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CpG- and LPS-activated MAPK signaling in *in vitro* cultured salmon (*Salmo salar*) mononuclear phagocytes

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ABSTRACT

The Mitogen-activated protein kinases (MAPK) are involved in transmitting intracellular signals downstream of diverse cell surface receptors and mediate the response to ligands such as growth factors, hormones and cytokines. In addition, MAPK are critically involved in the innate immune response to pathogen-derived substances, commonly referred to as pathogen-associated molecular patterns (PAMPs), such as bacterial lipopolysaccharide (LPS) and bacterial DNA rich in CpG dinucleotides. Currently, a great deal of knowledge is available about the involvement of MAPK in the innate immune response to PAMPs in mammals; however, little is known about the role of the different MAPK classes in the immune response to PAMPs in lower vertebrates. In the current study, p38 phosphorylation was induced by CpG oligonucleotides (ODNs) and LPS in primary salmon mononuclear phagocytes. Pre-treatment of the cells with a p38 inhibitor (SB203580) blocked the PAMP-induced p38 activity and suppressed the upregulation of most of the CpG- and LPS-induced transcripts highlighting the role of this kinase in the salmon innate immune response to PAMPs. In contrast to p38, the phosphorylation of extracellular signal-regulated kinase (ERK), a MAPK involved primarily in response to mitogens, was high in resting cells and, surprisingly, incubation with both CpG and control ODNs downregulated the phospho-ERK levels independently of p38 activation. The basal phospho-ERK level and the CpG-inducible p38 phosphorylation were greatly influenced by the length of *in vitro* incubation. The basal phospho-ERK level increased gradually throughout a 5-day culture period and was PI3K-dependent as demonstrated by its sensitivity to Wortmannin suggesting it is influenced by growth factors. Overall these data indicate that both basal and PAMP-induced activity of MAPKs might be greatly influenced by the differentiation status of salmon mononuclear phagocytes.

1. Introduction

Ligands for innate immune receptors such as the toll-like receptors (TLRs) activate leukocytes by triggering complex intracellular signaling cascades. In mammals, it has been shown that TLR ligands, such as LPS and DNA rich in unmethylated CpG dinucleotides activate all major groups of MAPK in leukocytes, including monocytes, macrophages and dendritic cells (DCs) [2–5]. The major groups of MAPK include the extracellular signal-regulated kinase (ERK), the c-Jun NH terminal kinase (JNK), and the p38 MAP kinase. Upon activation, MAPK are phosphorylated on the threonine (Thr) and tyrosine (Tyr) residues at their Thr–Xaa–Tyr (TXY) motif found in a highly conserved activation loop near the active site. The ERK signaling is activated mainly by mitogenic stimuli such as growth factors [4]. On the other hand, JNK and p38 pathways are activated by stress stimuli such as UV irradiation and osmotic shock, and by proinflammatory cytokines [6].

The MAPK proteins and functions are well conserved across vertebrates and it has been demonstrated that in lower vertebrates, TLR ligands such as LPS and CpG ODNs, activate the innate immune

Abbreviations: MAPK, mitogen-activated protein kinase; PAMPs, pathogen-associated molecular patterns; TLRs, the toll-like receptors; LPS, lipopolysaccharide; JNK, c-Jun NH terminal kinase; ERK, extracellular signal-regulated kinase; ODNs, oligodeoxynucleotides; IL-1β, interleukin-1β; MKK, MAPK kinase; MK2, MAPK-activated protein kinase2; DCs, dendritic cells; NFkB, nuclear factor kappaB; NRF2, nuclear factor (erythroid-derived 2)-like 2; PI3K, phosphatidylinositol 3-kinase; WB, Western blot; M-CSF, macrophage colony stimulating factor; HRP, horseradish peroxidase; eEF2, eukaryotic elongation factor 2; FBS, fetal bovine serum.

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response through MAPK. More specifically, LPS, CpG ODNs and recombinant trout interleukin-1β (IL-1β)-induced phosphorylation of endogenous p38 in salmon head kidney macrophages in a dose-dependent manner [7]. In addition, in whole salmon head kidney leukocyte cultures, p38 activation was shown to be crucial for LPS-induced upregulation of immune genes including IL1-β, TNF, COX2 and, to a lesser extent, CD83 [8].

A major goal of the current study has been to further investigate the involvement of p38 in the response of salmon mononuclear phagocytes to LPS and CpG ODNs. Cultures of primary cells were stimulated with *Escherichia coli* LPS and CpG ODNs alone or in the presence of a chemical inhibitor of p38 (SB203580). This inhibitor has been widely used in mammalian systems [9,10], however, data about its efficacy and specificity in lower vertebrates is scarce. In the current study, SB203580 was able to completely abolish the CpG- and LPS-induced p38 activity as determined by analysis of MAPK-activated protein kinase2 (MK2, a direct target of p38) phosphorylation. A microarray analysis demonstrated that the upregulation of most of the CpG and LPS-induced genes was p38-dependent, highlighting the importance of the p38 kinase for the innate immune response of salmon leukocytes against pathogens. Additional data indicate that the MAPK activation by PAMPs is affected by a PI3K-dependent mechanism and the differentiation of the cells.

2. Materials and methods

2.1. Fish and reagents

Non-vaccinated healthy Atlantic salmon, *Salmo salar* L., strain Aquagen standard (Aquagen, Kyrksæterøra, Norway), 500–1000 g, was obtained from Tromsø Aquaculture Research Station (Tromsø, Norway). The fish were kept at 10 °C in tanks supplied with running filtered sea water and were fed on commercial dry food. All experiments were performed according to the guidelines from the national committee for animal experimentation (Forsøksdyrutvalget, Norway).

The phosphorothioate-modified ODNs were purchased from Thermo Scientific. The sequences are as follows, where phosphorothioate modifications are marked with *: CpG-B: T*C*G*T*C*G*T*T*T*T*G*T*C*G*T*T*T*T*G*T*C*G*T*T; CpG-A: G*G*G*G*G*A*C*G*A*T*C*G*T*C*G*G*G*G*G*G. In the control, non-stimulatory ODN (GpC) the places of the cytosines and guanines in the CpG-B sequence are switched. LPS from *E. coli* 0111:B4 was obtained from Sigma Aldrich (cat.# L2630). The p38 inhibitor (SB203580) was purchased from Alexis Biochemicals. Wortmannin was purchased from Invivogen. Rabbit antibodies against phosphorylated p38 (p-p38; cat. # 9211), p-MKK3/6 (cat. # 9231), p-MK2 (cat. # 3041), p-ERK (cat. # 9101), p-Akt (cat. # 4060) and eukaryotic elongation factor 2 (eEF2; cat. # 2332) were obtained from Cell Signaling Technology. The secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG-HRP was purchased from Santa Cruz Biotechnology (cat. # sc-2004)

2.2. Isolation and stimulation of primary mononuclear phagocytes from Atlantic salmon

Head kidney (HK) leukocytes were isolated as described previously [11]. The organs were passed through a 100-mm pore size cell strainer (Falcon) in L-15 medium containing penicillin (60 mg/ml), streptomycin (100 mg/ml), 2% fetal bovine serum (FBS) and heparin (20 U/ml). The resulting suspension was placed on a 25/54% discontinuous Percoll gradient and centrifuged at 400 g for 40 min at 4 °C. The cells at the interface were collected and washed twice in L-15 medium. The cells were seeded in 24-well plates at a density of 7×10^6 cells per well. After 1 h of incubation in L-15, supplemented with 0.1% FBS at 14 °C, the cells were washed by vigorous pipetting with fresh L15 medium and the adherent mononuclear phagocytes were further incubated in L-15 supplemented with 5% FBS. The salmon head kidney leukocyte preparations from different fish may contain varying numbers of adherent mononuclear phagocytes. Under the experimental conditions used in the current study the percentage of confluent mononuclear phagocytes isolated from different individuals varied between 30 and 80%. Only cells from individuals giving 50–60% confluency were selected for further experiments. In the different experiments the cells were stimulated immediately after washing or after 1, 3 and 5 days as indicated in the Results section and in the figure legends. For the Western blot analysis (WB), the cells were lysed with 50 μl of NuPAGE LDS sample buffer, sonicated and heated for 10 min at 70 °C. For RT-PCR microarray analyses, the cells were lysed with TRIzol Reagent (Invitrogen).

2.3. Western blot analysis

Cell lysates were separated by SDS ⁄PAGE (4–12% precast NuPAGE; Invitrogen), followed by transfer to a 0.45 μm pore size polyvinylidene difluoride membrane (Millipore). The blots were pre-blocked for 1 h (Tris-buffered saline, 5% BSA, 0.1% Tween-20) and incubated for 24 h with 1:1000 dilution of the primary antibodies followed by 1 h incubation with 1:10,000 dilution of the secondary antibody in blocking solution. Detection was performed with Super-Signal West Pico substrate (Pierce Biotechnology). The membranes were stripped for 10 min in 0.2 M NaOH followed by washing, blocking and antibody incubation. The size of the proteins was estimated using the MagicMark Western protein standard (Invitrogen).

2.4. Real-time PCR

The RNA isolation and the cDNA synthesis were performed with RNeasy Mini Kit (Qiagen) and TaqMan Reverse Transcription Reagents kit (Applied Biosystems) as previously described [12]. The Real-time PCR reactions were assembled using Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences, the reaction conditions and the data analysis have previously been described [13]. Statistical analysis was carried out using the Student *t* test. The value of $p < 0.05$ was considered to be significant.

2.5. Microarray analysis

The cDNA microarray containing 1800 unique clones, the reagents and the procedures have been previously described [13].

3. Results

3.1. Time course of the MK2, p38, ERK and MKK3/6 phosphorylation in CpG and LPS-stimulated mononuclear phagocytes; the p38 inhibitor effectively inhibits the CpG- and LPS-induced MK2 phosphorylation

In order to analyze the efficacy of the p38 inhibitor, the cells were stimulated alone or in the presence of the inhibitor for 0.5, 2 and 4 h prior to assessment of the phosphorylation of MK2 which is a target of p38 (Fig. 1). The CpG-B and the LPS-induced MK2 phosphorylation were completely suppressed in the presence of the p38 inhibitor at all of the tested time points. These data also showed that the LPS stimulation induced a faster response and there was a good correlation between the levels of phospho-p38