JMLS 한국해양생명과학회지[Journal of Marine Life Science]
December 2018; 3(2): 45-50
http://jmls.or.kr

[Article]

Expressional Analysis of STAT2 Gene in Rock Bream, *Oplegnathus faciatus*, Under LPS or Poly I:C Stimulation and Megalocityvirus Infection

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Received : November 09, 2018
Revised : November 19, 2018
Accepted : December 10, 2018

Rock bream iridovirus (RBIV) is a megalocytivirus widely infected in various fish species in Korea, causing symptoms of acute inflammation and enlargement of spleen. In our previous study, RBIV induced the initial upregulation but later down-regulation of proinflammatory cytokines and IFN1 gene expression. Signal transducers and activators of transcriptions (STAT) are transcription factors involved in the regulation of immune genes including IFNs. This study was conducted to analyse the expression of STAT2. The expressional study of STAT2 gene was performed in head kidney and spleen upon RBIV infection and immune stimulants like LPS or poly I:C *in vitro*. Consequently, STAT2 gene expression pattern was different in head kidney and spleen as it was significantly up-regulated by LPS from 4 h to 8 h but down-regulated at 24 h while up-regulated by poly I:C at 8 h in head kidney while, in spleen, STAT2 gene expression was down regulated by LPS but significantly up-regulated by poly I:C. Upon RBIV stimulation, STAT2 gene expression was significantly down-regulated by high dose RBIV at 4 h but up-regulated at 8 h and 24 h in head kidney. In spleen cells, it was up-regulated by medium dose RBIV at 4 h and by high dose RBIV at 4 h and 8 h but down regulated later then. *In vivo*, STAT2 gene expression was not significantly affected by RBIV infection while significant up-regulated by vaccination at day 7 post-vaccination, indicating STAT2 gene can be involved in adaptive immune response in rock bream.

Keywords: Rock bream, Megalocytivirus, Cytokines, STAT2, Gene expression

Introduction

Signal transducers and activators of transcriptions (STAT) are transcription factors involved in the regulation of immune genes including IFNs. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators (Schindler et al., 2007). STAT1 and 2 mediate signaling by type I IFNs (IFN-alpha and IFN-beta). Following type I IFN binding to cell surface receptors, Jak kinases (TYK2 and JAK1) are activated, leading to tyrosine phosphorylation of STAT1 and STAT2 (Steen et al., 2013). The phosphorylated STATs form a complex with IFN regulatory factor family protein p48 (IRF9), in which this protein acts as a transactivator, but lacks the ability to bind DNA directly. The complex of STAT1, STAT2, and IRF9 associates with IFN-alpha-stimulated gene factor 3 (ISGF3G) and enters into the nucleus. ISGF3 binds to the IFN stimulated response element (ISsiRE) to activate the transcription of interferon stimulated genes, which drive the cell in an antiviral state (Bluyssen et al., 1997, Hambleton et al., 2013). Acts as a regulator of mitochondrial fission by modulating the phosphorylation of DNM1L at Ser-616 and Ser-637 which activate and inactivate the GTPase activity of DNM1L respectively (Shahni et al., 2015). Transcription adaptor P300/CBP (EP300/CREBBP) has been shown to interact specifically with STAT2, which is thought to be involved in the process of blocking IFN-alpha response by adenovirus (Mazzon et al., 2009).

Rock bream (*Oplegnathus fasciatus*) aquaculture in Korea has been threatened by Rock bream iridovirus (RBIV), a megalocytivirus widely infected in various fish species. Megalocityvirus is divided into four subgroups by phylogenetic similarities of the major capsid protein (MCP) gene. Subtype I is red sea bream irido-

virus (RSIV) cluster including ehime-1 strain (Inouye et al., 1992); subtype II is rock bream iridovirus (RBIV) (Jung and Oh, 2000); subtype III is infectious spleen and kidney necrosis virus (ISKNV) (He et al., 2000); subtype IV is turbot (*Scophthalmus maximus*) reddish body iridovirus (TRBIV) (Shi et al., 2004).

Major symptoms of RBIV infection is acute inflammation and enlargement of spleen. There is no commercially available vaccine for RBIV though closely related RSIV has been successfully prevented by a commercial vaccine. In our previous study, pro-inflammatory cytokines and IFN1 gene expression was initially up-regulated but later down-regulated by RBIV infection. The viral suppressor of cytokine signaling (SOCS) of megalocytivirus is known to play a key role to inhibit immune response by blocking STAT-JAK pathway. STATs transduce signals in response to a variety of cytokines and growth factors (Najjar and Fagard, 2010) and regulate a number of cellular functions, mainly in the immune system.

In rock bream, STAT2 was cloned and observed the ubiquitous expression of RbSTAT2 transcripts in all tested tissues in healthy fish by quantitative real-time PCR (qPCR), with the highest expression in blood cells (Bathige et al., 2017). Up-regulation of RbSTAT2 gene was found with immunostimulation of bacteria in vivo with antiviral potential confirmed by WST-1 assay in rock bream heart cells treated with RBIV. Subcellular localization studies by transfection of pEGFP-N1/RbSTAT2 into rock bream heart cells revealed that the RbSTAT2 was usually located in the cytoplasm and translocated near to the nucleus upon poly I:C administration. In this study, the expressional study of STAT2 gene was performed in head kidney and spleen upon RBIV infection and immune stimulants like LPS or poly I:C *in vitro* and *in vivo*.

Materials and Methods

1. Fish

Rock bream (50 ± 1.0 g) were obtained from marine fish farms located on the southern coast of Korea. After transfer to the laboratory at Gangneung-Wonju National University (Gangneung, Korea), the fish were kept in 100-ℓ tanks at 25℃ for 3 weeks with daily feeding of a commercial diet for acclimatization.

2. Virus

The virus used in this study was prepared from rock bream embryo cells experimentally infected with megalocytivirus sachun-1 (IVS-1; Genbank Access. No. AF487899) strain prepared by intraperitoneal injection with splenic homogenates (10 mg/fish) from IVS-1-infected rock bream (Jeong et al., 2003). The concentration of virus particles was 10^8 copies/ml estimated by quantitative real-time PCR (Q-PCR) method using the primers qM1F (5'-GGC GAC TAC CTC ATT AAT GT-3') and qM1R (5'-CCA CCA GGT CGT TAA ATG A-3') to amplify major capsid protein (MCP) gene (Jin et al., 2017). Q-PCR was performed using LightCycler 480II (Roche) in a 20 μl reaction volume containing 10 μl 2× LightCycler 480 SYBR Green I Master (Roche), 0.5 pM of each primer and 1 μl of template DNA. The amplification conditions were as follows: 94℃ for 10 min, followed by 40 cycles of 94℃ for 10 sec, 52℃ for 15 sec and 72℃ for 15 sec. As a standard, recombinant plasmid TOPO-TA containing 141 bp of the MCP gene was amplified and purified from transformed *Escherichia coli* (DH-5α). Serial 10-fold dilutions of the control plasmid were used to establish a standard curve (1.0×10^8 to 1.0×10^1 copies/μl). The standard curve was generated using the means of triplicate determinations.

3. Quantitative real-time RT-PCR analysis

Real-time RT-PCR was performed by the method modified from Hong et al. (2013). Briefly, total RNA was isolated using Qiazol (Qiagen) and reverse transcribed to cDNA. For reverse transcription, 3 μg of RNA in 12.5 μl DEPC-water was incubated with 1 μl of oligo(dT)18 primer (100 μM, Thermo) for 5min at 65℃. Then, 1 μl of RevertAid reverse transcriptase (100 U/μl, Thermo), 4 μl of 5×first strand buffer (Thermo) containing 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$ and 50 mM DTT, 1 μl of RiboLock RNase Inhibitor (20 U/μl; Thermo) and 1 μl of 10 mM dinucleoside triphosphate (dNTP) mix (Thermo) were added and the mixture incubated at 42℃ for 1h. The reaction was terminated by heating to 70℃ for 10 min and added 380 μl of TE buffer to make up the final volume to 400 μl.

Gene expression was analysed by Q-PCR using the LC96 real-time thermocycler (Roche). The Q-PCR reaction was performed in a 20 μl reaction containing 10 μl of SYBR Green Real time PCR Master Mix (TaKaRa, Japan), 0.4 mM of each forward and reverse primers and 4 μl of cDNA using the following protocol: 60 s at 95℃; the template was amplified for 40 cycles of denaturation for 15 s at 95℃, annealing and extension for 1 min at 60℃. Q-PCR was performed in duplicate for each sample, along with a serial dilution of references and transcript level was calculated using the integrated software as described previously (Hong et al., 2013).

Gene-specific primers of a forward primer (STAT2Q-F; 5'-GCC-

TATGGATGTCCGTCACT-3') and a reverse primer (STAT2Q-R; 5'-AGAACGACTCCTCCTGGACA-3') were used for Q-PCR. Serially diluted references were used for absolute quantification analysis. After normalization to the expression level of the house keeping gene, i.e. elongation factor (EF)-1α, fold change was calculated by dividing the ratio to EF-1α by negative control sample at each time point. Negative controls were unstimulated samples.

4. STAT2 gene expressions in spleen and head kidney cells *in vitro*

STAT2 gene expression was analysed in head kidney and spleen because the organs are main targets of RBIV and consist of different cell types, playing the different roles in immune response. Head kidney and spleen were aseptically taken from 4 fish and freshly prepared as primary cell cultures (Hong et al., 2013). Briefly, the organs were passed through a sterile mesh (BD, USA) in L15 medium (Sigma) containing penicillin (100 U/ml, Gibco) and streptomycin (100 μg/ml, Gibco). The cells were washed by centrifugation at 2,000 g for 5 min at 4℃ and adjusted to 2×10^6 cells/ml. One ml of the cells was added onto a well of 24 well culture plate, and stimulated with LPS (strain 055:B5, 1 μg/ml; Sigma-Aldrich), poly-I:C (1 μg/ml; Sigma-Aldrich) and different doses of IVS-1 (0.1, 1, 10×10^5 copies/ml) for 4 h, 8 h and 24 h at 25℃. The concentration chosen for LPS and poly I:C was known to be optimal for immune gene expression studies based on previous studies.

The first strand cDNA synthesis and real-time PCR were performed as described above. Real-time PCR quantification of expression was expressed as fold change relative to the time-matched negative controls.

5. STAT2 gene expression in head kidney and spleen after intraperitoneal injection of formalin-killed RBIV or live RBIV *in vivo*

Four healthy rock bream were challenged with intraperitoneal injection of 100 μl IVS-1 (corresponding to 1×10^5 copies/fish) whereas other four fish was injected with 100 μl of PBS as negative group. The amount of virus and challenging time was carefully chosen from our preliminary experiment. Fish was sacrificed 1, 3, 7 days later and taken tissues including the head kidney and spleen. Total RNA was isolated by Qiazol (Qiagen) after homogenising in TissueLyzer (Qiagen) following the manufacturer's instructions. The first strand cDNA synthesis and real-time PCR were performed as described above. The relative gene expression of STAT2 was calculated as fold change relative to the time-matched negative controls after normalised against the expression level of EF-1α.

To confirm the viral infection, genomic DNA was isolated from the head kidney and liver and PCR was performed to amplify MCP gene of IVS-1 using the primers qM1F and qM1R mentioned above in a 20-μl reaction mixture containing 2 μl DNA template (0.1 μg/μl), 10 mM Tris-HCl (pH 9), 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 500 μM each dNTP, 1 pM each primer, and 1 U ExPrime Taq DNA polymerase (Genet Bio, Korea), using a Perkin-Elmer 2400 thermal cycler with the following cycling condition; initial denaturing at 94℃ for 5 min, 30 cycles of denaturing at 94℃ for 30 sec, annealing at 60℃ for 30 sec and extension at 72℃ for 30 sec, and final extension at 72℃ for 5min. The PCR products were visualised under UV light after electrophoresis on a 1.5% agarose gel and staining with ethidium bromide.

6. Statistical analysis

In vitro Q-PCR data were analysed using the SPSS Statistics package 19.0 (SPSS Inc., Chicago, Illinois) as described previously [9]. One way-analysis of variance (ANOVA) and the LSD post hoc test were used to analyse expression data, with $p<0.05$ between treatment and control groups considered significant.

In vivo Q-PCR data were analysed using independent-samples *t*-test to compare the means of infected and non-infected groups on the same tissue, with $p<0.05$ between treatment and control groups considered to be significant. Results are expressed as means ± standard error.

Results

1. STAT2 gene expression in head kidney and spleen cells stimulated by PAMPs

Freshly prepared primary cultures of rock bream head kidney and spleen cells were stimulated with pathogen-associated molecular patterns (PAMPs; LPS and poly I:C) for 4, 8, or 24 h to analyse the STAT2 gene expression (Fig. 1). In head kidney, STAT2 gene expression was significantly up-regulated by LPS at 4 h and 8 h but down-regulated at 24 h. poly I:C up-regulated STAT2 gene expression at 8 h while down-regulated at 4 h and 24 h. In spleen STAT2 gene expression was gradually down-regulated by LPS from 4 h to 24 h but up-regulated by poly I:C from 4 h to 8 h, indicating differential expression of STAT2 gene in different organs

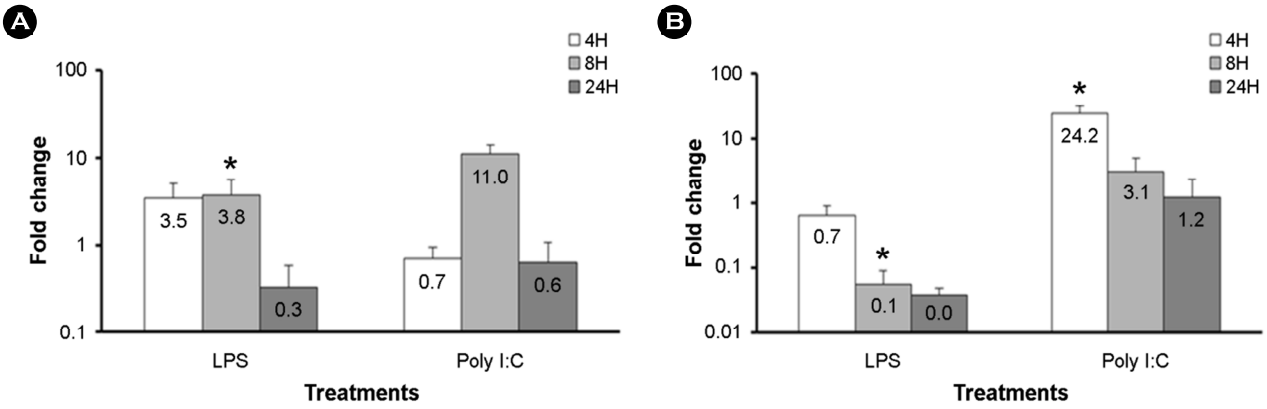

Fig. 1. Expression of STAT2 in head kidney **(A)** and spleen **(B)** cells stimulated with LPS (1 μg/ml), poly I:C (1 μg/ml) for 4, 8, 24 h at 25℃. The qPCR data are normalised relative to the expression of EF-1α and fold change was calculated by dividing the ratio to EF-1α by negative control sample at each time point. Negative controls were unstimulated samples. The results represent the mean ± SEM of STAT2 gene expression from primary cultured cells of 4 fish. The mean fold changes are shown above the bars. The asterisks (*) above the mean value are marked when the *p* values of ANOVA LSD post hoc test between stimulated samples and their time-matched controls are below 0.05.

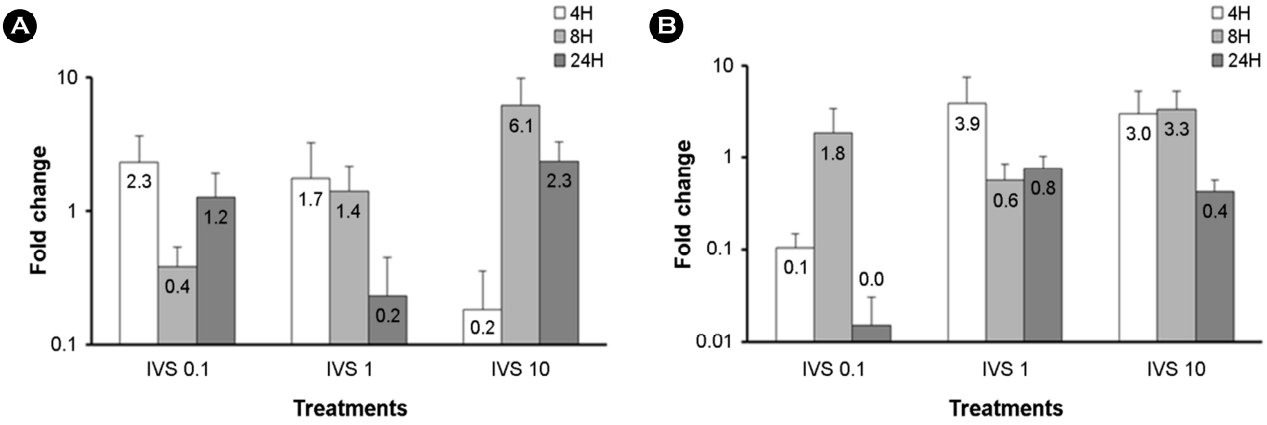

Fig. 2. Expression of STAT2 in head kidney **(A)** and spleen **(B)** cells stimulated with various doses of RBIV (0.1, 1, 10×10^5 copies/ ml) for 4, 8, 24h at 25℃. The qPCR data are normalised relative to the expression of EF-1α and fold change was calculated by dividing the ratio to EF-1α by negative control sample at each time point. Negative controls were unstimulated samples. The results represent the mean ± SEM of STAT2 gene expression from primary cultured cells of 4 fish. The mean fold changes are shown above the bars. The asterisks (*) above the mean value are marked when the *p* values of ANOVA LSD post hoc test between stimulated samples and their time-matched controls are below 0.05.

by different stimulation.

2. STAT2 gene expression in head kidney and spleen cells infected with RBIV

To analyse STAT2 gene expression under RBIV infection, rock bream head kidney and spleen cells were stimulated with various concentrations of RBIV (0.1, 1, 10×10^5 copies/ml) for 4, 8, or 24 h.

In head kidney, STAT2 gene expression was up-regulated by 10×10^5 copies/ml of RBIV at 8 h and then slightly decreased but sustained up-regulation at 24 h (Fig 2). However, in spleen, STAT2 gene expression was up-regulated by 10×10^5 copies/ml of RBIV at 4 h and 8 h but down-regulated at 24 h.

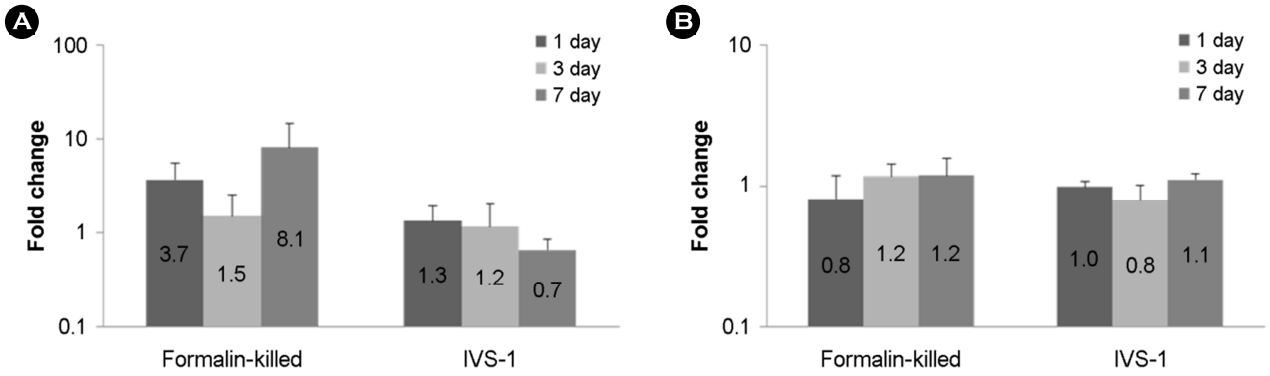

Fig. 3. Expression of STAT2 gene in head kidney **(A)** and spleen **(B)** of rock bream intraperitoneally injected with formalin-killed RBIV (1×10^8 copies/fish) or live RBIV (1×10^3 copies/fish). The data are normalised relative to the expression of EF-1α and fold change was calculated by dividing the ratio to EF-1α by negative control fish injected with PBS at each time point. The results represent the mean ± SEM of 8 fish. The asterisks (*) above the mean value are marked when the *p* values of ANOVA LSD post hoc test between stimulated samples and their time-matched controls are below 0.05.

3. STAT1 and 3 gene expression in head kidney and spleen injected with formalin-killed RBIV or infected with RBIV *in vivo*

To analyse STAT2 gene expression by vaccination or infection with RBIV in major immune organs, its expression was analysed at 1, 3 and 7 days after intraperitoneal injection of formalin-killed RBIV or RBIV (Fig 3). As a result, it was found that STAT2 gene expression was up-regulated only in spleen without any significant change in head kidney until day 7 post-vaccination. Meanwhile, there was no significant change found in both tissues after infection with RBIV (1×10^5 copies/fish) from day 1 to day 7 post-infection but there was a slight decrease observed in spleen at day 7 post-infection.

Discussion

In this study, it was analysed the gene expressions of transcription factor, STAT2, which may be involved in immune regulation during RBIV infection in rock bream. We have also STAT2 gene expression induced by PAMPs like LPS and poly I:C. We have found that STAT2 gene expression was differentially altered by LPS and poly I:C in the two major immune organs i.e., head kidney and spleen. This result shows that STAT2 is regulated by immune stimulant which is derived from bacteria or mimic viruses at transcriptional level. As a STAT family member responding to immune stimulants, up-regulated expression of STAT2 gene will produce STAT2 proteins at a higher level than before stimulation, regulating immune responses in combination with STAT1 and IRF9. STAT2 gene expression was different in spleen by LPS and poly I:C as showing significant down-regulation at 8 h post-stimulation while there was significant up-regulation by poly I:C at 4 h. It can be postulated that there might be a different immune response by LPS and poly I:C in spleen as LPS is a T cell independent antigen which can induce B cell response while poly I:C will induce antiviral response. Since STAT2 is involved in antiviral response which mediates signaling by type I IFNs (IFN-alpha and IFN-beta). The complex of STAT1, STAT2, and IRF9 form ISGF3 transcription factor, enter the nucleus and bind to the ISRE, activating the transcription of interferon stimulated genes and driving in an antiviral state (Bluyssen et al., 1997, Hambleton et al., 2013).

Upon RBIV infection *in vitro*, STAT gene expression was up-regulated or down-regulated at different time point in head kidney and spleen cells but there was no statistical significance. In a previous study, measles virus and lymphocytic choriomeningitis virus interfere with dendritic cell development through the generation of type I IFN that acts via STAT2-dependent, but STAT1-independent, pathway, indicating that viruses subvert the antiviral effect of type I IFN through STAT2-specific signalling for their survival (Hahm et al., 2005).

In vivo experiment, STAT2 gene expression was not significantly affected by RBIV infection while it was significant up-regulated by vaccination at day7 post-vaccination, indicating STAT2 gene can be involved in adaptive immune response in rock bream. There are evidences that adaptive immune cells like plasmacytoid dendritic cell (pDC) produces a high level of type I IFN responding

to viral exposure through TLR7/9 signalling (Boasso, 2013). After maturation into APC, pDC may promote efficient antiviral primary T cell responses by inducing CD4 T cell differentiation into Th1 cells and the activation and clonal expansion of virus-specific naive CD8 T cells (Boasso, 2013).

Acknowledgement

This work was supported by 2016 Academic Research Support Program in Gangneung-Wonju National University.

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