Ghrelin stimulates phagocytosis and superoxide production in fish leukocytes

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Abstract

To clarify the role of ghrelin in the fish immune system, the *in vitro* effect of ghrelin was examined in phagocytic leukocytes of rainbow trout (*Oncorhynchus mykiss*). Administration of trout ghrelin and des-VRQ-trout ghrelin, in which three amino acids are deleted from trout ghrelin, increased superoxide production in zymosan-stimulated phagocytic leukocytes from the head kidney. Gene expression of growth hormone (GH) secretagogue-receptor (GHS-R) was detected by RT-PCR in leukocytes. Pretreatment of phagocytic leukocytes with a GHS-R antagonist, [D-Lys³]-GHRP-6, abolished the

stimulatory effects of trout ghrelin and des-VRQ-trout ghrelin on superoxide production. Ghrelin increased mRNA levels of superoxide dismutase and GH expressed in trout phagocytic leukocytes. Immunoneutralization of GH by addition of anti-salmon GH serum to the medium blocked the stimulatory effect of ghrelin on superoxide production. These results suggest that ghrelin stimulates phagocytosis in fish leukocytes through a GHS-R-dependent pathway, and also that the effect of ghrelin is mediated, at least in part, by GH secreted by leukocytes.

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Introduction

Ghrelin was originally discovered in rat stomach as an endogenous ligand for the growth hormone (GH) secretagogue-receptor (GHS-R) (Kojima *et al.* 1999). Ghrelin is recognized as an important regulator not only of GH secretion but also of feeding, glucose homeostasis, gastric motility, the cardiovascular system and cell proliferation (Muccioli *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003). The effect of ghrelin on energy metabolism has suggested its potential use as a therapeutic target in disorders of GH secretion, feeding and nutritional condition (Muccioli *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003). Signal transduction mediated by G protein has been detailed in fish GHS-R (Chan *et al.* 2004). However, expression of GHS-R gene was detected in human T cells, B cells and neutrophils, suggesting the action of ghrelin on the immune system (Hattori *et al.* 2001). Recently, Dixit *et al.* (2004) revealed that ghrelin inhibits expression of proinflammatory cytokine in human T cells and monocytes, suggesting a role of ghrelin in the immune system. However, evidence of the immunomodulatory effect of ghrelin is limited, and the effect of ghrelin on the defense mechanism is still unclear.

It is well known that GH stimulates immune functions in various vertebrates (Sakai *et al.* 1996a, 1996b, 1997, Balm 1997, Clark 1997, Cohen & Kinney 2001, Yada & Nakanishi 2002, Yada *et al.* 2004a, 2004b). In fish, *in vivo* and *in vitro* administration of GH enhances specific and nonspecific immune functions, such as phagocytosis by neutrophils and macrophages (Sakai *et al.* 1996a, 1997, Balm 1997, Yada & Nakanishi 2002, Yada *et al.* 2004a, 2004b). Extrapituitary expression of GH gene in fish leukocytes suggests autocrine or paracrine action of GH in the modulation of immune functions (Yada & Azuma 2002, Yada & Nakanishi 2002, Yada *et al.* 2005). Thus, ghrelin seems to be a likely candidate for modulator of the GH gene expression in the fish immune system.

We hypothesized that phagocytosis, one of the major defense mechanisms in primitive vertebrates, is activated by ghrelin in fish. Ghrelin has been isolated in rainbow trout, and its stimulatory action on GH secretion has been demonstrated in the same species (Kaiya *et al.* 2003a). This study examined the *in vitro* effects of ghrelin on the activity of phagocytic leukocytes isolated from the head kidney of trout, which is equivalent to the bone marrow in higher vertebrates. The effects of ghrelin on superoxide dismutase (SOD) and GH gene expression were also examined in trout phagocytic leukocytes by real-time PCR.

Materials and Methods

Fish

Rainbow trout (*Oncorhynchus mykiss*), each weighing about 500 g, were reared at the National Research Institute of Fisheries Science at Nikko (Japan) for successive generations in outdoor concrete ponds supplied with a continuous flow of spring water at 10 °C under natural photoperiod. They were fed commercial dry diet (Oriental, Chiba, Japan).

Hormones and reagents

Rainbow trout ghrelin and des-VRQ-trout ghrelin were synthesized as described previously (Kaiya *et al.* 2003a). Salmon GH was isolated from chum salmon (*O. keta*) pituitaries, as described by Kawauchi *et al.* (1986). GHS-R-specific antagonist, [D-Lys³]-GHRP-6, was purchased from Phoenix Pharmaceuticals (Belmont, CA, USA). The specificity of a polyclonal antisalmon GH antiserum (AS9-2) was validated by immunocytochemistry and RIA (Bolton *et al.* 1986). Nitroblue tetrazolium (NBT) and zymosan A were purchased from Sigma.

Isolation of leukocytes

Head-kidney leukocytes (HKL) were isolated as described by Sakai *et al.* (1996a) with slight modifications. Six fish were used for each experiment. Fish were anesthetized in 3-aminobenzoic acid ethyl ester (MS222; Sigma), killed by decapitation and the head kidney was placed in Eagle's minimum essential medium (MEM) with Earle's salt (Sigma), containing 0.2% heparin sodium and buffered with 7.5% NaHCO₃ (pH 7.6); minced with forceps; and filtered by nylon mesh (37 µm). The dissociated cells were placed on 34/51% Percoll (Pharmacia) cushions and centrifuged at 400 g for 25 min. The leukocyte band was harvested, washed with PBS (pH 7.6) and suspended in MEM containing 0.5% trout serum. Viable leukocytes were counted by trypan blue exclusion (viability of >90%), and were used for *in vitro* experiments, as described below. To isolate peripheral blood leukocytes (PBL) for RT-PCR, blood was collected from the caudal vessels with a heparinized syringe. Blood was diluted 1:2 in MEM containing 0.2% heparin sodium. The mixture was placed on a 54% Percoll cushion, and centrifuged at 400 g for 25 min, and the leukocyte band was harvested.

Superoxide production in phagocytic cells

Quantification of superoxide anion production by adherent and phagocytic leukocytes as a killing mechanism after phagocytosis was used to examine the effects of trout ghrelin, des-VRQ-trout ghrelin and salmon GH.

The superoxide production in adherent cells isolated from HKL was determined as the reduction of NBT (Sakai *et al.* 1996a). In brief, isolated leukocytes from each fish were suspended in MEM containing 0.5% trout serum at a density of 10⁷ cells/ml, and 100 µl were seeded onto 96-well microplates in duplicate. The cells were preincubated overnight in an atmosphere of 95% O₂/5% CO₂ at 15 °C. After removal of nonadherent cells, adherent cells on the bottom of the well (about 10⁵ cells) were incubated in MEM with or without hormones for 4 h at 15 °C. The adherent leukocytes from trout head kidney contain about 90% macrophages and 10% granular neutrophils (Sakai *et al.* 1996a). After incubation, the medium was aspirated, and the cells were further incubated in 100 µl MEM containing NBT (1 mg/ml) with zymosan A as a stimulant for superoxide production. After incubation for 1 h at 15 °C, the medium was aspirated, and the cells were fixed with methanol for several minutes. They were air-dried, and dissolved in 120 µl of 2 M KOH and 140 µl dimethyl sulfoxide (DMSO; Sigma). Absorbance at 620 nm was measured with a microplate reader (SpectraMax 190; Nihon Molecular Devices, Tokyo, Japan).

RNA extraction and RT-PCR for GHS-R mRNA

Tissues (stomach, intestine, gills, skin, body and head kidney, spleen and pituitary) were frozen in liquid nitrogen immediately after isolation, and stored at -80 °C until use for RNA extraction. Skin was taken from the dorsal side of the body. Isolated PBL and HKL were seeded onto 24-well microplates, and incubated overnight in an atmosphere of 95% O₂/5% CO₂ at 15 °C. Nonadherent cells from PBL were harvested by centrifugation at 500 g for 5 min and immediately used for RNA extraction.

Total RNA was extracted from tissues and cells by the guanidium-isothiocyanate-phenol method (Chomczynski & Sacchi 1987), and treated with RNase-free DNase I (Takara, Shiga, Japan). After inactivation of DNase, reverse transcription was carried out with the SuperScript First-Strand Synthesis System (Invitrogen). PCR was performed with AmpliTaqGold DNA Polymerase (Applied Biosystems, Foster City, CA, USA) and the GeneAmp 9700 PCR System (Perkin Elmer, Norwalk, CT, USA). Nucleotide sequences of full-length rainbow trout GHS-R 1a and 3'-end truncated form (GHS-R 1b) have recently been determined (H Kaiya, unpublished data), and PCR primers were designed to amplify trout GHS-R 1a (5'-TGCCTTTCCACTTGCATCGT-3', forward; 5'-TTCCCCCTCCAAATTGGCT-3', reverse) and GHS-R 1b (5'-GCGGTTGAGTCTGGGCTTCTA-3', forward; 5'-TTATGCAGTGGTTAAAATAGT-3', reverse). The amplification condition was 40 cycles of 94 °C for 1 min, 57 °C for 1 min, and 72 °C for 1 min. The amplified products were analyzed by a 1.5% agarose gel and stained with ethidium bromide.

Quantification of SOD and GH mRNA levels by real-time PCR

To determine absolute amounts of mRNA, standard partial cDNA of rainbow trout SOD were cloned and sequenced. The SOD 1 fragment was purified with rainbow trout HKL cDNA with AmpliTaq Gold DNA Polymerase (Applied Biosystems) and primers (5'-GGCTTCCACGTCCATGCTTA-3', forward; 5'-CCCAGATCATCAGCCTTCTCAT-3', reverse), as described by GeneBank accession no. AF469663. The amplification regime was 40 cycles consisting of 94 °C for 1 min, 54 °C for 30 s and 72 °C for 1 min. The products were purified and subcloned into pT7 Blue (Merck). Cloned cDNA fragments were digested by KpnI at the ends of the insert, separated by agarose gel electrophoresis, and purified with GENECLEAN (Qbiogene, Carlsbad, CA, USA).

The number of HKL from each fish was adjusted to 10^7 cells/ml in MEM containing 0.5% trout serum, and 300 µl were seeded onto 24-well microplates. The cells were cultured overnight in an atmosphere of 95% O₂/5% CO₂ at 15 °C. Nonadherent cells were removed, and adherent cells (about 3×10^5 /well) were incubated in MEM with or without hormones for 4 h at 15 °C. Total RNA was extracted and treated with RNase-free DNase I, and reverse transcription of RNA was carried out as described above. Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems), as described previously (Yada *et al.* 2005). The PCR mixture (20 µl) contained 1X TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM each forward and reverse primers, 100 nM fluorogenic probe, and standard (6×10^2 – 6×10^7 copies/reaction) or template cDNA (0.04–40 ng/reaction). After denaturation at 95 °C for 10 min, PCR amplification of 50 cycles of 95 °C for 15 s and 60 °C for 1 min was conducted. The sequences of the primers and probe were as follows: 5'-GACAACACCAACGGCTGTATGA-3', forward; 5'-CTCCGTGGGTCTGGTTGTG-3', reverse; 5'-TGCCGGACCCCACTTCAACCC-3', probe for trout SOD 1, 5'-GGAGCGGAGCAGCAAGAG-3', forward; 5'-TGCAGGAAAGGTCTCATCTG-3', reverse; 5'-CTGTC TCCAGGGTTTCGGTTTCCCAG-3', probe for trout GH 2, and 5'-TTCAACACCCCTGCCATGTA-3', forward; 5'-ACGGCCAGAGGCGTATAGG-3', reverse; 5'-TGGCCATCCAGGCCGTGTTGT-3', probe for trout β-actin. SOD and GH mRNA levels were standardized with β-actin mRNA levels in each sample.

Statistical analysis

The significance of differences between control and experimental groups was evaluated by analysis of variance followed by paired Student's *t*-test for parametric groups or Wilcoxon's rank sum test for nonparametric groups.

Calculations were performed with a computer program, STATISTICA (Statsoft, Tulsa, OK, USA).

Results

Rainbow trout ghrelin and des-VRQ-trout ghrelin (10 nM) produced significant increases in superoxide production in phagocytic HKL (Fig. 1). Although the stimulatory effects of ghrelin were significant without stimulation by zymosan A, the largest responses were observed in the presence of zymosan A at 2 mg/ml. Salmon GH used as a positive control of enhancement of superoxide production produced a tendency similar to that of ghrelin. Then, experiments of superoxide production were done with that condition of stimulation. Trout ghrelin and des-VRQ-trout ghrelin at concentrations from 1 pM to 10 nM increased superoxide production in phagocytic HKL in a dose-dependent manner (Fig. 2).

Figure 3 shows the expression of GHS-R genes in various tissues and lymphoid cells. Both forms of GHS-R mRNAs (GHS-R 1a and 1b) were expressed ubiquitously in lymphoid tissues and cells, such as head kidney, spleen, nonadherent and adherent PBL, and HKL. The stimulatory effects of trout ghrelin and des-VRQ-trout ghrelin on superoxide production in phagocytic HKL were abolished by pretreatment with a GHS-R antagonist, [D-Lys³]-GHRP-6 (Fig. 4). That inhibitory effect of GHS-R antagonist was not significant in GH-enhanced superoxide production. Quantification of SOD mRNA levels revealed that both trout ghrelin and des-VRQ-trout ghrelin, and salmon GH stimulated gene expression of SOD in phagocytic HKL (Fig. 5).

Effects of trout ghrelin and des-VRQ-trout ghrelin on GH mRNA levels in HKL are shown in Fig. 6. Both ghrelins produced significant increases in GH gene expression. In Fig. 7, immunoneutralization of GH by addition of anti-salmon GH serum to the medium resulted in significant inhibition of superoxide production in HKL enhanced by two trout ghrelins.

Discussion

The present study demonstrated that ghrelin stimulates superoxide production associated with phagocytosis in trout leukocytes. The effect of ghrelin was abolished by a GHS-R antagonist, [D-Lys³]-GHRP-6, which is known to inhibit signal transduction mechanism also in fish GHS-R (Chan *et al.* 2004). Those facts suggest that ghrelin activates trout phagocytes through a GHS-R-mediated mechanism. Administration of ghrelin increased mRNA levels of SOD, which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. This result suggests that ghrelin and GH stimulate both

Figure 1 Dose-related influence of zymosan A on the effects of trout ghrelin, des-VRQ-trout ghrelin and salmon GH on superoxide production in phagocytic head-kidney leukocytes. Cells were incubated with each hormone (10 nM) for 4 h, and then treated with zymosan A for 1 h. Data are expressed as means $\pm$ S.E.M. ($n=6$). *, **Significantly different from the control at $P<0.05$ and $P<0.01$ respectively.

superoxide and hydrogen peroxide as reactive oxygen species during the process of phagocytosis in fish phagocytic leukocytes (Secombes 1996). To our knowledge, this is the first report to demonstrate the effect of ghrelin

in enhancing the immune function of phagocytic leukocytes in vertebrates.

The present study revealed that trout ghrelin and des-VRQ-trout ghrelin are equipotent in stimulating

Figure 2 Dose-related effects of trout ghrelin and des-VRQ-trout ghrelin on zymosan A-stimulated superoxide production in phagocytic head-kidney leukocytes. Cells were incubated with each hormone (10 nM) for 4 h, and then treated with zymosan A (2 mg/ml) for 1 h. Data are expressed as the percentage of the control in each individual (mean $\pm$ S.E.M., $n=6$). ***, **Significantly different from the control at $P<0.05$ and $P<0.01$ respectively.

Figure 3 Expression of GHS-R mRNAs (GHS-R 1a and 1b) detected by RT-PCR in tissues and cells of trout.

superoxide production in phagocytic leukocytes from head kidney. The two trout ghrelin isoforms are derived from a single gene by alternative splicing, and show similar effects on GH secretion from trout pituitary (Kaiya *et al.* 2003a). A similar isoform of ghrelin is also present in rat and man as des-Gln¹⁴-ghrelin, which is also a ligand for GHS-R (Hosoda *et al.* 2000, 2003). Signal transduction mediated by G protein has been detailed also in fish GHS-R (Chan *et al.* 2004). Carboxyl-terminus is amidated in teleost ghrelins, but not in tetrapod ghrelins

(Kaiya *et al.* 2003a). It is known that the active core of ghrelin is the amino-terminal tetrapeptide including acylation (Bednarek *et al.* 2000, Matsumoto *et al.* 2001). Thus, results in this study indicate that the conserved structure at the N-terminus is important for immunomodulatory activity, the amide structure at the C-terminus being unrelated to its biologic activities.

The stimulatory effect of ghrelin on GH secretion from the pituitary is well established in mammals, birds, amphibians and several species of fish, including rainbow trout (Kojima *et al.* 1999, Kaiya *et al.* 2001, 2002, 2003a, 2003b, Muccioli *et al.* 2002, Riley *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003, Unniappan & Peter 2004). Poppi *et al.* (2002) reported that hexarelin, a synthetic GHS-R ligand, stimulates GH release from bovine and

Figure 4 Effects of a GHS-R-specific antagonist, [D-Lys³]-GHRP-6, on superoxide production in phagocytic head-kidney leukocytes. Cells were preincubated with [D-Lys³]-GHRP-6 (10 µM) for 1 h, incubated with trout ghrelin (10 nM) or des-VRQ-trout ghrelin (10 nM) for 4 h, and then treated with zymosan A (2 mg/ml) for 1 h. Data are expressed as the percentage of the control in each individual (mean ± S.E.M., n=6). *Significantly different from control at P<0.05. †Significant difference between columns at P<0.05.

Figure 5 Effects of trout ghrelin, des-VRQ-trout ghrelin and salmon GH on SOD mRNA levels in phagocytic head-kidney leukocytes. Cells were incubated with each hormone (10 nM) for 4 h. Data are expressed as the percentage of the control in each individual (mean ± S.E.M., n=6). * **Significantly different from control at P<0.05 and P<0.01 respectively.

Figure 6 Effects of trout ghrelin and des-VRQ-trout ghrelin on GH mRNA levels in phagocytic head-kidney leukocytes. Cells were incubated with each hormone (10 nM) for 4 h. Data are expressed as the percentage of the control in each individual (mean $\pm$ S.E.M., $n=6$). *Significantly different from the control at $P<0.05$.

porcine lymphocytes. In this study, stimulation of GH gene expression by trout ghrelin and des-VRQ-trout ghrelin was observed in phagocytic leukocytes of trout. Furthermore, the stimulatory effect of ghrelin on superoxide production was abolished by immunoneutralization with anti-GH serum added to the medium, suggesting the

Figure 7 Effects of immunoneutralization against GH with anti-salmon GH serum on the superoxide production in phagocytic head-kidney leukocytes. Cells were incubated with trout ghrelin (10 nM) or des-VRQ-trout ghrelin (10 nM) in the presence of antisalmon GH for 4 h, and then treated with zymosan A (2 mg/ml) for 1 h. Data are expressed as the percentage of the control in each individual (mean $\pm$ S.E.M., $n=6$). ***Significantly different from control at $P<0.05$ and $P<0.01$ respectively. ††Significant difference between columns at $P<0.01$.

importance of GH secreted by leukocytes. GH is now known to be produced in many immune tissues in tetrapods (Venters *et al.* 2001, Jeay *et al.* 2002). Distribution of GH mRNA in lymphoid tissues and leukocytes has also been demonstrated in fish, including rainbow trout (Calduch-Giner & Pérez-Sánchez 1999, Mori & Devlin 1999, Yada & Azuma 2002, Yada & Nakanishi 2002, Yada *et al.* 2005). GH is known to stimulate phagocytosis in both mammals and fish (Edwards *et al.* 1988, Fu *et al.* 1991, Sakai *et al.* 1996a, 1997, Yada & Nakanishi 2002). In rainbow trout, *in vivo* administration of GH enhances serum bactericidal activity and resistance to artificial infection of *Vibrio anguillarum* (Sakai *et al.* 1997). Ghrelin may enhance phagocytosis in part by stimulation of the autocrine pathway of GH. Hypothalamic and extrahypothalamic regulation of GH gene expression in the pituitary is well documented also in teleost fish (Melamed *et al.* 1998, Argenton *et al.* 2002). Our recent study revealed that GH mRNA levels in trout leukocytes are increased by *in vitro* administration of cortisol (Yada *et al.* 2005). However, regulation of GH gene expression in the fish immune system has not been fully elucidated. Further studies are needed on the immunomodulatory effects of other endocrine factors regulating GH secretion in the pituitary and lymphoid cells.

Apart from the stimulation of GH secretion, ghrelin has been shown to regulate prolactin and adrenocorticotropin secretion, feeding, glucose homeostasis, gastric motility, the cardiovascular system and cell proliferation in mammals (Muccioli *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003). In fish, ghrelin is known to stimulate not only GH but also prolactin and luteinizing hormone secretion (Riley *et al.* 2002, Unniappan & Peter 2004). Expression of ghrelin in the stomach of female tilapia (*Oreochromis niloticus*) is higher than that in male fish, suggesting involvement of ghrelin in sexual dimorphism (Parhar *et al.* 2003). Ghrelin also stimulates food intake in goldfish (*Carassius auratus*) (Unniappan *et al.* 2004). The multifunction of ghrelin coincides well with distribution of GHS-R in various tissues (Muccioli *et al.* 2002). The present study revealed that two genes of GHS-R are ubiquitously expressed in trout tissues. Distribution of GHS-R mRNAs in the adherent leukocytes coincides with the *in vitro* stimulation of phagocytosis by ghrelin. The nonadherent leukocytes separated from peripheral blood also express GHS-R genes. The nonadherent leukocytes were microscopically identified as 95% lymphocytes (Yada *et al.* 2004b). Lymphocytes seem to be targets of ghrelin in teleost fish, as shown in human lymphocytes (Hattori *et al.* 2001, Poppi *et al.* 2002). In trout, GHS-R gene expression is also detected in the skin and osmoregulatory tissues, such as the gills and body kidney. Unlike mammals, fish show distinct endocrine regulation of pigmentation and body color change (Bentley 1998). Hormonal control of osmoregulation is also characteristic of euryhaline fish.

GH and insulin-like growth factor-I play important osmoregulatory roles in stimulating secretion of excess ions in several fish species, including rainbow trout (Sakamoto *et al.* 1993, McCormick 1995). Expression of GHS-R in the skin, gills and kidney of trout suggests that ghrelin is involved in the regulation of pigmentation and osmoregulation in teleost species.

It is becoming clear that ghrelin, like GH, is an important regulator of energy balance (Muccioli *et al.* 2002, Yoshihara *et al.* 2002, Broglio *et al.* 2003). GH is known to enhance proliferation of fish lymphocytes (Sakai *et al.* 1996a, 1996b, Yada *et al.* 2004b). However, ghrelin causes inhibition of cell proliferation in the human thyroid tumor cell line (Muccioli *et al.* 2002), although it stimulates the proliferation of prostate cancer cell lines (Jeffery *et al.* 2002). These contradictory results in the role of ghrelin in cell proliferation could be due to differences in the type of cell lines. Recently, De Vriese *et al.* (2005) revealed that administration of anti-ghrelin serum inhibits proliferation of erythroleukemic cells, suggesting that the autocrine pathway of ghrelin can stimulate the proliferation of immune cells. Studies are needed on the proliferative effect and autocrine pathway of ghrelin in the immune system in fish.

Interactions between the endocrine and immune systems via hormones and cytokines are important to adjust defense mechanisms in both mammals and fish (Weyts *et al.* 1999, Yada & Nakanishi 2002). Administration of homologous interleukin (IL)-1 β activates the hypothalamo-pituitary-interrenal axis in rainbow trout (Holland *et al.* 2002). In man, ghrelin inhibits expression of IL-1 β , IL-6, and tumor necrosis factor (TNF)- α in T cells and monocytes, suggesting that ghrelin modulates cell-to-cell interaction in leukocytes during inflammatory processes (Dixit *et al.* 2004). Although several cytokine genes, including IL-1 β and TNF- α , are known to be expressed in PBL and/or HKL of rainbow trout, endocrine regulation of cytokine production in fish has yet to be determined (Yada & Nakanishi 2002). GH affects the production of several cytokines in human leukocytes (Derfalvi *et al.* 1998, Malarkey *et al.* 2002, Uronen-Hansson *et al.* 2003), and it seems to play a role in inhibiting the inflammatory response accompanied by increased levels of plasma ceruloplasmin, an acute-phase protein, in rainbow trout (Yada *et al.* 2004a). These results imply that ghrelin is involved in regulation of inflammation through cytokines and/or GH production in trout leukocytes. The role of ghrelin in the regulation of cytokine production in the fish immune system should be clarified in future studies.

In summary, the present study revealed that ghrelin stimulates phagocytosis, superoxide production and GH gene expression in rainbow trout leukocytes. Fish are considered to be the most primitive vertebrates possessing an immune system similar to that of mammals, characterized by lymphocytes, immunoglobulin, major histocompatibility

complex (MHC) and T-cell receptor (TCR) (Yada & Nakanishi 2002). Even in fish, phagocytosis by macrophages, after antigen presentation through cell-to-cell interaction with the MHC/TCR system, is necessary for production of specific antibodies (Manning 1994). Besides the energy homeostasis shown in mammals, ghrelin may, like GH, possess multiple functions in the immune system (Clark 1997, Cohen & Kinney 2001, Yada & Nakanishi 2002, Yada *et al.* 2004b).

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