Molecular Immunology 63 (2015) 227–234

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

ISA virus regulates the generation of reactive oxygen species and p47phox expression in a p38 MAPK-dependent manner in *Salmo salar*

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ARTICLE INFO

Article history:
Received 23 April 2014
Received in revised form 1 July 2014
Accepted 5 July 2014
Available online 12 August 2014

Keywords:
ISA virus
MAPK
ROS production
Innate immunity

ABSTRACT

Several viruses, including Orthomyxovirus, utilize cellular reactive oxygen species (ROS) for viral genomic replication and survival within host cells. However, the role of ROS in early events of viral entry and signal induction has not been elucidated. Here, we show that ISA virus (ISAV) induces ROS production very early during infection of CHSE-214 and SHK-1ϒcells, and that production is sustained over the observed 24 h post-infection. The mitogen-activated protein kinase (MAPK) family is responsible for important signaling pathways. In this study, we report that ISAV activates ERK and p38 in *Salmo salar*. In salmonid macrophages, while ERK was required for SOD, GLURED, p47phox expression, p38 regulated the ROS production by the NADPH oxidase complex activation. These results, together with the presence of several consensus target motifs for p38 MAPK in the promoter of the *S. salar* p47phox gene, suggest that p38 MAPK regulates p47phox gene expression in fish through the activation of this key transcription factor.

1. Introduction

Phagocytic cells generate reactive oxygen species (ROS) as a mechanism to eliminate invading pathogens. Oxygen-containing free radicals such as superoxide (O^{2-}), the hydroxyl radical (OH), and hydrogen peroxide (H_2O_2), are highly reactive molecules due to the presence of unpaired valence shell electrons. Additionally, these molecules mediate important roles in cell signaling, homeostasis, and immunity. The imbalance between the generation of ROS and the cell's ability to detoxify these same mediators produces a state known as oxidative stress (Valko et al., 2007).

The infectious salmon anemia virus (ISAV) is an aquatic Orthomyxovirus (genus Isavirus) that causes a multisystemic disease characterized by high mortality with pale gills, severe anemia, decreased hematocrit value and severe hemorrhagic necrosis of internal organs (Kibenge et al., 2004; Rimstad and Mjaaland, 2002). Several ISAV isolates with different virulence properties and pathogenicity *in vivo* are described as acute or protracted variants (Kibenge et al., 2006). However, *in vitro* correlation between replication properties of different ISAV isolates and the development of cytopathic effect (CPE) is less clear (Mjaaland et al., 2002). Therefore, *in vitro* studies are important for understanding mechanisms of pathogenesis and resistance to ISAV at the cellular level, particularly on oxidative stress caused by enhanced production of ROS and reduced capacity for their neutralization in virus infected cells (Hasnain et al., 2003). Recently, it was shown that during ISAV infection, several genes that are directly or indirectly involved in metabolism of ROS were down-regulated and there was a reduced level of glutathione as well (Schiøtz et al., 2008). ROS are generated by the activity of the NADPH oxidase family of enzymes in the membrane of phagocytic cells. These NADPH oxidases generate ROS by carrying electrons across membranes from NADPH in the cytosol to an electron acceptor (i.e., oxygen) in the extracellular space or phagosome (Bedard and Krause, 2007). The NADPH activation occurs after the phosphorylation of p47phox by different types of protein kinases, such as PKC α (Olavarría et al., 2010) and MAPK, among others. Particularly, mitogen-activated protein kinase (MAPK) pathways are central to the control of cellular responses including host defense, inflammatory responses and cell death. In fact, MAPKs can be activated by a wide variety of different stimuli, but in general, ERK1 and ERK2 are preferentially activated in response to growth factors and phorbol esters, while p38 kinase is more responsive to stress stimuli ranging from osmotic shock and ionizing radiation to cytokine stimulation (Pearson et al., 2001). Once activated, MAPKs phosphorylate target substrates on serine or threonine

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http://dx.doi.org/10.1016/j.molimm.2014.07.016
0161-5890/

residues however; substrate selectivity is often conferred by specific interaction motifs located on physiological substrates. MAPKs catalyze the phosphorylation and activation of several protein kinases, termed MAPK-activated protein kinases (MKs), which represent an additional enzymatic and amplification step in the MAPK catalytic cascades: 90-kDa ribosomal S6 kinases (RSKs), the mitogen- and stress-activated kinases (MSKs), and MAPK-activated protein kinase 5 (MK5, formally termed MAPKAP-K5). The MKs are related kinases that mediate a wide range of biological functions in response to mitogens and stress stimuli. Interestingly, H9N2 avian influenza virus (Orthomyxovirus) activates ERK and p38 in avian species; specifically, ERK was required for to suppress TNF-α and FasL and inhibited TNF-family-mediated extrinsic apoptosis in H9N2-infected chicken macrophages. Similarly, infection with an influenza virus leads to activation of a variety of intracellular signaling pathways including different MAP-kinase (MAPK) cascades (Ludwig et al., 2003, 2006). In this regard, Luig and colleagues recently demonstrated that influenza A viruses are able to activate both members of the MAPKAP-kinase family, MK2 and MK3, and that this activation is beneficial to the formation of mature virus particles, because it involves inhibition of the dsRNA-activated antiviral protein kinase, PKR. This information strongly suggests that MAPK signaling may play an important role in the host response to infection by orthomyxovirus and specifically ISAV.

In the present study, we examined the effect of the activation of NADPH oxidase on ISAV-induced intracellular signal transduction pathways, as well expression of genes involved in cellular responses to oxidative stress during the 24 h post-infection.

2. Materials and methods

2.1. Animals

Specimens (30 g mean weight) of Atlantic salmon (*Salmo salar*) were obtained from Novartis Puerto Varas, Chile. The fish were certified to be free of infectious salmon anemia virus and *Piscirickettsia salmonis*. Tissue samples were directly collected in the fish farm and immersed in TRIzol reagent (Invitrogen) for further analysis.

2.2. Cell culture

SHK-1 is a cell line from *S. salar* head kidney, which exhibits macrophage properties. The cells were cultured at 18 °C in 75 cm^2 tissue-culture-treated flasks (Costar), in L-15 medium (500 ml with 300 mg/l L-glutamine) supplemented with 500 μl gentamicin sulfate (50 mg/ml in distilled water), 365 μl 2-mercaptoethanol (55 mM in Dulbeco's phosphate buffered saline) and 5% fetal bovine serum (FBS). All media components were purchased from Gibco. Confluent flasks were subcultured weekly by dividing cells and medium evenly between two flasks and adding an equal volume of new media to each flask. The cells used in this study were between 55 and 60 passages.

2.3. Viral propagation

A CHSE-214 cell line was prepared in MEM growth medium at a concentration of 4×10^8 cells/l and inoculated with ISAv designated ISAv-Austral D08I1055-1 (21) at a concentration of 10^6 50% tissue culture infective doses (TCID50)/L at a ratio of 1 ml of virus per 50 ml of cell suspension. Inoculated cells were seeded into a 175-cm^2 tissue culture flask and incubated for 24 h. ISAv- infected CHSE-214 cells were then frozen/thawed twice, harvested and pelleted by centrifugation at $3000 \times g$ for 10 min. Supernatant containing the propagated virus was titrated and utilized in the viral experiments set. The supernatant from uninfected PK-15 cells served as a sham control. The presence or absence of ISAV in the virus preparation was confirmed by IFA and PCR before the challenge. The virus used in this study represented 12 *in vitro* passages in cell culture.

2.4. Cell culture and treatments

S. salar head kidney (bone marrow equivalent in fish) leukocytes, obtained as described elsewhere, were maintained in sRPMI (RPMI 1640 culture medium (Invitrogen) adjusted to serum osmolarity (353.33 mOsm) with 0.35% NaCl) and supplemented with 5% fetal calf serum (FCS, Invitrogen), 100 IU/ml penicillin and 100 lg/ml streptomycin (P/S; Biochrom). Leukocytes from *S. salar* head kidney were incubated for 1, 4, 8, 16 and 24 h at 15 °C with ISAV in RPMI 1640 culture medium supplemented with 5% FCS and P/S (Olavarría et al., 2010). In some experiments, cells were pretreated for 1 h with 10 μM SB203580 (p38 MAPK inhibitor), 10 μM apocynin (NADPH oxidase inhibitor) or 10 μM PD98059 (ERK 1/2 inhibitor) (all from Sigma–Aldrich). None of the treatments used in this study affected cell viability, as determined by trypan-blue exclusion and light microscopy. In all the experiments, cell viability was >95% at the end of the incubation period.

2.5. Respiratory burst assays

Respiratory burst activity was measured as the luminol-dependent chemiluminescence produced by *S. salar* head kidney leukocytes. This was brought about by adding 100 μM luminol (Sigma–Aldrich) and one l μg/ml PMA (Sigma–Aldrich), while the chemiluminescence was recorded every 117 s for 1 h in a FLUOstart luminometer (BMG Labtechnologies). The values reported are the average of quadruple readings, expressed as the slope of the reaction curve from 117 to 1170 s, from which the apparatus background was subtracted.

2.6. Analysis of gene expression

To quantify virus production, RNA was isolated from serial dilutions of 1 ml supernatants containing known amounts of ISAV (by titration and determination of infective dose, TCID50/ml) and cDNA produced as described. Total RNA was extracted from tissues or cell pellets with TRIzol reagent (Invitrogen), following the manufacturer's instructions, and treated with DNase I, Amplification grade (1 U/mg RNA, Invitrogen). The SuperScript III RNase H Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNAwith oligo-dT18 primer from 1 mg total RNA at 50 °C for 50 min. Real-time PCR was performed in an ABI PRISM 7500 instrument, using SYBR Green PCR Core Reagents (both from Applied Biosystems, Foster City, CA). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. For each mRNA, gene expression was normalized to the ribosomal protein S18 (rps18) content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$).

The primers used were p47phox (FJ594435.1): forward 5′-GAGGAGCCTGAAGAAGCTGA-3′ and reverse 5′-TCCAGCAGCTTGTGAATGAC-3′; SOD (BT046917.1): forward 5′-GGAGACAACGAGGAGAGTCG-3′ and reverse 5′-GGTAGAGTTCGGGGGTAAGC-3′; NRF-2 (BT059007.1) forward 5′-TTCCCATTGGTAGAGGCAAC-3′ and reverse 5′-CAGCTCAGGAAGGGACAAAG-3′; GLURED (BT045539.1) forward 5′-TTCCCACGGTAGTTTTCAGC-3′ and reverse 5′-TGCACTGAGTCTTCCTGGTG-3′ and BETA ACTIN (NM_001123525.1) forward 5′- ACTGGGACGACATGGAGAAG-3′ and reverse 5′-GGGGTGTTGAAGGTCTCAAA-3′ and S11 (NM 001141447) forward 5′-A CAGAAATGCCCCTTCACTG-3′ and reverse 5′-GCCTCTTCTCAAAACGGTTG-3′.