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Proinflammatory Effects of Bacterial Lipopolysaccharide (LPS) in Rainbow Trout (*Oncorhynchus mykiss*) Macrophage Cells

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Proinflammatory effects of bacterial lipopolysaccharide (LPS) have been assessed by analysing the induction of two inflammatory genes, interleukin-1β (IL-1β) and cyclooxygenase-2 (COX-2), in rainbow trout (*Oncorhynchus mykiss*) macrophage cells. Production of a metabolite of arachidonic acid by COX-2, prostaglandin E_2 (PGE_2), was also analysed in macrophage cells after LPS stimulation. Northern blot analysis revealed that LPS (5 μg/mL) significantly upregulated IL-1β (54 times) and COX-2 (40.7 times) gene expression in macrophage cells after 4 h stimulation. According to RT-PCR (Reverse Transcription Polymerase Chain Reaction) analysis, IL-1β gene induction in LPS stimulated macrophage cells was started within 1 h and significantly increased thereafter until 4 h. Meanwhile, COX-2 gene induction by LPS was delayed in comparison with IL-1β gene induction as a faint band was observed after 4 h stimulation in head kidney macrophage cells. LPS also significantly increased PGE_2 production in head kidney leucocytes, presumably via activating COX-2 expression that metabolites arachidonic acid to PGE_2. In conclusion, it was demonstrated that LPS could induce two main inflammatory and immune related genes, IL-1β and COX-2, and increase PGE_2 production in trout head kidney macrophage cells, representing a strong inflammatory activity.

Key words: Lipopolysaccharide, Macrophage, Interleukin-1β, Cyclooxygenase-2, Prostaglandin E_2, *Oncorhynchus mykiss*

Introduction

Macrophage cells are an ancient defence against infectious pathogens and are widely distributed throughout the bodies of all multicellular animals (Bayne, 1990). In addition to ingesting microbes they can secrete a wide range of biologically active molecules such as hydrolases, complement components, enzyme inhibitors, binding proteins, cytokines (e.g. interleukin-1, tumor necrosis factor a), eicosanoids (e.g. prostagladins and leukotrienes) and reactive oxygen and nitrogen species (ROS and RNS) which are involved in inflammatory response and ultimately bactericidal activity. They also possess numerous ligands for immunologically relevant molecules, including immunoglobulins, cytokines and lipoprotein, and they can present processed antigens, in association with class II MHC molecules, to helper T cells (Auger and Ross, 1992). Many of the functions carried out by macrophages, including the destruction of potential pathogens, are enhanced through activation of the cells, induced by a variety of host and pathogen-derived products including LPS (Auger and Ross, 1992).

LPS is an endotoxic component of the outer membrane in gram-negative bacteria, consisting of three covalently linked regions: lipid-A-coreoligosaccharide-O-specific chain and known to induce strong inflammatory and immuno stimulatory responses. According to Sergey et al. (1998), LPS is a potent inducer of endogenous IL-1, a proinflammatory cytokine, and the effects induced by LPS are similar to those observed following IL-1β administration. Indeed, LPS-induces effects can be blocked or ameliorated by the IL-1 receptor antagonist and IL-1β-deficient mice exhibit lower fever following LPS administration.

In the present study, proinflammatory effects of LPS have been assessed by analysing the induction

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of related-genes, and PGE_2 in rainbow trout (*Oncorhynchus mykiss*) macrophage cells. The expression of IL-1β and COX-2 genes, which are important in the regulation of inflammatory and immunological events, has been investigated. Production of PGE_2, a metabolite of arachidonic acid by COX-2, was also analysed in macrophage cells after LPS stimulation.

Materials and Methods

Isolation of head kidney macrophage cells

From anaesthetized rainbow trout (*O. mykiss*) head kidney was aseptically taken and washed several times with L15 medium (Leibovitz, Gibco) to remove blood. Leucocytes enriched total head kidney cells were prepared by pushing head kidney through a 100 μm nylon mesh in the presence of L15 containing 2% FCS (Fetal Calf Serum) and 0.1% heparin, then layered onto 51% percoll gradient and centrifuged at 1600 rpm for 30 min at 4℃. Cells at the interface were collected, washed twice with L15 medium containing 0.1% FCS and 0.1% heparin, and then adjusted to 0.8×10^7 cells/mL in L15 medium containing 10% FCS. Attached macrophages were obtained by washing out unattached leucocytes after overnight incubation at 18℃. For the gene induction experiment, 3 mL of L15 containing 2% FCS was added into macrophages, and incubated with or without LPS (*Escherichia coli* 0127:B8 saccharide, Sigma) at 18℃ for 0, 1 or 4 h. IL-1β and COX-2 gene expression following to LPS stimulation was analysed by RT-PCR. Macrophage cells were also incubated with or without LPS at 18℃ for 4 h and IL-1β and COX-2 gene expression was observed by Northern blot analysis.

RTG-2 cell line culture

RTG-2 cells, gonad fibroblast cell line, were also cultured until confluent 80% at 18℃ in a 25 cm^2 flask containing 15 mL of L15 plus 10% FBS and antibiotics. RTG-2 cells were incubated with or without LPS at 18℃ for 0, 1 or 4 h and IL-1β and COX-2 gene expression was analysed by RT-PCR.

Preparation of total RNA

Total RNA was extracted by using RNAzol B (Biogenesis, CS-104) as followed by manufacturer's instruction. Briefly, the cell lysates were prepared by repeated pipetting in RNAzol B, extracted by one tenth volume of chloroform, then precipitated with equal volume of chilled isopropanol and washed by 75% chilled ethanol. The RNA pellet was resuspended in DEPC-treated water and stored at -70℃ until analysed by Northern blot or RT-PCR.

Northern blot analysis

IL-1β and COX-2 gene expression in macrophage cells following to LPS addition was analysed by Northern blot. For Northern blot analysis, 7-10 μg of total RNA/lane was size-fractionated by electrophoresis through a 1.1% formaldehyde agarose gel and blotted onto a nylon membrane (Amersham Hybond-N+) by vacuum pump. Two membranes were prepared for the detection of IL-1β and COX-2 genes and sequentially hybridized with the ^{32}P labeled cDNA probes at 65℃ for 4 h. Each membrane was first hybridised with the rainbow trout IL-1β or COX-2 gene probe, then rehybridized with β-actin. The rainbow trout IL-1β, COX-2 and β-actin cDNA probes used in these experiments were prepared by the random primer labelling of fragments from the RT-PCR. The β-actin mRNA expression level, which is constitutively expressed in the rainbow trout macrophages, was used as a control.

RT-PCR analysis

Cell specific effect of LPS on IL-1β and COX-2 gene expression was observed by RT-PCR analysis in the rainbow trout macrophage cells and RTG-2 cells. The primers were designed (IL-1β: 5'-CTGGAGAGTGCTGTGGAA-3' and 5'-TTCAGCAGTACATCACAGTAT GAG-3' and COX-2: 5'-CTTACTCACTACAAAGGG-3' and 5'-GACTTCCATGAAAGGACCAGCT-3') based on the nucleotide sequences of the IL-1β and COX-2 gene of rainbow trout (GenBank Accession numbers: AJ278242 and AJ238307, respectively). The reaction was run for 20 cycles and the cycling conditions were 95℃ for 45 sec, 58℃ for 45 sec and 72℃ for 45 sec, with the denaturation step prolonged to 5 min in the first cycle and the DNA synthesis step to 10 min in the last cycle. For each amplification procedure, a negative control was run using sterile water in place of cDNA template, to ensure that any amplified products generated from cDNA samples were not a result of any contamination i.e. DNA from pipettes or air. The PCR products were resolved on a 2% (W/V) agarose gel containing 0.1 μg/mL ethidium bromide. The expected sizes of the products for IL-1β and COX-2 genes were 99 and 397 bp, respectively.

PGE_2 assay

Head kidney macrophage cells were incubated in L15 medium with or without LPS at 18℃ for 48 h at a density of 5×10^5 cells mL^{-1} in 96 well culture plates (Nunclon). Cell culture supernatants are collected and stored at -80℃. The levels of PGE_2 were determined by EIA (Enzyme ImmunoAssay) Kit (Amersham) as followed by the manufacturer's instruction.

Results and Discussion

In the present study, IL-1β and COX-2 gene induction was investigated, since they are known to be main inflammatory genes in mammals (Dinarello, 1984). It was revealed in Northern blot analysis that LPS (5 μg/mL) significantly enhanced IL-1β (54 times compared to control) and COX-2 (40 times compared to control) gene expression in macrophage cells after 4 h stimulation (Fig. 1). It is known in mammals that transcription of the IL-1 genes can be initiated by LPS, phorbol esters, calcium ionophores, exposure to UV light, complement components, autoreactive T-cells, and adhesion of cells to a surface or the cross-linking of cellular adhesion molecules (Fenton, 1992). In fish, previous

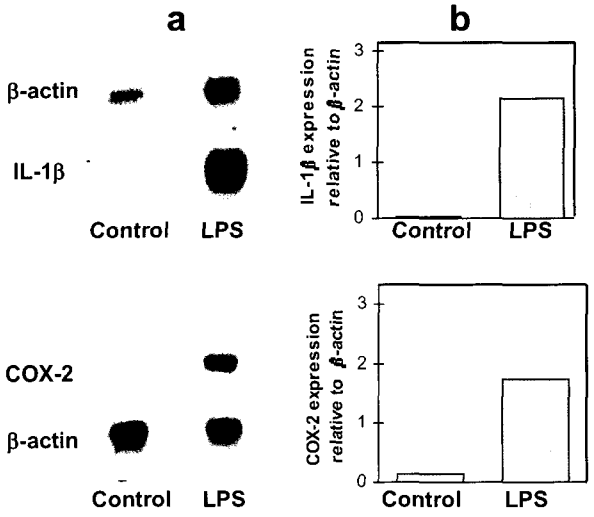

Fig. 1. Effect of LPS on COX-2 and IL-1β gene expressions in macrophage cells. a. Northern blot analysis. Rainbow trout macrophage cells were stimulated without (lane 1) or with 5 μg/mL of LPS (lane 2) for 4 h prior to RNA extraction. 10 μg RNA was loaded per lane and the blot hybridized with ^{32}P labelled IL-1β and COX-2 cDNA probes, then rehybridized with a β-actin probe. b. The relative levels of IL-1β and COX-2 mRNA were quantified by densitometric scanning of exposed film and expressed as a ratio relative to the values obtained for β-actin expression with the same samples.

studies looking at factors affecting the expression of these genes have focused on bacterial and mitogen induced expression. For example, the tissue distribution of IL-1β expression in bacterially challenged fish has been demonstrated by Zou et al. (1999) by RT-PCR analysis in blood, gill, liver, kidney and spleen. Expression of the COX-2 gene is also known to be exquisitely sensitive to LPS in mammals (Dinarello, 1984). COX-2 produces prostanoids, particularly PGE_2, that mediate many IL-1β-induced changes including ACTH (Adreno Cortico Tropic Hormone) release (Komaki et al., 1992). As in mammals, the increased generation of prostanoid seen after exposure of fish macrophages to proinflammatory signals, including LPS and leucocyte-derived activating factors (LAF) has been attributed to the induction of COX-2 (Holland, 1997). Therefore, the induction of this gene could be a good indicator for inflammatory activity in the rainbow trout, followed by the observation of PGE_2 production induced by LPS.

To study kinetics of IL-1β and COX-2 gene expression and tissue specific induction of these genes after LPS stimulation, RTG-2 cell line and head kidney macrophage cells were stimulated with 10 μg of LPS for 0, 1, and 4 h prior to RNA extraction and IL-1β and COX-2 gene expressions were examined by RT-PCR. The results from RT-PCR analysis revealed that IL-1β gene induction in macrophage cells was started within 1 h and significantly increased thereafter until 4 h. This is in agreement with Fenton et al. (1988) and Jarrous and Kaempfer (1994) who demonstrated in mammals that IL-1β mRNA levels rapidly raise within 15 min but started to fall after 4 h depending on the stimulant.

Meanwhile, COX-2 gene induction by LPS was delayed in comparison with IL-1β gene induction since no band was seen at 1 h and a faint band was only observed after 4 h stimulation in head kidney macrophage cells (Fig. 2). It was proposed that COX-2 gene expression might be induced via IL-1β gene expression in trout macrophage cells as in mammals. Diaz et al. (1998) have also reported that IL-1β and TNF-α can induce PGE_2 production by transcriptional stimulation of the COX-2 gene in human fibroblasts. It was also demonstrated that phytohemagglutinin (PHA) is a stronger inducer for COX-2 gene expression compared with LPS. As seen in Fig. 2,

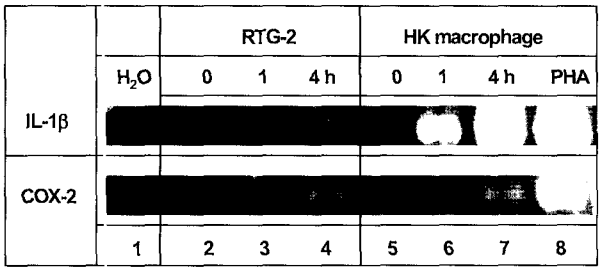

Fig. 2. Effect of LPS on IL-1β and COX-2 gene expressions in RTG-2 cells and head kidney macrophage cells. 1. water; 2. RTG-2 cells before stimulation; 3. RTG-2 cells stimulated with LPS 10 μg/mL for 1 h; 4. RTG-2 cells stimulated with LPS 10 μg/mL for 4 h; 5. Head kidney macrophage cells before stimulation; 6. Head kidney macrophage cells stimulated with LPS 10 μg/mL for 1 h; 7. Head kidney macrophage cells stimulated with LPS 10 μg/mL for 4 h; 8. Positive control cDNA from PHA stimulated head kidney

stimulation with PHA (5 μg/mL) induced much higher level of COX-2 gene expression than that of LPS (10 μg/mL) in head kidney macrophage cells.

On the other hand, IL-1β gene expression was not significantly increased by LPS stimulation in RTG-2 cells, revealing that RTG-2 cells are much less sensitive to LPS than macrophage cells (Fig. 2). Wang et al. (2002) has reported that 25 μg/mL of LPS is required to induce IL-1β gene in RTG-2 cell line and this is 5 or 2.5 times higher LPS concentration than that used in this study (5 or 10 μg/mL). LPS concentration influence on different cell types could be studied in further analysis.

It has been reported that IL-1β markedly enhances the metabolism of arachidonic acid, in particular of prostacyclin and PGE_2, in inflammatory cells such as fibroblasts, synovial cells, chondrocytes, endothelial cells, hepatocytes, and osteoclast (Dinarello, 1984). Komaki et al. (1992) reported that many IL-1β -induced changes were mediated by prostanoids, particularly prostaglandin (PG) E_2, by mediating ACTH response. In terms of this, along with the effects on nflammatory gene induction, regulatory activity of LPS on a prostanoid (i.e.PGE_2) production in the rainbow trout was also analysed. Treatment of macrophage cells with LPS (100 or 10 μg/mL) for 48 h significantly increased PGE_2 production, presumably via activating COX-2 expression that metabolites arachidonic acid to PGE2 (Fig. 3).

In conclusion, LPS could induce two main

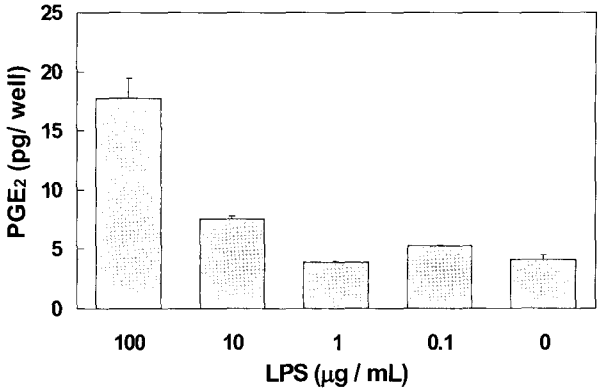

Fig. 3. Effect of LPS on PGE_2 production. Head kidney leucocytes were incubated in L15 medium with LPS (0.1, 1, 10, 100 μg/mL) or without LPS for 48 h at 18℃. The levels of PGE_2 produced were determined by EIA.

inflammatory genes, IL-1β and COX-2, and increase PGE_2 production in the rainbow trout head kidney macrophage cells, representing inflammatory activity.

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