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Stimulated mRNA Expression of the Second Glyceraldehyde 3-Phosphate Dehydrogenase in the Barred Knifejaw *Oplegnathus fasciatus* Spleen during Bacterial and Viral Injection

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Transcriptional response of the second isoform of glyceraldehyde (GAPDH-2) to infectious challenges using various bacterial species and the rockbream iridovirus (RBIV) was examined in the spleen of the barred knifejaw (*Oplegnathus fasciatus*). Bacterial challenges of the juvenile barred knifejaws with *Escherichia coli*, *Edwardsiella tarda*, *Vibrio anguillarum* and *Streptococcus iniae* resulted in the significant elevation of the GAPDH-2 transcripts in the spleen up to 12-fold relative to that in the non-challenged controls (PBS-injected). In addition, the barred knifejaw fingerlings injected with RBIV stock also represented the highly upregulated mRNA expression of the GAPDH-2 up to more than 20-fold when compared to that of control fingerings. Results obtained from this study strongly suggest that the GAPDH-2 is no longer a housekeeping glycolytic protein and rather than that it might be associated with immune-relevant cellular activities. From this finding, the traditional dogma for the use of GAPDH as an invariant standard for gene expression assays should be carefully revised depending on the kinds of biological stimulations applied in this species.

Keywords: GAPDH-2, Inflammatory infection, mRNA expression, Barred knifejaw

Introduction

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a key enzyme playing a crucial role in glycolytic pathway (energy production). Due to its housekeeping function, the structure and function of this housekeeping enzyme have been known to be fairly conserved in a wide spectrum of organisms. Consequently, it has led to a classical use of GAPDH mRNA expression as an invariant standard for various gene expression assays (Barber et al., 2005). However, a number of recent articles have claimed that mammalian GAPDH is no longer a traditional housekeeping protein. Instead, this protein is a multifunctional player involved in various cellular pathways (Sirover, 2005).

Mammalian species express two distinct GAPDH isoforms, in which the second GAPDH isoform (GAPDH-2) composed of a long prolin (Pro)-rich peptide at N-terminus. Moreover, mammalian GAPDH-2 is expressed in a sperm-specific fashion (so called GAPDH-S) (Miki et al., 2004). On the other hand, teleost GAPDH-2s lack the Pro-rich domain and show a wide array of mRNA tissue distribution (Manchado et al., 2007; Cho et al., 2008). Our recent work on the molecular phylogeny of vertebrate GAPDHs also undoubtedly suggested that teleost GAPDH-2, a potential orthologue of mammalian GAPDH-S experienced a different evolutionary history from the mammalian counterpart (Kim and Nam, 2008).

More importantly, we also found that the teleost GAPDH-2 isoform from the barred knifejaw *Oplegnathus fasciatus* displayed a significant change of its mRNA expression levels in liver, resulting from bacterial and/or viral infections (Cho et al., 2008), suggesting that the barred knifejaw GAPDH-2 could also be potentially involved in non-glycolytic pathways. In this study, we extended our examination on the GAPDH-2 expression to the spleen, one of the most important immune-relevant organs in fish. We tested altered mRNA expression of the barred knifejaw GAPDH-2 in response to various infectious treatments.

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Materials and Methods

Gene sequences

Sequence information of the barred knifejaw GAPDH-2 cDNA that had been deposited to GenBank is available under the accession number (EU828450), and detailed structural

characteristics of its cDNA and deduced amino acids can be referred to Cho et al. (2008). A hepcidin gene from the barred knifejaw (EU809940) was employed as a positive control to validate the induction of inflammation conditions resulting from the experimental challenges applied in this study. A partial fragment of barred knifejaw 18S rRNA (unpublished data) was used as an internal standard to normalize the gene expression levels across samples.

Preparation of bacterial and viral stocks

Bacterial suspensions were prepared with three Gram (−) and two Gram (+) bacterial species as follow: *Escherichia coli* XLI-blue MRF' strain (Gram -; Stratagene, USA), *Edwardsiella tarda* (Gram -; FSW910410), *Vibrio anguillarum* (Gram -; KFCC11377P), *Lactococcus garviae* (Gram +; ATTC49156) and *Streptococcus iniae* (Gram +; JSL0108). Bacteria were freshly cultured using each appropriate medium up to an optical density ($OD_{600\ nm}$) of 0.8. Grown bacterial cells were washed twice with a cold phosphate buffered saline (PBS; pH 7.6) and resuspended in the PBS at the concentration of 1×10^6 cells/ml. The suspension was immediately used for injection. On the other hand, rockbream iridovirus (RBIV) stock was obtained by preparing a homogenate with the spleen from juvenile barred knifejaws that had been artificially overloaded with the RBIV according to the procedures described by Choi et al. (2006). The viral stock was suspended in PBS (pH 7.6) and its potency was tested prior to being used by examining the nearly 100% mortality of the barred knifejaw fingerlings within 2 weeks (data not shown).

***In vivo* experimental challenge**

For bacterial challenge, six individuals (average body weight = 120 g) per group were injected intraperitoneally with 1×10^5 cells in a 100 μl of injection volume. The same amount of PBS was also injected into the same-sized juveniles in order to prepare a non-challenged control group. Fish belonging to each group (five bacterial groups and one PBS control in total) was stocked into a 50 litter tank containing 40 litter of well-aerated seawater at 20°C. For each group, two replicate tanks were prepared. Fish were maintained for 48 hours with a daily water exchange rate of 100%. After 48 hours, spleen was surgically removed from four randomly selected individuals from each replicate tank. Tissues were stored in RNA*later* solution (Ambion, USA) at -20°C until used. On the other hand, viral challenge was conducted with fingerlings (average body weight = 4 g). Fish (n = 24) were given an intraperitoneal (IP) injection of 50 μl of a RBIV stock (approximately 1×10^7 particles) and transferred to a 50 litter tank. The same number of fingerlings was also given an IP injection of 50 μl of PBS for a preparing control group. Experimental tanks were managed similarly as above. At nine days post-injection, clinical sign of RBIV syndrome began to occur in several fingerlings injected with RBIV. At that time, ten individuals were randomly obtained from either RBIV- or PBS-injected group. Spleen was surgically removed for RNA analysis.

Nucleic acid preparation

Total RNA from spleen was isolated using RNeasy midi kit (Qiagen, Germany) according to the manufacturer's instruction. The total RNA sample was treated with DNase I to remove the possible contamination of DNA using the RNeasy mini clean-up kit (Qiagen). Integrity of total RNA was checked by examining 28S:18S rRNA ratios on a MOPS/formaldehyde agarose gel. To prepare template for RT-PCR assays, two μg of purified total RNA was reverse transcribed into cDNA using Omniscript RT kit (Qiagen) and oligo-$d(T)_{20}$ primers according to the recommendation by the manufacturer. A barred knifejaw 18S rRNA reverse primer (RB18S RV: 5'-AGAATTTCACCTCTAGCGGC-3') was also included in the reaction at the final concentration of 0.1 μM for preparing the internal control to normalize the expression levels across samples.

RT-PCR analysis

End-point semi-quantitative RT-PCR was performed in order to examine first potential changes of GAPDH-2 mRNAs in response to experimental challenges, and then the fold increase of mRNA expression was estimated using real-time RT-PCR. The RT-product prepared as above was two-fold (for target genes) or 10-fold (for 18S rRNA) diluted with sterile distilled water. One μl of diluted cDNA was used as a template for PCR amplification. Based on our preliminary experiments to optimize the PCR amplification in a semi-quantitative fashion, GAPDH-2transcripts (amplicon = 397 bp) were amplified using a pair of oligonucleotide primers, RBGAPDH2-1F (5'-GTCTGTCCAAGCCTGCATCT-3') and RBGAPDH2-1R (5'-GTGTAGTAGAGCTAAGGGGT-3') under the following thermal cycling conditions: 28 cycles at 94°C for 30 s, 58°C for 20 s and 72°C for 30 s with an initial denaturation step at 94°C for 3 min. In addition, hepcidin, a

known inflammation modulating, anti-microbial peptide was also included in the semi-quantitative RT-PCR assays in order to validate if the present bacterial and viral injections would raise inflammatory conditions in those tissues. The internal fragment of the barred knifejaw hepcidin precursor (expected size = 265 bp) was amplified using two oligonucleotide primers (qRBHEPC-F: 5'-GAAGACATTCAGTGTTGCAG-3' and qRBHEPC-R: 5'-TCAGAATCTGCAGCACAGTC-3') under the following conditions: 30 cycles at 94°C for 20 s, 58°C for 20 s and 72°C for 20 s. On the other hand, the internal standard 18S rRNA (expected size = 407 bp) was amplified using a pair of primers, qRB18S 1F (5'-TACCACATCCAAGGAAGGCA-3') and qRB18S 1R (5'-TTCCTAGCTGCGGTATTCAG-3'). Thermal cycling conditions were 20 cycles at 94°C for 30 s, 58°C for 20 s and 72°C for 30 s. Amplification products were separated on a 1.5% agarose gel, and visualized by ethidium-bromide (Et-Br) staining. Et-Br stained bands were analyzed using the image analysis software, Quantity-One™ implemented in Versa-Doc 4000 (Bio-Rad, USA). Expression level of GAPDH-2 was normalized against that of 18S rRNA control in order to estimate the relative mRNA levels among groups.

After the semi-quantitative RT-PCR assays, real-time RT-PCR analysis was carried out in order to estimate fold increases of GAPDH-2 mRNAs resulting from the infection treatments. Amplification was performed using 2X iQ SYBR Supermix (Bio-Rad, USA) and iCycler iQ Real-Time Detection System (Bio-Rad, USA) to calculate average threshold cycles (Ct) for both GAPDHs and 18S rRNA in the spleen cDNAs. PCR amplification was carried out using the same primer pairs as described above, RBGAPDH2-1F/1R and qRB18S 1F/1R. Reaction (25 μl) was 40 cycled at 94°C for 20 s, 58°C for 20 s and 72°C for 30 s and fluorescence signal was taken after each elongation step. Dissociation and melting curves were also examined after the final elongation step in order to confirm the specificity of PCR. Standard curves for both GAPDH-2 and 18S rRNA control were prepared using serially diluted positive cDNAs to estimate the PCR efficiency ($E = 10^{-1/slop}-1$) for each gene. Expression levels of GAPDH-2 in each cDNA sample were normalized against its own level of 18S rRNA, and then fold increases of GAPDH transcripts in challenged groups as relative to non-challenged groups were estimated using the comparative ΔCt method (Schmittgen and Livak, 2008). All of RT-PCR assays were carried out in triplicates and differential expression levels between and/or among groups were assessed by student's t-test and/or ANOVA followed by Duncan's multiple range tests at $P = 0.05$.

Results and Discussion

No mortality was found during bacterial challenges whereas an outbreak of mortality up to 30% was detected in RBIV-injected fingerling group immediately after the sampling at 9 days-post injection. Ultimately there was no survivor in the RBIV-injected group after 2 weeks of the challenge, although the control group (PBS-injected) exhibited a good survival rate higher than 95%. From the bacterial challenge, the expression level of 18S rRNA judged by semi-quantitative RT-PCR was fairly constant regardless bacterial challenge ($P>0.05$) (Fig. 1). A positive amplification using hepcidin displayed a dramatic increase of its transcripts in response to exposures to microbes. It was not surprising since many previous studies have already reported the similar findings with experimentally designed infection treatments (for review, see Hilton and Lambert, 2008). Hence, our finding

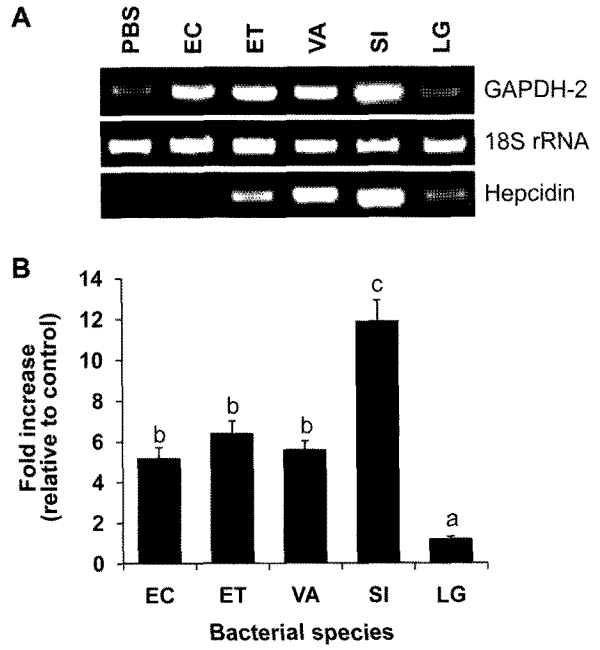

Fig. 1. Stimulated mRNA expression of barred knifejaw GAPDH-2 in spleen after experimental challenges with *E. coli* (EC), *E. tarda* (ET), *V. anguillarum* (VA), *S. iniae* (SI) and *L. garviae* (LG) along with PBS-injected control. Relative expression levels were examined both by semi-quantitative RT-PCR (A) and real-time RT-PCR (B) assays. The 18S rRNA is used as an internal standard while hepcidin as a quality control to validate the inflammation states. The same letters on histograms indicate that means are not significant different based on ANOVA ($P<0.05$). T bars indicate standard deviations based on triplicate analyses.

undoubtedly indicates that the inflammatory states could be effectively induced in those individuals by the present experimental challenges. RT-PCR analysis indicated that the transcription of the GAPDH-2 was significantly modulated toward upregulation in the spleen by bacterial injection, although the amount of elevation was variable among experimental groups ($P<0.05$) (Fig. 1). However, unlike other bacterial species, the group injected with *L. garviae* did not show any significant alteration of the GAPDH-2 mRNA levels, suggesting the regulation of the GAPDH-2 could be affected by bacterial species. The increased amount of GAPDH-2 in response to bacterial challenge was clearly validated again by real-time RT-PCR amplification. Average threshold cycle numbers (Ct) for internal control (18S rRNA) were constant again between samples, however Ct values for GAPDH-2 was significantly decreased from PBS-injected group, consequently resulting in the increased gene expression up to 12-fold in *S. iniae*-injected fish ($P<0.05$) (Fig. 1). As well as the bacterial challenges, viral infection also considerably elevated the GAPDH-2 transcripts in spleen of this species ($P<0.05$). Furthermore as shown in Fig. 2, the amount of fold increase was much higher than that observed in bacterial challenged groups. Relative mRNA level of GAPDH-2 in spleen of RBIV-injected fingerlings was more than 20-fold when compared to the basal expression in PBS-injected group ($P<0.05$).

For a long time, GAPDH mRNA level has been commonly used as an invariant standard for a number of gene expression assays based on the belief that this gene is constitutively expressed irrespective of most biological conditions (Barber et al., 2005; Filby and Tyler, 2007). However, recent studies strongly suggest that this traditional dogma should be carefully revised. In particular, mammalian GAPDHs are known to be involved in a number of disease-related pathways including induced apoptosis and neurodegenerative disorders (Chuang et al., 2005). In teleost species also, potential association of GAPDH with non-glycolytic function has been recently proposed (Manchado et al., 2007). Although there was only quite a limited knowledge is available for the physiological role of teleost GAPDH-2, the expression pattern of teleost GAPDH-2 was shown not to be same with that in mammals. Since the physiological function of the mammalian GAPDH-2 (GAPDH-S) is tightly linked only with the spermatogenesis process (Miki et al., 2004), the primary isoform (GAPDH-1) seems to be responsible for multivalent roles required in diverse cellular pathways. However in contrast, teleost GAPDH-2s, which are expressed ubiquitously across tissue, are proposed not only to share non-glycolytic roles with GAPDH-1 but also to display a certain unique function that is distinct from that of GAPDH-1 (Cho et al., 2008). However unfortunately, the specific criteria which the teleost GAPDH-2s function in have not been fully explored yet.

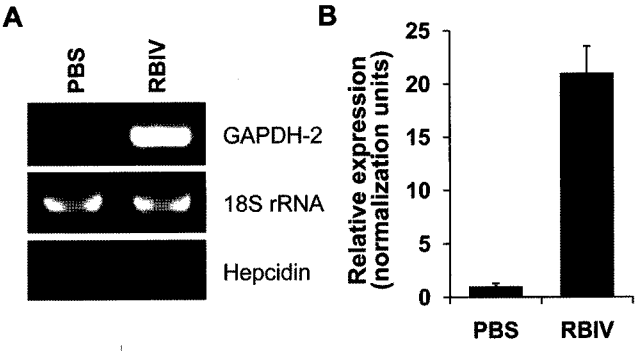

Fig. 2. Upregulated expression of barred knifejaw GAPDH-2 in spleen after RBIV infection as assessed by semi-quantitative RT-PCR (A) and real-time RT-PCR (B) assays. The 18S rRNA is used as an internal standard while hepcidin as a quality control to validate the inflammation states. Histograms in (B) were prepared by normalization against the 18S rRNA control based on triplicate examinations. T bars indicate standard deviations.

The spleen is one of the most important immune organs and houses immune cells such as B and T cells in fish. For this reason, the spleen has been a frequent target in various studies for survey of immune-related genes from fish. Our data on the stimulated expression of GAPDH-2 transcripts in spleen upon infectious treatments is supported by the previous observations made with tumor-bearing mice where the GAPDH mRNA levels were markedly increased in spleen by tumor implantation (Aledo et al., 1999). Also it was reported that GAPDH transcripts were significantly stimulated in the murine cells by the activation with tumor-necrosis factor alpha (TNF-α) and interferon gamma (INF-γ) (Bereta and Bereta, 1995). On the other hand, other findings on the stimulated GAPDH expression in rat and chicken by an injection of lipopolysaccharide (LPS) are also generally in agreement with our results (Xie et al., 2006; De Boever et al., 2008). Currently the detailed mechanism behind the induction of teleost GAPDH-2 transcripts upon infection is not clearly understood yet. However, one possible explanation is that proapoptotic cascade triggered by the inflammation-mediated tissue damage might be responsible for this phenomenon as similarly in mammals (Xie et al., 2006). However the possibility of direct interaction between fish GAPDH-2 proteins and the invading microbes could not be ruled out (see

also Petrik et al., 1999). Further study is needed to extend the examinations of the GAPDH-2 expression with various biological treatments which potentially cause cytotoxicity and/or inflammation.

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References

Aledo, J. C., J. A. Seura, L. G. Barbero and J. Marquez, 1999. Upregulation of glyceraldehyde-3-phosphate dehydrogenase mRNA in the spleen of tumor-bearing mice. Biochimie, 81, 1109–1113.

Barber, R. D., D. W. Harmer, R. A. Boleman and B. J. Clark, 2005. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. Physiol. Genomics, 21, 389–395.

Bereta, J. and M. Bereta, 1995. Stimulation f glyceraldehyde-3-phosphage dehydrogenase mRNA levels by endogenous nitric oxide in cytokine-activated endothelium. Biochem. Biophys. Res. Commun., 217, 363–369.

Cho, Y. S., S. Y. Lee, K. H. Kim and Y. K. Nam, 2008. Differential modulations of two glyceraldehyde 3-phosphate dehydrogenase mRNAs in response to bacterial and viral challenges in a marine teleost *Oplegnathus fasciatus* (Perciformes). Fish Shellfish Immunol., 25, 472–476.

Choi, S. K., S. R. Kwon, Y. K. Nam, S. K. Kim and K. H. Kim, 2006. Organ distribution of red sea bream iridovirus (RSIV) DNA in asymptomatic yearling and fingerling rock bream (*Oplegnathus fasciatus*) and effects of water temperature on transition of RSIV into acute phase. Aquaculture, 256, 23–26.

Chuang, D. M., C. Hough and V. V. Senatorov, 2005. Glyceraldehyde-3-phosphate dehydrogenase, apoptosis, and neurodegenerative diseases. Annu. Rev. Pharmacol. Toxicol., 45, 269–290.

De Boever, S., C. Vangestel, P. De Backer, S. Croubels and S. U. Sys, 2008. Identification and validation of housekeeping genes as internal control for gene expression in an intravenous LPS inflammation model in chickens. Vert. Immunol. Immunopathol., 122, 312–317.

Hilton, K. B. and L. A. Lambert, 2008. Molecular evolution and characterization of hepcidin gene products in vertebrates. Gene, 415, 40–48.

Filby, A. L. and C. R. Tyler, 2007. Appropriate 'housekeeping' genes for use in expression profiling the effects of environmental estrogens in fish. BMC Mol. Biol., 8, 10.

Kim, K. Y. and Y. K. Nam, 2008. Evolutionary history of two paralogous glyceraldehyde 3-phosphate dehydrogenase genes in teleosts. J. Fish. Sci. Tech., 11, 177–181.

Manchado, M., C. Infante, E. Asensio and K. P. Canavate, 2007. Differential gene expression and dependence on thyroid hormones of two glyceraldehyde-3-phosphate dehydrogenases in the flatfish Senegalese sole (*Solea senegalensis* Kaup). Gene, 400, 1–8.

Miki, K., W. Qu, E. H. Goulding, W. D. Willis, D. O. Bunch, L. F. Strader, S. D. Perreault, E. M. Eddy and D. A. O'Brien, 2004. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proc. Natl. Acad. Soc., 101, 16501–16506.

Petrik, J., H. Parker and G. J. M. Alexander, 1999. Human hepatic glyceraldehyde-3-phosphate dehydrogenase binds to the poly(U) tract of the 3' non-coding region of hepatitis C virus genomic RNA. J. Gen. Virol., 80, 33109–33113.

Schmittgen, T. D. and K. J. Livak, 2008. Analyzing real-time PCR data by the comparative Ct method. Nature Protoc., 3, 1101-1108.

Sirover, M. A., 2005. New nuclear functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells. J. Cell. Biochem., 95, 45–52.

Xie, W., N. Shao, X. Ma, B. Ling, Y. Wei, Q. Ding, G. Yang, N. Liu, H. Wang and K. Chen, 2006. Bacterial endotoxin lipopolysaccharide induces up-regulation of glyceraldehyde-3-phosphate dehydrogenase in rat liver and lung. Life Sci., 79, 1820–1827.

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