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Short communication

Molecular responses of calreticulin genes to iron overload and bacterial challenge in channel catfish (*Ictalurus punctatus*)

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ABSTRACT

Infection and inflammation are often accompanied by oxidative stress caused by the accumulation of reactive oxygen species which can be deleterious to the health of the host. Antioxidant defense mechanisms and components are crucial in limiting cellular and tissue-level damage and restoring homeostasis. In mammals, calreticulin is a 46-kDa multifunctional calcium binding protein of the endoplasmic reticulum that has many critical functions in the eukaryotic cell including regulation of intracellular calcium homoeostasis, lectin binding and chaperoning, and oxidative stress responses. In previous studies from our lab, the calreticulin gene was observed to be strongly upregulated in catfish during challenge with infectious Gram-negative bacteria. However, little is known about the function of this gene in teleost fish. The objective of this study, therefore, was to characterize the calreticulin gene from channel catfish, to determine its genomic organization, to profile its patterns of tissue expression, and to establish its potential for physiological antioxidant and immune responses in catfish after bacterial infection with *Edwardsiella ictaluri* and iron treatment. Our results indicate that there are at least three calreticulin related genes in the catfish genome. The three calreticulin genes are widely expressed in various tissues under homeostatic conditions and their expression showed significant upregulation following infection and/or iron level changes.

1. Introduction

Fish are subject to a host of environmental stressors, particularly in culture conditions where they are regularly exposed to increased crowding, handling, altered water chemistries, and a variety of infectious pathogens. These stressors can upset delicate balances in cellular metabolic responses, increase levels of reactive oxygen species (ROS), and lead to oxidative stress and tissue damage (Victor et al., 2004). Intracellular iron accumulation leads to oxygen radical formation (Toyokuni, 2002) and regulation of cellular iron levels is crucial in mediating oxidative stress responses (Braun, 1998). During bacterial infection, iron metabolism is particularly regulated as host and pathogen compete for control of iron stores (Ganz, 2003; Liu et al., 2010a,b). Iron binding proteins and iron transport and regulatory proteins can play dual roles in both antioxidant and immunomodulatory responses.

Calreticulin is a 46-kDa endoplasmic reticulum (ER) luminal Ca^{2+}-binding chaperone protein (Baumann and Walz, 2001; Corbett and Michalak, 2000; Gelebart et al., 2005; Michalak et al., 2009), which was first characterized in rabbit (Ostwald and MacLennan, 1974). Working together with calnexin and ERp57, it is involved in the chaperoning of nascent polypeptides that traverse through the ER (Hebert and Molinari, 2007). Calreticulin is composed of three distinct structural and functional domains: a globular N-domain, an extended P-domain and an acidic C-domain. *In vitro* studies indicated that the polypeptide- and oligosaccharide-binding regions are located in the N-domain of calreticulin (Leach et al., 2002). The N-domain contains the double cysteine residues that are involved in the S–S bonds of the secondary structure of the protein. The protein contains an N-terminal cleavable signal sequence that directs it to the ER, and an ER retention/retrieval signal KDEL (Lys-Asp-Glu-Leu) in the C-domain. Between the N- and C-domains of calreticulin is the proline-rich P-domain, which binds Ca^{2+} with a relatively high affinity ($Kd = 1\ \mu M$), but low capacity (1 mol of Ca^{2+} per mol of protein) (Baksh and Michalak, 1991). The calreticulin P-domain contains pairs of triple repeats A (amino acid sequence PXXIXDPDAXKPEDWDE) and B (amino acid sequence GXWXPPXIXNPXYX)

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(Michalak et al., 2009). The C-domain of calreticulin contains a large number of negatively charged residues that are responsible for the Ca^{2+} buffering function of the protein. The C-domain binds over 50% of ER luminal Ca^{2+} (Nakamura et al., 2001) with high capacity (25 mol of Ca^{2+} per mol of protein) and low affinity (*Kd* = 2 mM). Calreticulin is implicated in many cellular functions, including lectin-like chaperoning, Ca^{2+} storage and signaling, regulation of gene expression, cell adhesion, wound healing, cancer and autoimmunity (Gelebart et al., 2005; Michalak et al., 2009). Calreticulin has documented functions in oxidative stress responses caused by hydrogen peroxide, hypoxic injury and iron overload in mammals (Ihara et al., 2006; Jia et al., 2008; Núñez et al., 2001), and was also found to have a protective role in hereditary hemochromatosis, an iron-overload disease (Pinto et al., 2008).

Compared with intensive studies of the gene in mammals, studies of calreticulin gene in teleost fish to date have been limited. It has been identified in zebrafish (Rubinstein et al., 2000) and characterized in rainbow trout (Kales et al., 2004, 2007). Channel catfish (*Ictalurus punctatus*) is the most important aquaculture species in the United States, accounting for more than 60% of all U.S. aquaculture production (USDA, 2006). In previous microarray studies of catfish transcriptomic responses to enteric septicemia disease (Peatman et al., 2007, 2008), calreticulin was found to be upregulated three days post infection. To better characterize and analyze catfish calreticulin gene in relation to antioxidant and immune responses, here we have generated full genomic sequences of the calreticulin gene, determined its genomic organization, localized the gene on the catfish physical map, and characterized patterns of calreticulin-related gene expression. Additionally, we have analyzed calreticulin-related gene transcriptional responses to bacterial infection and iron overload.

2. Materials and methods

2.1. Identification of ESTs, BAC library screening and genomic sequencing of the catfish calreticulin gene

BLAST searches were used to identify partial cDNAs for calreticulin using channel catfish expressed sequence tags (ESTs) from previous sequencing efforts (Wang et al., 2010). All channel catfish ESTs were assembled into contiguous sequences (contigs) using the sequence assembly program CAP3 (http://deepc2.psi.iastate.edu/aat/cap/cap.html). Three contigs of calreticulin were identified by BLAST analyses, referred to here as calreticulin, calreticulin like and calreticulin like 2 according to the zebrafish nomenclature.

To identify positive BAC clones for channel catfish calreticulin gene, the CHORI-212 BAC library (Wang et al., 2007) was screened as previously described (Liu et al., 2010a,b). In brief, screening for calreticulin gene positive BAC clones was conducted using catfish gene-specific probes listed in Supplementary Table 1. Labeled probes were denatured and added into hybridization tubes containing BAC filters. Positive clones were identified and cultured in 2× YT medium for 20 h. BAC DNA was isolated and sequenced on an ABI 3130XL automated DNA sequencer as previously described (Xu et al., 2006).

2.2. Southern blot analysis

In order to determine the genomic copy number of the calreticulin-related genes in channel catfish, Southern blot analysis was conducted as previously described (Liu et al., 2010a,b). Briefly, genomic DNA was isolated from three individual adult channel catfish and 10 μg was digested with the restriction endonucleases *Eco*RI, *Hin*dIII or *Pst*I (New England Biolabs, Beverly, MA). The digested DNA samples were electrophoresed on a 0.7% agarose gel. The DNA was transferred to an Immobilon positively charged nylon membrane (Millipore, Bedford, MA) by capillary transfer for 18 h using 20× SSC buffer. The DNA was fixed to the membrane using a UV cross-linker (Stratagene, La Jolla, CA) and the auto-crosslink setting. The membrane was hybridized with cDNA probes amplified using primers listed in Supplementary Table 1, and as described for BAC-based screening.

2.3. Phylogenetic analysis

Calreticulin protein coding sequences from various species were retrieved from GenBank and Ensembl databases for multiple sequence alignment using ClustalW. Based on the multiple sequence alignment, a phylogenetic tree was constructed using the neighbor-joining method within the Molecular Evolutionary Genetics Analysis 4 (MEGA 4) package (Tamura et al., 2007). Data was analyzed using Poisson's correction, and gaps were removed by complete deletion. The topological stability of the trees was evaluated by 10,000 bootstrap replications.

2.4. Bacterial challenge and iron-dextran treatment

Bacterial challenge and iron-dextran treatment were conducted as previously described (Liu et al., 2010a,b). Briefly, a total of 960 channel catfish fingerlings were distributed into each of four groups: (1) control group (phosphate-buffered saline, PBS (pH 7.4) injected); (2) *Edwardsiella ictaluri* challenged group; (3) iron-dextran treated group; and (4) iron-dextran treated and *E. ictaluri* challenged group. The bacterial *E. ictaluri* cultured from a single colony grown on brain heart infusion (BHI) broth at 28 °C overnight was diluted with PBS, and 1×10^5 CFU of bacteria in 100 μL PBS were injected into each fish intraperitoneally. For the iron-dextran group, 100 μL of the iron-dextran solution (Sigma, St. Louis, MO) diluted in sterile PBS to a final concentration of 12.5 mg/mL was injected intraperitoneally. The fish in the combination group were injected with both bacteria and iron-dextran.

2.5. Tissue sampling and RNA extraction

A pool of tissues from organs including brain, gill, heart, head kidney, trunk kidney, intestine, liver, muscle, skin, spleen, and stomach of channel catfish from the control group were dissected and immediately immersed in RNA*later* (Invitrogen, Carlsbad, CA) to determine calreticulin genes, expression in various healthy catfish tissues. Similarly, the liver tissue from 45 fish (3 pools of 15 fish each) at 4h, 24h, 3d and 7d post-treatment in each group of the bacterial challenge/iron-dextran experiment were pooled and stored in RNA*later* (Invitrogen) for RNA extraction. Correspondingly, uninfected control samples were taken at each time interval. All samples were stored at −80 °C until RNA extraction.

Pooled tissue samples were homogenized under liquid nitrogen using a mortar and pestle, and then total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to manufacturer's protocol. The concentration of the total RNA was quantified using an Ultrospec 1100 pro (Amersham Biosciences, Fairfield, CT).

2.6. Quantitative real-time RT-PCR analysis

One-step quantitative real-time reverse transcription PCR (qRT-PCR) analysis was conducted on a LightCycler 1.0 (Roche Applied Science) as described by Liu et al. (2010a,b). Concentration of total RNA (100 ng) was determined by spectrophotometry and qRT-PCR reactions (10 μL each) were performed in triplicate using the Fast Start RNA Master SYBR Green I Reagents Kit (Roche Applied Science) following manufacturer's instructions with modifications as

previously described (Peatman et al., 2007, 2008). The 18S rRNA gene was used as a reference gene for comparative normalization of expression levels. The primers that were used in qRT-PCR are listed in Supplementary Table 1.

To assess the relative expression of calreticulins in various catfish tissues, the spleen RNA sample was arbitrarily chosen as the calibrator (1×) and the relative abundance of calreticulin genes was calculated for the remaining 10 tissues by ratio with the calibrator. The fold induction of the channel catfish calreticulin genes after different treatments was quantified in the liver. PCR specificity was assessed by melting curve analysis. Cycle threshold (Ct) values were generated and converted to fold differences by the relative quantification method using the Relative Expression Software Tool version 2009 (REST 2009) (Pfaffl et al., 2002). The fold-change of the channel catfish calreticulin genes after different treatments was made into a graphical representation; differential regulation was considered significant when $p < 0.05$.

3. Results and discussion

3.1. The channel catfish genome contains at least three calreticulin-related genes

Initially, calreticulin-related cDNAs were identified from ESTs. Sequence analysis indicated the presence of three distinct clusters of cDNAs. To determine the identities of the calreticulin-related genes, phylogenetic analysis was conducted based on amino acid sequences. As shown in Fig. 1, the three catfish cDNAs fell in different clades: one (calreticulin; CALR) is most similar to the salmonid and zebrafish calreticulin genes; the second calreticulin-related cDNA of catfish, herein referred to as calreticulin like (CALRL; following the nomenclature of zebrafish) is most similar to that of zebrafish; and the third catfish calreticulin-related cDNA, herein referred to as calreticulin like 2 (CALRL2; also following the nomenclature of zebrafish), is most similar to the zebrafish calreticulin like 2.

Phylogenetic analysis using our catfish sequences along with a broad group of teleost sequences indicated that the zebrafish nomenclature may be incorrect. The catfish CALRL2 (following the zebrafish nomenclature) and the zebrafish CALRL2 both fall into a well-supported clade containing mammalian CALR genes. The other catfish calreticulin sequences (CALR and CALRL following zebrafish nomenclature) appear to be teleost-specific, and are grouped with other fish CALRs. Of note, calreticulin gene numbers and patterns of divergence appear to vary among the analyzed species. For example, zebrafish, appears to have highly similar, duplicated copies of CALR genes, whereas the three calreticulin genes from catfish are well-distributed across the tree in distinct clades. No CALRL2 genes were identified from fugu or medaka. Additionally, the number of calreticulin genes differs among the well-studied mammalian genomes with two, three, and four genes found in human, rat, and mouse respectively. Little research has been conducted on the function of these additional calreticulin family members. Further work is clearly needed to determine functional orthologies between vertebrate calreticulin genes.

In order to further characterize the catfish CALR-related genes, genomic Southern blot analysis was conducted. As shown in Supplementary Figure 1, the catfish CALR gene exists in the catfish genome as a single copy gene because the restriction banding patterns can all be explained from a single copy gene. For instance, *Eco*R1 digest produced a single band. Although *Hin*dIII and *Pst*I digestions produced two and three bands, respectively, these bands can be explained by the presence of two internal *Hind*III (in the same intron) and two internal *Pst*I sites within the gene sequence. This result was confirmed by the fact that only one CALR-positive BAC clone was identified from a screen of the catfish BAC library (CHORI-212) which falls into contig 355 of the catfish physical map and linkage group 6 in the linkage map (Xu et al., 2007; Lu et al., 2010; www.catfishgenome.org). Similarly, the catfish CALRL gene appeared to exist in the catfish genome as a single copy gene. It is not conclusive at present whether a single copy or two copies of the catfish CALRL2 gene exist in the catfish genome as all three restriction endonucleases generated at least two bands. In zebrafish, two copies of the CALRL2 gene exist as head to tail tandem duplicates. It is possible that the CALRL2 gene in catfish is also duplicated. Taken together, these results demonstrated the presence of at least three CALR-related genes in catfish genome.

3.2. Structural features of the catfish CALR genes

The organization of the CALR gene was initially approached by traditional cloning and sequencing. One BAC clone (18_H18) containing the CALR gene was identified by screening of a channel catfish CHORI-212 BAC library (Wang et al., 2007). This clone was then used for sequencing channel catfish CALR. The complete genomic sequence of channel catfish CALR was obtained with 8446 bp and was deposited to GenBank with accession number GU936971. Alignment of the genomic sequence with the known cDNA sequence of the catfish CALR revealed the presence of 9 exons and 8 introns, similar to those in other fish and mammalian species. The number of exons and the sizes of exons are both well conserved among all analyzed species ranging from fish to mammals (Fig. S2).

As we are now in the midst of sequencing the catfish genome, the classical cloning followed by sequencing approach becomes extremely laborious. As a result, for the structural analysis of the catfish CALRL and CALRL2 genes, we have aligned the cDNA sequences with existing genomic sequence contigs (unpublished) to infer the gene structures. As shown in Supplementary Figure 2, the number and sizes of exons were well conserved among CALR, CALRL, and CALRL2 genes, although the size of the introns were highly variable.

The channel catfish CALR cDNA contains an open reading frame (ORF) of 1269 bp encoding a putative protein of 423 amino acids with a 103 bp 5′-untranslated region (UTR) and a 393 bp 3′-UTR. The channel catfish CALRL and CALRL2 genes have predicted ORF of 1272 and 1263 bp encoding putative proteins of 424 and 421 amino acids, respectively. Analysis of the deduced amino acid sequence by multiple sequence alignment indicated that the CALR gene is highly conserved throughout vertebrate evolution, with a minimum of at least 69% amino acid identity among all analyzed CALR genes (Fig. S3). The catfish CALR genes contains typical N-, P- and C-domains, triple repeats A (amino acid sequence PXXIXDPDAXKPEDWDE) and B (amino acid sequence GXWXPPXIXNPXYX), and the cysteines involved in the S–S bonds (Fig. S3). The catfish CALRL and CALRL2 genes are structurally extremely similar to the catfish CALR gene, with all conserved structural features. Amino acid identities among the three genes were between 67% and 74%.

3.3. Tissue expression and expression of CALR genes after bacterial infection and/or administration of iron-dextran

Quantitative real time RT-PCR was used to determine relative tissue distribution of CALR, CALRL and CALRL2 expression in 11 catfish tissues including brain, gill, head kidney, trunk kidney, heart, intestine, liver, muscle, skin, spleen and stomach. As shown in Fig. 2, expression of the catfish CALR genes was detected in all tissues, although the three genes exhibited highly different expression profiles in these 11 tissues. High CALR expression was found in spleen and liver and the lowest expression was found in muscle. For CALRL, the highest expression was found in spleen and lowest expression in skin. For the catfish CALRL2 gene, the highest expression was

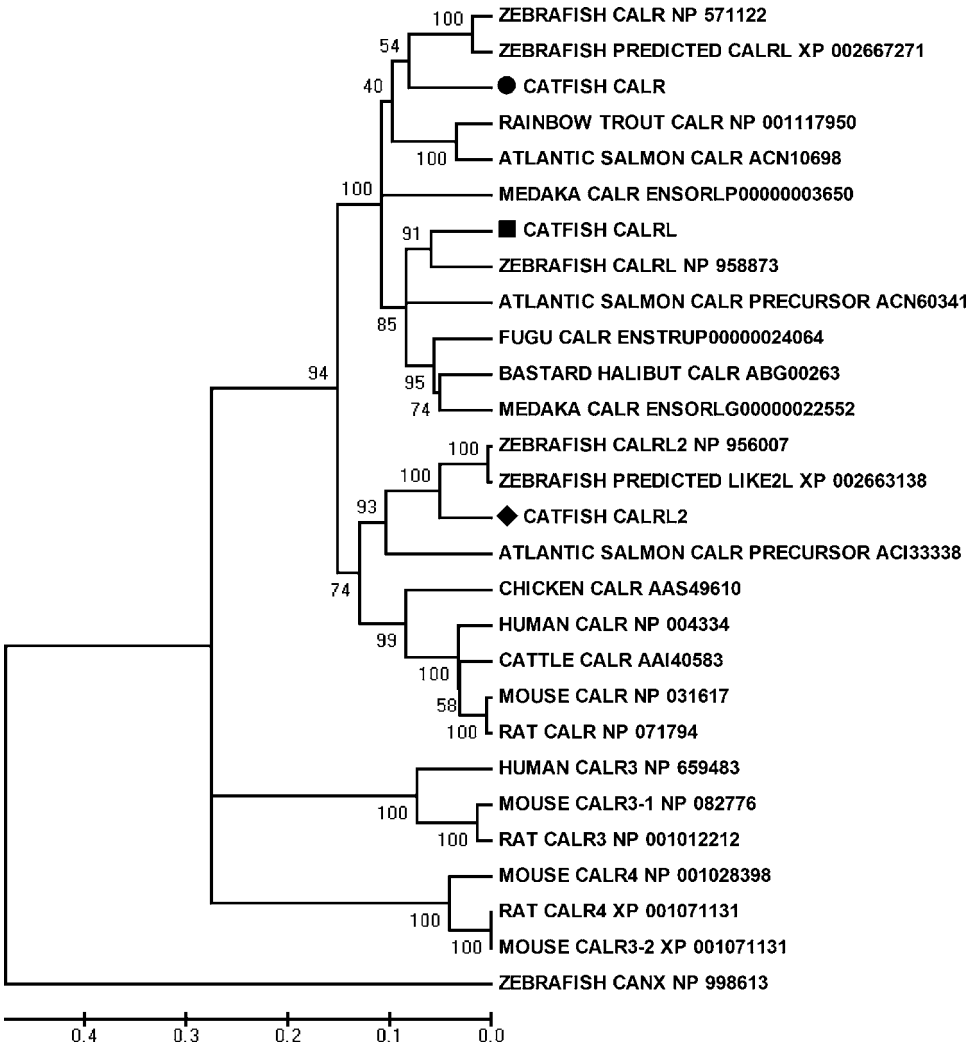

Fig. 1. Phylogenetic analysis of catfish calreticulin (CALR), calreticulin like (CALRL) and calreticulin like 2 (CALRL2) genes with other vertebrate calreticulin genes. The phylogenetic tree was constructed using the neighbor-joining method, based on a ClustalW multiple sequence alignment of amino acid sequences. The topological stability of the tree was evaluated by bootstrapping; bootstrap values are indicated by numbers at the nodes. GenBank accession numbers for the retrieved sequences are listed in the figure.

found in the head kidney and trunk kidney, the lowest expression was found in the skin, with very similar expression levels in all the other tissues tested.

Given our previous observation of calreticulin induction in liver at a single timepoint following infection (3d; Peatman et al., 2007, 2008), and the importance of liver in iron metabolism and storage, we examined expression of CALR, CALRL and CALRL2 genes in the liver of channel catfish after *E. ictaluri* challenge and/or iron-dextran treatment using qRT-PCR. As shown in Fig. 3, the three genes were regulated differentially by infection, iron-dextran, or the combination of infection and iron-dextran. However, some commonalities are obvious from all three treatments: (1) infection by *E. ictaluri* induced high level expressions of all three genes 24 h after infection, then levels of expression declined rapidly by 3d after infection; (2) this pattern held true when the fish were challenged with both the infection and iron dextran; (3) after exposure to iron dextran alone, both CALRL and CALRL2 increased significantly at 24 h after exposure and slowly decreased through 7 d post exposure, whereas CALR did not change significantly over time (Fig. 3).

CALR is reported to play a role in many cellular functions including lectin-like chaperoning, Ca^{2+} storage and signaling, regulation of gene expression, cell adhesion, wound healing, and autoimmunity (Gelebart et al., 2005; Michalak et al., 2009). Recently the CALR gene was also reported to play a protective role in the iron-induced oxidative stress (Núñez et al., 2001; Pinto et al., 2008). Recent research in mammalian species has emphasized the role of CALR as a receptor for C1q, mannose-binding lectins, and ficolins (Pagh et al., 2008; Lacroix et al., 2009). It has been suggested that a complex of CD91 with calreticulin serves as a scavenger receptor complex for apoptotic cells and micro-organisms (Walters and Berwin, 2005). These observations would closely connect CALR to innate immune processes mediated through the lectin and classical complement pathways. Recent research by Oladiran and Belosevic (2010) connect binding of CALR and C1q even in immune evasion strategies of extracellular parasites. If teleost CALRs play similar roles in lectin/C1q binding, the early induction of catfish CALR expression observed following bacterial infection may be due to increased pathogen binding/elimination via lectin-complement-mediated processes.

Research on teleost CALR genes has been limited, and has not previously described infection-mediated upregulation of CALR (Rubinstein et al., 2000; Kales et al., 2004, 2007). Kales et al. (2007), however, have reported the presence of CALR on the surface of PHA-

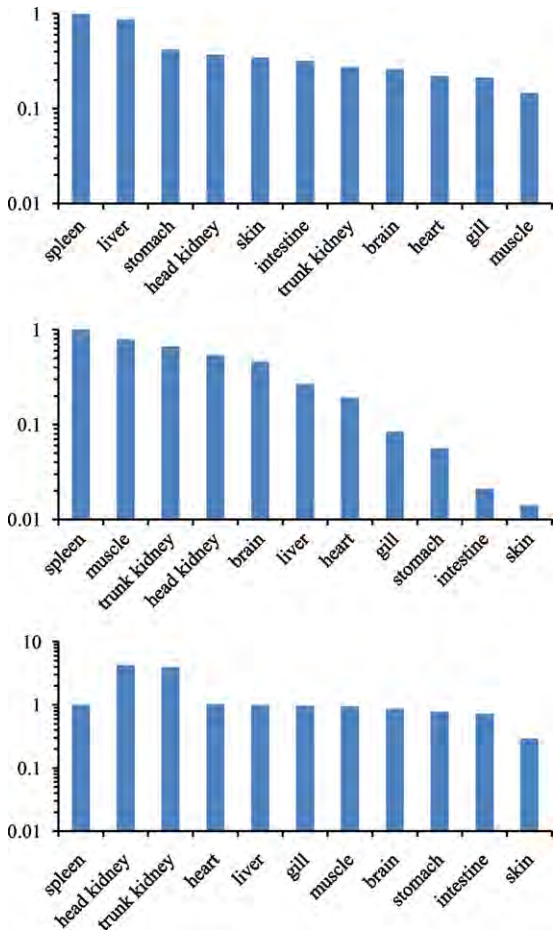

Fig. 2. Relative expression of calreticulin (CALR), calreticulin like (CALRL) and calreticulin like 2 (CALRL2) genes in channel catfish determined using qRT-PCR (log 10). Tissue RNA samples are labeled along the *X*-axis. Expression levels in all tissues are presented relative to that in the spleen tissue (1×).

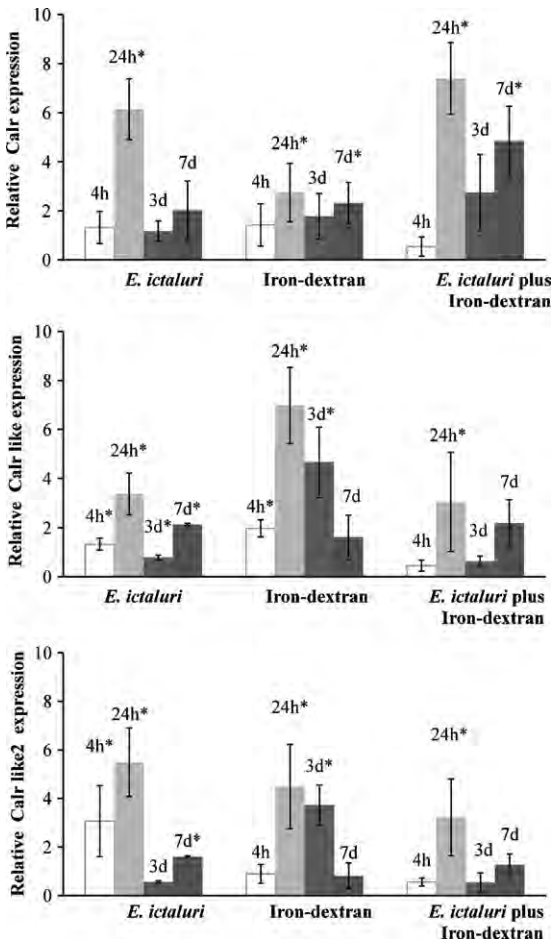

Fig. 3. Fold induction of channel catfish calreticulin (CALR), calreticulin like (CALRL) and calreticulin like 2 (CALRL2) genes after *E. ictaluri* and/or iron-dextran treatment in the liver. Relative calreticulin expression was expressed as fold change over control samples taken at the same time interval as normalized to change in expression in the 18S control. The bars indicate mean expression of 3 tested pools (15 fish each) ± SE. Asterisks indicate statistical significance at the level of $p < 0.05$ relative to appropriate control.

stimulated leukocytes, again suggesting potential immune roles. In this study, three catfish CALR genes (CALR, CALRL, and CALRL2) were found to be widely expressed in various healthy tissues and were significantly induced in the liver after intraperitoneal injection of *E. ictaluri*, suggesting potentially protective roles in disease response and roles in repairing the tissue damage caused by the *E. ictaluri* or in *E. ictaluri*-stimulated ROS responses. The catfish CALR genes were also significantly induced by iron overload, suggesting potential protective roles in iron-induced oxidative stress as similarly observed in mammals (Núñez et al., 2001; Pinto et al., 2008). However, future studies are warranted to further explore the functions of these genes in host–pathogen interactions.

Following up on our observation of induction of CALR expression following infection (Peatman et al., 2007, 2008), we have here further characterized CALR genes in channel catfish. Based on novel sequencing, Southern blotting, and *in silico* scans of extensive EST collections and preliminary genome sequence contigs, at least three CALR genes are present in the catfish genome. The gene structure (exon size) of CALRs across vertebrate evolution is remarkably conserved, indicating functional restraints on sequence divergence. Transcript profiling in healthy tissues indicated widespread expression, although levels differed noticeably between the different catfish genes. Expression of the catfish CALR genes after injection of *E. ictaluri* and/or iron significantly increased. Further study is needed to determine conclusively whether fish CALR genes may play roles in eliminating the incursive pathogen (via lectin/C1q binding) and in reducing excess ROS so as to protect host cells from oxidative damage.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2010.11.009.

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