 10 min in TBST (Tris Buffered Saline with Tween 20) buffer, detection was performed using the

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upm (long)</td>
<td>CTAATACGACTCACTATAGG</td>
<td>RACE – PCR</td>
</tr>
<tr>
<td>Upm (short)</td>
<td>CTAATACGACTCACTATAGG</td>
<td>Universal primers mix</td>
</tr>
<tr>
<td>Csp8F</td>
<td>TGCTACAGAAGAAATAG</td>
<td>Conserved region cloning</td>
</tr>
<tr>
<td>Csp8R</td>
<td>TGGATATAGATAGAGCCTTC</td>
<td></td>
</tr>
<tr>
<td>Csp8–5–1</td>
<td>TACGTTGCGGAGAGGA</td>
<td>5′ RACE PCR</td>
</tr>
<tr>
<td>Csp8–5–2</td>
<td>CGGTTATGAACGATCTGCTGA</td>
<td></td>
</tr>
<tr>
<td>Csp8–5–3</td>
<td>TCCCGTTGCTATACATCTT</td>
<td></td>
</tr>
<tr>
<td>Csp8–3–1</td>
<td>GTATGGACGCGCCCTACGGAATG</td>
<td></td>
</tr>
<tr>
<td>Csp8–3–2</td>
<td>CTACGAGCTTCTCGCCACACTAGGA</td>
<td></td>
</tr>
<tr>
<td>Csp8–E</td>
<td>ATGCAATTCTAGCTAAAGAGATTTCAAGCG</td>
<td></td>
</tr>
<tr>
<td>Csp8–X</td>
<td>ATTCAGCTAAGTCCATGCGGACACC</td>
<td></td>
</tr>
<tr>
<td>Csp8–RTF</td>
<td>TTTCTTGGTGAAGCTTGTG</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>Csp8–RTR</td>
<td>GTATTACGCTAGCTTGGG</td>
<td>Real time PCR</td>
</tr>
<tr>
<td>β–ActinF</td>
<td>TTTCTTGGTGAAGCTTGTG</td>
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</tr>
<tr>
<td>β–ActinR</td>
<td>GTATTACGCTAGCTTGGG</td>
<td>Real time PCR</td>
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</tbody>
</table>
Pierce Plus enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Fisher Scientific, USA) and X-ray film. Densitometric analysis of protein bands was performed using Gel-Pro Analyzer (Media Cybernetics, USA).

2.9. Immunohistochemical identification of caspase-8

Paraffin-embedded, 4 μM-thick sections of kidney samples from all the groups were obtained by the standard techniques. Sections were mounted on aminopropyltriethoxysilane-treated slides. The slides were routinely deparaffinized with xylene and rehydrated with a series of ethanol washes. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in absolute methanol. After washing with PBS, the sections were subjected to antigen retrieval in boiling sodium citrate buffer (0.01 mM, pH 6.0) for 10 min in a microwave oven set at 95–100 °C. After cooling for 20 min and washing 3 times with 0.1 M Tris–HCl, 5% BSA was added, and the sections were incubated for 20 min. The polyclonal antibodies were diluted 1:300 and added to slides, and non-immune serum was used as a negative control. The slides were then maintained at 4 °C overnight, following which they were again washed 3 times. The slides were further incubated with a polymerized HRP-anti Ms/Rb IgG (Maxim, China) and washed 3 times. At room temperature, DAB was added to the organ sections, followed by incubation for 10 min without direct light. The slides were washed 3 times with reagent quality water and then counterstained with Mayer’s hematoxylin (Boster, China) for 10 min and washed under running water. Gradient ethanol was utilized for dehydration, and dimethylbenzene, for clearing the sections, which were then mounted in neutral resin. The slides were observed under an Olympus BX51 light microscope (Olympus Corp., Japan).

2.10. Statistical methods

The statistical analyses were performed by SPSS software, Version 11.5. The percentage of TUNEL-positive cells, activities of caspase-3, -8 and -9, and the mRNA and protein expression were analyzed by a factorial ANOVA model, represented by 2 independent variables (time, dose) and their interaction. An F-test was performed, followed by a post hoc LSD test and pairwise comparison. Statistically significant difference was accepted at P < 0.05. All results are expressed as mean ± SD (standard deviation).

3. Results

3.1. Molecular characterization and homology analysis of common carp caspase-8

In this work, we identified and characterized the common carp caspase-8 gene. The common carp caspase-8 mRNA has 2019-nt in length (GenBank accession No. KC822471), encoding a hypothetical protein of 475 amino acids with a calculated molecular weight of 54.66 kDa. It exhibited a typical caspase-8 domain architecture (GenBank accession No. KC822471), encoding a hypothetical protein of 475 amino acids with a calculated molecular weight of 54.66 kDa. It exhibited a typical caspase-8 domain architecture.

3.2. Detection of apoptosis by TUNEL assay

To confirm and consolidate the occurrence of apoptosis, TUNEL analysis was employed to evaluate the DNA fragmentation. Hardly any TUNEL-positive nuclei were present in control conditions [Fig. 3A(a and d)], whereas Cd treatment increased the ratio of TUNEL-positive nuclei [Fig. 3A(b, c, e and f)]. TUNEL-positive staining was detected mainly in kidney interrenal cells, including hematopoietic cells. The positive cells were not detected in renal tubules or glomeruli. According to the factorial ANOVA analysis, the percentage of apoptotic cells was enhanced with increasing Cd exposure time, and the difference was statistically significant (P = 0.000) between fish groups exposed for 96 h and those exposed for 168 h. Further analysis indicated that only 2.5 μM Cd-treated groups revealed the marked difference (P = 0.051) between the two sampling time points (Fig. 3B), and the percentage of TUNEL-positive cells reached a peak at 168 h time point. In addition, significant increase of apoptotic cells could be observed with increasing Cd concentrations (P = 0.000). The following LSD Test indicated that after 96 h of Cd exposure, the percentage of apoptotic cells with different Cd treatment concentrations followed the hierarchical pattern: group exposed to 10 μM Cd²⁺ → group exposed to 2.5 μM Cd²⁺ → control group (P = 0.000) (Fig. 3B). Whereas after exposure for 168 h the number of apoptotic cells in different Cd-treated conditions followed another hierarchical pattern: group exposed to 2.5 μM Cd²⁺ → group exposed to 10 μM Cd²⁺ → control group (P = 0.000). In addition, there was significant interaction between the two factors (time and dose) (P = 0.000). Taken together, these data support the idea that Cd effectively triggers apoptosis in the kidney tissue of common carp.

3.3. Analysis of caspase-3, -8 and -9 activities

The initiator caspases, caspase-8 and caspase-9, are implicated in the two separate but well-defined pathways of caspase-3 activation. To confirm the caspase activations, caspase-3, -8, and -9 activities were determined to investigate whether apoptosis was induced by Cd. The activities of caspase-3 and -9 were significantly increased with increasing Cd exposure time (P = 0.044, P = 0.005, respectively) between groups exposed for 96 h and those exposed for 168 h. Further analysis indicated that only 2.5 μM Cd-treated groups significantly increased the caspase activations (P = 0.000) and peaked. Similar effects were also observed when fish were exposed to 10 μM Cd (P = 0.000, P = 0.001, respectively) [Fig. 4(A and C)]. In contrast, although 2.5 μM Cd treatment enhanced the activities of caspase-3 and -9, no significantly differences were detected when compared to their control groups (P = 0.228, P = 0.158, respectively). At the 96 h time point, exposure to Cd at 10 μM markedly increased the activities of caspase-3 and -9 (P = 0.000, P = 0.001, respectively) [Fig. 4(A and C)]. In contrast, although 2.5 μM Cd treatment enhanced the activities of caspase-3 and -9, no significantly differences were detected when compared to their control groups (P = 0.228, P = 0.158, respectively). At the 168 h time point, 2.5 μM Cd treatments significantly increased caspase-3 and -9 activities (P = 0.000) and peaked. Similar effects were also observed when fish were exposed to 10 μM Cd (P = 0.000, P = 0.001, respectively). However, caspase-9 activity was lower in 10 μM Cd-treated group than that in 2.5 μM Cd-treated group (P = 0.000), whereas caspase-3 activity in 10 μM Cd-treated group did not markedly differ from that in 2.5 μM Cd-treated group (P = 0.370).

In fish, exposed to 2.5 μM Cd, the activities of caspase-3 and -9 were dramatically elevated with increasing exposure time (both have a P = 0.000). In contrast, the activities of caspase-3 and -9 were stable in control group and 10 μM Cd-treated group with increasing exposure time.
The activity of caspase-8 was not affected by exposure time ($P = 0.666$) and concentrations ($P = 0.378$), and no significant interaction was found between the two factors ($P = 0.958$) (Fig. 4B).

Fig. 1. Multiple alignment of the caspase-8 amino acid sequence of common carp and other animals. Identical amino acids among all sequences are indicated by asterisks, whereas those with high or low similarity are indicated by semicolons and dots. The pentapeptide active-site motif (QACRG) is boxed. The putative cleavage site at aspartic acid residues, which separates large and small subunits, are shaded in gray. The GenBank accession numbers of caspase 8 amino acid sequences used here are as follows: zebrafish NP_571585.2, mouse (Mus musculus) NP_033942.1, hamster (Cricetulus griseus) NP_001233725.1, human (Homo sapiens) NP_001085751.1, chicken (Gallus gallus) NP_001220, medaka (Oryzias latipes) NP_001098258.1 and seabass (Dicentrarchus labrax) ACO53630.1.
3.4. Effect of Cd exposure on caspase-8 mRNA and protein levels

Real-time quantitative PCR was used to analyze the transcriptional level of caspase-8 in kidney of Cd-treated fish and their control groups. Cd exposure induced an increase in mRNA expression of caspase-8 (Fig. 5). The group D (exposure to 10 μM Cd²⁺ for 168 h) had a maximum relative mRNA level, which was about 2.34-fold of control group. But according to the factorial ANOVA analysis, the mRNA level of caspase-8 showed no significant effect as a function of time period between the groups exposed for 96 h and those exposed for 168 h (P = 0.586). Meanwhile, the mRNA expression of caspase-8 did not differ significantly among fish groups exposed to different Cd concentrations (P = 0.284). The interaction between the two factors (time and dose) was also not significant (P = 0.571).

To generate recombinant protein for producing polyclonal antibodies, the expression plasmid pET-32a-CSP8 was transformed into *E. coli* BL21 (DE3) strain and the pET-32a-CSP8 expressed as recombinant fusion proteins containing a His tag. After IPTG induction, the pET-32a-CSP8 fusion proteins, which were produced and mainly expressed in precipitate (Fig. 6A), were used to immunize rabbits lately.

To determine whether caspase-8 activation is involved in Cd-induced apoptosis of the kidney tissue, time and concentration course experiments were performed by analyzing the activation status of caspase-8 using Western blot analysis. Changes of caspase-8 cleavage were used as a marker for the activation of extrinsic apoptosis pathway. The result showed that no cleaved fragment was detected and only an approximately 55 kDa procaspase-8 fragment was detected in all groups (Fig. 6B). The protein expression of procaspase-8, which presents a similar expression manner to that of mRNA, was not affected by exposure time (P = 0.727) and concentrations (P = 0.900) (Fig. 6C). In addition, the interaction between the two factors (time and dose) was not significant (P = 0.861).

3.5. Immunohistological localization of caspase-8 in kidney

Immunohistochemical examination indicated that negative controls showed no positive reaction (Fig. 7(A–C and G–I)).
expression of caspase-8 mainly was localized on tubular epithelial cells [Fig. 7(D–F and J–L)]. In control groups, the weak immunoreaction of caspase-8 was observed in the cytoplasm of the collecting duct epithelium, proximal and distal tubular epithelium [Fig. 7(D and J)]. No staining was detected in the nuclei of these cells. There was a weak to moderately strong stronger staining about these positive cells in the experimental groups [Fig. 7(E, F, K and L)].

4. Discussion

The partial cDNA sequence of common carp caspase-8, which does not include stop codon, was isolated by Vidal et al. (2008). To explore the relationship between caspase-8 and Cd-induced apoptosis, we firstly isolated the complete caspase-8 cDNA sequence. The common carp caspase-8 appears homologous nearly identical to that of vertebrates, especially for fish caspase-8 based on identity, similarity and phylogenetic analysis. Furthermore, comparison of the amino acid sequence with caspase-8 sequences from other vertebrates revealed a conserved and typical caspase-8 domain architecture including two putative DEDs followed by caspase family p20 and p10 domain (large and small subunits). In mammals, DEDs are able to form intracellular filaments and functions as a crucial nexus to transmit the extrinsic death signal to downstream effectors through the cleavage of caspase-8 (Tibbetts et al., 2003). Therefore, the DED is pivotal to cellular homeostasis by coregulating proliferation and apoptosis. The large subunit contained a unique pentapeptide motif QACQG, which is a conserved active site in all animals’ caspase-8 genes. The evolutionary conservation and structure similarity of common carp caspase-8 gene with other known vertebrate ones reveal that we have obtained a caspase-8 homologous gene.

The potential involvement of apoptosis as a mechanism for Cd toxicity in vivo and in vitro has been demonstrated by many previous studies (Hossain et al., 2009; Liu et al., 2011). In this study, apoptosis mainly occurred in interrenal cells using TUNEL assay, and the percentage of apoptotic cells was significantly elevated in a time- and concentration-dependent manner after Cd exposure. The increased frequency of apoptotic response indicates increased renal cell turnover (Lundebye et al., 1999). Overall, the result reveals that Cd can effectively trigger apoptosis in common carp.

Many reports show that caspase activities are increased following the occurrence of apoptosis. For example, the activities of caspase-3, -8 and -9 are significantly elevated after Cd treatment in gills of freshwater crab Sinopotamon henanense (Wang et al., 2012) and in rainbow trout hepatocytes (Rioso-de Faverney et al., 2004). However, most of the other studies indicate that Cd only stimulates rise in the activities of caspase-3 and -9, not caspase-8 (Hossain et al., 2009; Lasfer et al., 2008; Lee et al., 2006; Liu et al., 2011; Xie and Shaikh, 2006). In agreement with most of the above reports, our present study also reveals that the activities of caspase-3 and -9 were increased, whereas caspase-8 activity was not affected by Cd treatment. The common results of TUNEL assay and enzymatic activation indicate that Cd may induce apoptosis through intrinsic pathway, which further confirms our previous report where cleaved fragments of caspase-9 are detected in kidney after Cd exposure using Western blot analysis (Gao et al., 2013).
The activation of caspase-8 that we cloned was supported by the observation that the cleaved form was detected in the kidney tissue of purse red common carp after stimulation with poly(I:C) (Supplementary Fig. 1). However, in the present study, neither mRNA nor zymogen level of caspase-8 varied significantly with Cd treatment, and no cleaved fragment of caspase-8 was detected, which reveals that Cd failed to activate caspase-8. Our finding is consistent with a previous report (Li and Lim, 2007), where Cd-induced liver cell apoptosis in the rat is not related to the death receptor pathway. However, some other studies show that Cd exposure results in the production of cleaved fragments of caspase-8 (Son et al., 2010; Song and Koh, 2012). The apparent controversial results may be caused by difference of species, mixed sex sample, small sample size, cell type and the non-optimal exposure (cell treatment) conditions (Kim et al., 2008).

To date, only a few studies on zebrafish have been performed about caspase-8 immunolocalization in fish (Yeh et al., 2008). In this study, the immunoreactivity staining of caspase-8 in common carp was predominantly limited to cytoplasm of tubular epithelial cells, which is similar to a previous report (Elrod et al., 2010). It did not make a remarkable difference in variable Cd exposure conditions. Therefore, the immunopositive staining of caspase-8 may be not influenced by Cd exposure. Furthermore, most of the caspase-8 positive cells are different from TUNEL positive cells, which indicates that the occurrence of apoptosis may be not related to caspase-8. As an initiator caspase known to be involved in death receptor-mediated apoptotic pathways, caspase-8 kept stable immunoreactivity after Cd treatment, which indicates that the extrinsic apoptotic pathway was not possibly activated during this period of Cd treatment.

In contrast to effector caspases, the activation of initiator caspase-8 and its regulation are quite complex. In the death receptor pathway, binding of death ligand to its specialized membrane death receptor induces receptor trimerisation, and then results in the formation of a death-inducing signaling complex (DISC), which induces dimerization and subsequently activates caspase-8 (Bao and Shi, 2007). In T cell, the short form of cellular caspase-8 (FLICE)-like inhibitory protein (cFLIPs) can block death-receptor signaling through competition with caspase-8 for recruitment to FADD which is a component of the DISC (Budd et al., 2006). Moreover, other regulators such as viral and mammalian inhibitors of apoptosis protein (IAP) also inhibit caspase-8 with various
patterns: cowpox virus product cytokine response modifier A (Crm A) prevents caspase-8 activity by direct interaction with the active enzyme; the cleaved subunits of baculoviral protein p35 may form an inhibitory complex with caspase-8 (LeBlanc, 2003). In addition, in mammals, Cd can activate caspase-8 in an indirect way by the phosphorylation of ERK1/2, p38 and JNK MAPK (Brama et al., 2012). Therefore, in this study, that Cd did not activate caspase-8 is possibly attributable to the following factors: Cd could not induce the interaction of death receptors with their ligands; Cd triggered negative regulators of caspases such as cFLIPs, Crm A and p35; Cd failed to induce phosphorylation of ERK1/2, etc. However, most of these crucial apoptotic regulators in common carp have not been identified, and how Cd affects these molecules remains to be discovered.

5. Conclusions

The present result suggests that the cloned caspase-8 gene is homologous to the ones in other vertebrates. The results of TUNEL-positive staining, altered caspase-3 and -9 activities, and stable caspase-8 activity, mRNA level, protein expression and immunoreactivity reveal that Cd can trigger apoptosis, but it is not able to activate caspase-8 in purpe red common carp. The Cd-induced apoptosis may occur through a mitochondrial mediated pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dci.2013.08.004.

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