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Effects of recombinant trout leptin in superoxide production and NF-κB/MAPK phosphorylation in blood leukocytes

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ABSTRACT

Studies in mammals indicate that leptin is a multifunctional cytokine involved in regulation of energy metabolism and the modulation of the immune function. However, evidence for an immunomodulatory effect of leptin in fish is still missing. At least in part, this lack of knowledge is due to the absence of materials and models. In this study, we produced trout recombinant leptin (rt-lep) and tested its capacity to trigger cellular pathways, usually active in mammal immune system cells. STAT3, NF-κB, and the three major MAPK cascades (JNK, p38 and ERK), were activated by rt-lep in in vitro incubations with blood leucocytes of the rainbow trout *Oncorhynchus mykiss*. We also showed that rt-lep causes a decrease in superoxide anion production in trout blood leucocytes. Thus our data indicate that as in mammals also in teleosts leptin plays pleiotropic activities. Importantly, its actions in fishes do not always conform to the picture emerging for mammals.

1. Introduction

Leptin is an adipocyte-derived hormone discovered in 1994 [74]. The finding that the leptin concentration in the plasma is proportional to the body adiposity led to the theory that such hormone behaves as an "adipostat", a humoral signal carrying information regarding energy reserves [38]. In fact, leptin acts on the central nervous system (CNS) to regulate food intake and metabolism [48]. Though discovered only recently, leptin has been shown to be an extremely powerful hormone involved in several aspects of body functioning such as reproduction [44,47], haematopoiesis [16], angiogenesis [57], wound healing [14], cardiovascular pathophysiology [11] and immune response [31].

Leptin three-dimensional structure is similar to that of a cytokine consisting of a four α-helix bundle motif which is common to the IL-6 family of cytokines [72]. In mammalian innate immunity, leptin upregulates phagocytic function via phospholipase activation [39] as well as proinflammatory cytokine secretion, such as TNF-α (early), IL-6 (late), and IL-12 [16,36]. Leptin stimulates proliferation of human circulating monocytes in vitro [68], increases immature dendritic cells migratory performance [30,36], stimulates reactive oxygen species production [3] and chemotaxis [4] via a mechanism that may involve interaction with monocytes [69]. Leptin is also involved in development, differentiation, activation, proliferation and cytotoxicity of natural killer cells [58]. In adaptive immunity, leptin increases proliferation of naive T cells and provides a survival signal for double positive $CD4^+CD8^+$ and single-positive $CD4^+CD8^-$ thymocytes during T lymphocyte maturation [22]. Moreover, leptin promotes survival of T cells inhibiting apoptosis and stimulates B cell proliferation and their cytokines production [1].

The effect of leptin on immune cells is mediated by leptin receptor (ObR), which is expressed on peripheral blood monocytes and T lymphocytes (both CD4 and CD8) [6]. Leptin binding to its cognate receptor leads to NF-κB and MAP kinase (JNK, p38 and ERK) signaling pathways activation, which promote production of proinflammatory cytokines and anti-apoptotic effects in mammalian immune cells [49].

Abbreviations: CD4, cluster of differentiation 4; CD8, cluster of differentiation 8; ERK, extracellular-signal-regulated kinase; ERK1/2, extracellular signal-regulated kinase 1/2; IL-6, interleukin 6; IL-12, interleukin 12; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK, mitogen-activated protein kinases; p38 MAP kinase, protein 38 mitogen activated kinase; TNF-α, tumor necrosis factor alpha.

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The identification of leptin-like molecules in fish was firstly reported by Johnson et al. [25]. Thus far, many studies have examined the relationship between leptin, feeding behavior and metabolism in fish [59–61,66]. Recently, leptin research in fish has received a strong impulse and a cDNA coding for a homologue to mammalian leptin has been identified in pufferfish (*Takifugu rubripes*) [29], zebrafish (*Danio rerio*) [20], common carp (*Cyprinus carpio*) [23], rainbow trout (*Oncorhynchus mykiss*) [45], yellow catfish (*Pelteobagrus fulvidraco*) [19], orange-spotted grouper (*Epinephelus coioides*) [73]. Leptin sequence in fish is highly divergent and recently two different leptins have been identified in several teleosts species [50]. However, the three dimensional structure seems to be highly conserved among vertebrates [8,12].

The low degree of primary sequence conservation in teleosts may partially explain why leptin studies in lower vertebrates, such as fish, are still relatively few, due to the limited availability of homologous leptin to be employed in physiological studies. Therefore, in this study, recombinant rainbow trout (*Oncorhynchus mykiss*) leptin (rt-leptin) was produced in an *Escherichia coli* expression system and its role in rainbow trout immune response was analyzed.

The combination of the knowledge on teleosts immune system and the development of the rainbow trout *Oncorhynchus mykiss* homologous leptin make it a suitable animal model to significantly widen our knowledge of leptin and immune system relationship.

2. Materials and methods

2.1. Animals

Adult rainbow trout (*Oncorhynchus mykiss*), weighing 500–600 g, were used for this study. Fish were obtained from a local dealer (Di Mella, Santacroce del Sannio, Benevento, Italy) and allowed to acclimate in recirculating water system tank at 12 °C for 24 h before sacrifice. Fish were euthanized by immersion in MS-222 (MP Biomedicals, LLC, Aurora, OH, USA) 80 mg/L (LC50 >200 mg/L). Fish used in this study were treated in accordance with the European Commission recommendation 2007/526/EC and 2010/63/UE on revised guidelines for the accommodation and care of animals used for experimental and other scientific purposes. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Biogem Consortium, Ariano Irpino, Italy. The protocol was approved by the Committee on the Ethics of Animal Experiments of the same Consortium. Water parameters and environmental conditions were as stated in the European Commission recommendation 2007/526/EC and 2010/63/UE. All efforts were made to minimize fish suffering.

2.2. Isolation of leucocytes and in vitro cell culture

Blood was withdrawn from the caudal vein using a syringe previously rinsed with heparin. Leucocytes were isolated according to Galeotti et al. [17] with few modifications. Blood was diluted 1:5 with RPMI (Lonza 1640) and centrifuged at 200 × *g* for 10 min at 4 °C. The pellet was diluted 1:50 with RPMI and layered onto Histopaque (1.077 g/l) (Sigma–Aldrich, St. Louis, MO, USA) and then centrifuged at 300 × *g* for 25 min at 4 °C. Leucocytes were harvested from the interface and washed with RPMI by centrifugation at 300 × *g* for 10 min at 4 °C. Isolated leucocytes were counted with the trypan blue exclusion test and seeded in 6 ml plate at the density of 6,000,000 cells/well in triplicate. Leucocytes were cultured with L-15 (Liebovits Medium, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco, Germany), 2 mM L-glutamine, 100 μU/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsband, CA, USA), and incubated at 18–20 °C overnight.

2.3. Cloning of rainbow trout recombinant leptin (rt-lep) cDNA

Total liver RNA was isolated using SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Total RNA was used as template for the single strand cDNA synthesis, employing oligodT primers and Super-ScriptTM III Reverse Transcriptase (Invitrogen). The complete trout leptin cDNA sequence was amplified from total liver cDNA with PCR using the high fidelity Platinum Taq DNA Polymerase (Invitrogen). Leptin primers were designed according to trout leptin sequence reported in data bank (Accession no. AB354909.1). The primers designed were 5′ AAGAATTCGCTTCATCTCTCATTG 3′ (primer forward) and 5′ AACTCGAGGTAACAGTAATTCAG 3′ (primer reverse). The forward primer and the reverse primer contain an EcoRI and an XhoI restriction site, respectively, to facilitate cloning in the pET28 expression vector. The PCR thermal conditions were: denaturation at 95 °C for 5 min, then 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 10 min. The PCR product was separated by 1% agarose gel electrophoresis and purified from agarose gel using phenol-chloroform method [53].

The PCR product was sequenced by the Molecular Biology Service (www.sbmweb.it) as follows: sequence reactions were obtained with the *BigDye Terminator Cycle Sequencing* technology (*Applied Biosystems, Foster City, CA)*, purified in automation using the*Agencourt CleanSEQ Dye terminator removal Kit* (*Agencourt Bioscience Corporation, 500 Cummins Center, Suite 2450, Beverly MA 01915, USA*) and a robotic station *Biomek FX* (*Beckman Coulter, Fullerton, CA*). Products were analysed on an Automated Capillary Electrophoresis Sequencer *3730 DNA Analyzer* (*Applied Biosystems*). The PCR product sequence resulted 100% homologous to the trout leptin sequence reported in data bank (Accession no. AB354909.1).

2.4. Expression in E. coli and purification of rt-lep

Purified leptin cDNA coding sequence was cloned into the plasmid vector pET 28 (a) (PET System Vector, Novagen), which contains a six histidines tag (His-tag) at the N-terminus for purification of the recombinant protein, according to the manual instructions (pET System Manual, Novagen). pET 28-Lep plasmid was transformed into *E. coli* DH5α, and plasmid DNA subsequently extracted using a mini prep plasmid extraction and sequenced. After the confirmation of the gene sequence, the pET 28-Lep plasmid was transformed into *E. coli* BL21 according to Sambrook et al. [53]. A single bacterial colony was inoculated into 200 ml of LB medium (1% Tryptone, 0.5% yeast extract, 1% NaCl pH 7.0) containing 200 μl of kanamycin (100 mg/ml) and cultured at 37 °C with shaking overnight, prior to culture in 4 l of LB containing 4 ml of kanamycin at 37 °C with shaking. When absorbance at 600 nm reached between 0.5–0.6 nm, 1 M IPTG (isopropyl-beta-D-thiogalactopyranoside, Sigma), used 1:1000, was added to the culture, to induce the expression of His-tag rt-lep. 1 ml of cells not IPTG induced was used as negative control. Cells were grown for an additional 1 h at 37 °C with shaking. IPTG induction was confirmed by SDS-PAGE 15%, stained with Comassie Brilliant blue G (Sigma). Running buffer was Tris–Glycine (25 mM Tris, 250 mM glycine, 0.1% SDS, pH8.3). Gel was the cells induced with IPTG were then harvested by centrifugation at 3700 × *g* for 20 min at 4 °C and the cell pellet was resuspended in 25 ml of Binding Buffer 1 × pH 8.0 (5 mM Imidazole, 500 mM NaCl, 20 mM Tris–HCl). The cell lysate was sonicated on ice five times and the soluble fraction was collected after centrifugation at 10,000 × *g* for 30 min at 4 °C. The insoluble fraction was obtained after resuspending the pellet in 10 ml of Binding Buffer