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Protective effects of leucine against lipopolysaccharide-induced inflammatory response in *Labeo rohita* fingerlings

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ABSTRACT

The present study investigated the protective effects of leucine against lipopolysaccharide (LPS)-induced inflammatory responses in *Labeo rohita* (rohu) *in vivo* and *in vitro*. Primary hepatocytes, isolated from the hepatopancreas, were exposed to different concentrations of LPS for 24 h to induce an inflammatory response, and the protective effects of leucine against LPS-induced inflammation were studied. Finally, we investigated the efficiency of dietary leucine supplementation in attenuating an immune challenge induced by LPS *in vivo*. Exposure of cells to 10–25 μg mL^{-1} of LPS for 24 h resulted in a significant production of nitric oxide and release of lactate dehydrogenase to the medium, whereas cell viability and protein content were reduced ($p < 0.05$). LPS exposure (10 μg mL^{-1}) increased mRNA levels of the pro-inflammatory cytokines TNF-α, IL-1β and IL-8 *in vitro* ($p < 0.05$). However, pretreatment with leucine prevented the LPS-induced upregulation of TNF-α, IL-1β and IL-8 mRNAs by downregulating TLR4, MyD88, NF-κBp65, and MAPKp38 mRNA expression. Interestingly, mRNA expression of the anti-inflammatory cytokine, IL-10, which was increased by LPS treatment, was further enhanced ($p < 0.05$) by leucine pretreatment. The enhanced expression of IL-10 might inhibit the production of other pro-inflammatory cytokines. It was found that leucine pretreatment attenuated the excessive activation of LPS-induced TLR4-MyD88 signaling as manifested by lower level of TLR4, MyD88, MAPKp38, NF-κBp65 and increased level of IκB-α protein in leucine pre-treatment group. *In vivo* experiments demonstrated that leucine pre-supplementation could protect fish against LPS-induced inflammation through an attenuation of TLR4–MyD88 signaling pathway. Taken together, we propose that leucine pre-supplementation decreases LPS-induced immune damage in rohu by enhancing the expression of IL-10 and by regulating the TLR4-MyD88 signaling pathways.

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1. Introduction

Lipopolysaccharide (LPS), a component of the cell envelope of Gram-negative bacteria, is often associated with disease processes [1]. Few reports suggested that LPS at small doses can enhance non-specific immune responses in fish [2]. LPS has been considered to be a mitogen of B lymphocytes and highly bactericidal [3]. Dietary administration of LPS at lower doses provided higher protection to rainbow trout fingerlings against *Aeromonas hydrophila*, compared to higher doses of LPS [4]. Recently, Hang et al. [2] reported that catfish (*Pangasianodon hypophthalmus*) injected with LPS at 3 mg kg^{-1} exhibited higher protection against *Edwardsiella ictaluri*, and at elevated doses (15 or 45 mg of LPS Kg^{-1}) immune suppressive response was recorded. Further, LPS extracted from a virulent strain of *A. hydrophila* effectively stimulated immune responses in carp (*Cyprinus carpio*) and protected the fish from this bacterial infection [5]. Those authors suggested that origin of LPS may explain its potential immunostimulatory activities. However, the overstimulation of defense cells (monocytes, macrophages, and

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polymorphonuclear leukocytes) by LPS incites unregulated cytokine secretion, such as of tumor necrosis factor-α (TNF-α), interleukins, and prostaglandins, which limits inflammation [6]. LPS, which is one of the most potent initiators of inflammation, activates several signaling pathways, including inhibitory κB (IκB)/nuclear factor-κB (NF-κB) and mitogen-activated protein kinases (MAPKs) by acting on toll-like receptor (TLR) 4, to induce the expression of inflammatory genes and the release of mediators such as nitric oxide (NO), TNF-α, and interleukin-6 (IL-6) [7]. Therefore, inhibitors of the production of these inflammatory mediators and cytokines have been considered as candidates for anti-inflammatory agents. Among various pattern recognition receptors (PRRs), TLRs are important because they recognize diverse pathogen-associated molecular patterns (PAMPs) and activate signaling to induce innate immunity and protect against infectious diseases [8]. LPS stimulation of TLR4 involves the participation of several molecules. Bacterial LPS induced significant upregulation of IL-8 and IL-1β in head kidney leukocytes of Atlantic salmon [9]. TNF-α and IL-6 are pivotal pro-inflammatory cytokines involved in a variety of immune responses that lead to inflammation [10]. The improper regulation of LPS/TLR4 signaling has the potential to induce massive inflammation and cause acute sepsis or chronic inflammatory disorders in organisms [10]. Hence, it is important to further understand this pathway and identify novel targets to protect organisms against these pathological conditions.

Leucine is an essential amino acid for the optimal growth of fish [11]. Leucine deficiency causes the loss of weight, feed efficiency and protein content in carp, *Cirrhinus mrigala* [12], and catfish, *Heteropneustes fossilis* [13]. The disease resistance of fish is associated with immune defense mechanisms, which include innate as well as adaptive immunity. Recently, we have demonstrated that dietary leucine supplementation enhances the growth performance of *Labeo rohita* and modulates the expression of antioxidant and immune-related genes in the head kidney [14]. Recently, Ren et al. [15] observed that dietary administration of optimum level of leucine could significantly improve the growth, and down-regulate the expression of TNF-α in juvenile blunt snout bream, *Megalobrama amblycephala*. Furthermore, leucine intake improved the growth performance, intestinal antioxidant status and Nrf2 gene expression in *Ctenopharyngodon idella* [11]. These studies explored the potential role of leucine in modulating fish immunity. Recently it was reported that leucine can modulate airway inflammation in human lung epithelial cells [16]. Hirai et al. [10] demonstrated that pyroglutamyl-leucine (pyroGlu-Leu) inhibited LPS-induced inflammatory responses by blocking NF-κB and MAPK pathways in RAW 264.7 macrophages. In addition, pyroGlu-Leu was reported to be hepatoprotective [17].

Rohu, *L. rohita* is an economically important freshwater fish with high nutritional value in India. An understanding of the regulation of inflammatory responses in fish is very limited. We investigated the effects of leucine on LPS-induced inflammatory responses in *L. rohita* fingerlings *in vivo* and in hepatocytes *in vitro* for the first time. Based on our results, we propose a potential mechanism underlying the effects of leucine on LPS-induce immune-damage in fish.

2. Materials and methods

2.1. In vitro studies

2.1.1. Primary hepatocyte culture

Healthy *L. rohita* carps having a mean body weight of 56.3 g were collected from a local fish farm (Thanjavur, Tamil Nadu, India) and acclimatized (at 25 ± 2 °C) to laboratory conditions for two weeks, as described earlier [18]. Fish were fasted for 24 h before the experiment. Carps were sacrificed by decapitation and the hepatopancreas was rapidly removed. Hepatopancreases were washed with ice-cold phosphate buffer solution (PBS, pH 7.2) to remove as much blood as possible. Hepatocytes were isolated by 0.25% trypsin digestion in DMEM (Dulbecco Modified Eagle medium), following the methods of Li et al. [19]. Cells were suspended in DMEM followed by repeated washing (with DMEM) to remove enzymes and blood cells. A polyurethane membrane filtration technique was used to separate hepatocytes from hepatic fibroblasts [20].

Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 μg streptomycin sulfate/mL and 100 IU penicillin/mL at a concentration of 1×10^6 cells mL^{-1}. Cultures were maintained in an incubator at 26 ± 0.5 °C in 5% CO_2. Cells were allowed to attach to 24-well plates for 72 h.

2.1.2. Effect of LPS on cell viability, protein content, and inflammatory response in carp hepatocytes

Isolated hepatocytes (1×10^6 cells mL^{-1}) were incubated for 24 h in fresh medium containing 0, 5, 10, 15, or 25 μg mL^{-1} of LPS (LPS from *Escherichia coli* 0111:B4; Sigma-Aldrich, USA).

After 24 h, cell viability was determined by MTS [3-(4, 5-dimethylthiazol-2-yl)-5-3(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assays [21]. Nitric oxide (NO) production was assayed by measurement of the nitrite concentration using Griess assay as described earlier [22]. The absorbance was measured at 550 nm using a microplate reader. Nitrite concentrations were calculated with a sodium nitrite standard curve as reference [23].

Cells were lysed with 0.5% Triton X-100 (Sigma-Aldrich, USA) for 10 min on ice. Cell lysates were collected to detect protein content (PC) and the medium supernatant was collected to detect lactate dehydrogenase (LDH) activity. PC content was measured following the method of Bradford [24] using bovine serum albumin as a standard. LDH activity was measured according to a previously described method [25]. All experiments were performed at least three times.

2.1.3. Effect of leucine on LPS-induced inflammatory responses in carp hepatocytes

To study the protective effect of leucine against LPS exposure, hepatocytes were pretreated with 0 (not treated with LPS, negative control, group I), 100 (group II), 150 (group III), 200 (group IV), 250 (group V), 300 (group VI), or 350 (group VII) μg mL^{-1} of leucine for 72 h prior to a 24 h treatment with 10 μg mL^{-1} of LPS at 26 °C. The LPS concentration was chosen based on preliminary experiments that showed that 10 μg mL^{-1} of LPS could induce an inflammatory response in fish hepatocytes. Group I cells were incubated in DMEM. The groups (pretreatment + LPS exposure) were treated in the following manner: Control (Cntl) + Control (group I); 100 μg mL^{-1} of Leu + LPS (group II); 150 μg mL^{-1} Leu + LPS (group III); 200 μg mL^{-1} of Leu + LPS (group IV); 250 μg mL^{-1} of Leu + LPS (group V); 300 μg mL^{-1} of Leu + LPS (group VI); 350 μg mL^{-1} of Leu + LPS (group VII). After treatments, excreted media were collected to analyze LDH release. Cell lysates were collected to detect TNF-α, IL-1β, IL-8, IL-10, TLR4, MyD88, NF-κBp65 and MAPKp38 mRNAs ($n = 3$).

2.2. In vivo studies

2.2.1. Protective effect of leucine against LPS induced inflammatory response in carp

Recently, our group demonstrated that dietary administration of leucine at 4.7 g kg^{-1} is optimal for growth and enhanced immunity in *L. rohita* fingerlings [14]. Thus, we prepared the diet as previously described [14]. A total of 180 carps (*L. rohita*; average body weight

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