Down-regulation of corticosteroid receptor in leucocytes of stressed rainbow trout

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ABSTRACT

The relationship between stress and immunosuppression was investigated in peripheral blood leucocytes (PBL) in rainbow trout, with reference to corticosteroid receptor (CR) expression and responses to cortisol- and/or lipopolysaccharide (LPS)-administration. Confinement stress in shallow water resulted in a sustained elevation of plasma cortisol, whereas lysozyme and immunoglobin levels were suppressed. Significant increases in mRNA levels of caspase-6 and insulin-like growth factor (IGF)-I were observed in PBL isolated from stressed fish. Confinement stress also suppressed proinflammatory cytokine, interleukin (IL)-1β, expression in PBL. There were decreasing tendencies for the mRNA levels of CRs in PBL of stressed fish. In-vitro treatment of cortisol and LPS on isolated PBL from unstressed trout increased both IL-1 β and CR mRNA expression. However, in PBL from stressed fish, cortisol and LPS treatment increased IL-1 β but not CR mRNA levels. Proliferative activities estimated as in-vitro incorporation of bromodeoxyuridine (BrdU) were decreased by cortisol in PBL from the unstressed and stressed fish groups; however, LPS-stimulated proliferation was observed only in the unstressed fish. Ratios of apoptotic PBL quantified as cell fragmentation using an automated cell counter were increased by cortisol in both groups; however, LPS-stimulated apoptosis was observed only in the stressed fish. Our study reveals cortisol has immune-suppressive effects in stressed fish, irrespective of CR down-regulation and desensitization. The complexity of immune-endocrine interaction is shown by the stress-induced attenuation of LPS effects.

1. Introduction

Stress-induced secretion of cortisol leads to impairments of fish immune functions (Schreck, 1996; Mommsen et al., 1999; Wendelaar Bonga, 1997; Verburg-van Kemenade et al., 2009; Yada and Tort, 2016). While in-vivo and in-vitro administration of cortisol generally suppresses proliferation, it stimulates cell death of leucocytes, and decreases the number of antibody-producing cells and circulating immunoglobulin M (IgM) levels, which affects both adaptive and innate immune functions (Kaattari and Piganelli, 1996; Mashoof and Criscitiello, 2016; Nakanishi et al., 2018). Cortisol also impairs innate immune functions such as phagocytic, cytotoxic, and lysozyme activities in fish (Saurabh and Sahoo, 2008; Yada and Tort, 2016). Three distinct receptors for corticosteroids, glucocorticoids receptor (GR)-1 and -2, and mineralocorticoid receptor (MR) have been identified in several fish species including rainbow trout (*Oncorhynchus mykiss*) (Ducouret et al., 1995; Colombe et al., 2000; Bury et al., 2003). Much previous research has focused on the differential regulation of expression and signaling pathways among subtypes of fish corticosteroid

receptors (CRs), because only cortisol is produced as corticosteroid in most teleost fish, and therefore, has both mineralocorticoid and glucocorticoid actions (Prunet et al., 2006; Bury and Sturm, 2007; Stolte et al., 2008; Aluru and Vijayan, 2009).

Regarding immunosuppression by cortisol, a transient increase in mRNA levels of GR-2 in peripheral blood leucocytes (PBL) was observed in rainbow trout after acute netting stress (Yada et al., 2007). That up-regulation of CR gene expression coincided with elevated cortisol secretion and seemed to be followed by decreased levels of plasma IgM and a reduced number of IgM-positive leucocytes (Yada et al., 2007). Although there was a rapid increase in GR-2, there were gradual and sustained decreases in GR-1 and MR mRNA levels in PBL in trout from 24 h to 7 days after acute stress (Yada et al., 2007). These results indicate that CR expression in fish leucocytes is down-regulated during the chronic phase of immunosuppression following stress. Down-regulation of CR has been predicted during chronic stress in fish (Wendelaar Bonga, 1997; Yada and Tort, 2016). However, there are few studies that have examined the possible involvement of CR down-regulation in the regulation of immune functions during chronic stress.

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Most fish studies have been conducted to examine endocrine control of immune function, and knowledge of endocrine modification by immunity is limited (Nakanishi et al., 2018). The regulation of the endocrine system by immune components, especially in inflammatory cytokines, has been well examined in fish (Balm et al., 1995; Engelsma et al., 2001, 2002; Verburg-van Kemenade et al., 2009). Conversely, possible involvements of cytokines in stress-induced immunosuppression is poorly understood in fish.

This study aimed to assess the effects of chronic stress on CRs expressed in the fish immune system, with reference to endocrine regulation by immune components. Confinement and crowding stress in shallow flowing water up to 24 h was applied as a physical and social confrontational stress without change to environmental factors in water quality. Plasma levels of cortisol, lysozyme, and IgM, and mRNA levels of caspase-6 related to apoptotic cell death were examined in PBL of rainbow trout. To clarify the difference in stress responses between the immune and endocrine systems, changes in mRNA levels of a pro-inflammatory cytokine, interleukin (IL)-1 β, and CRs after in-vitro administrations of an endotoxin, lipopolysaccharide (LPS) from *Escherichia coli* O55:B5, and cortisol were examined in PBL isolated from the control and stressed trout. Furthermore, the proliferative activity and apoptotic cell death of PBL responding to LPS and cortisol were compared between the unstressed and stressed fish.

2. Materials and methods

2.1. Animals

Rainbow trout (*Oncorhynchus mykiss*), weighing about 200 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 a natural photoperiod. They were fed a commercial dry diet (Oriental Yeast, Chiba, Japan). The fish used in this study were transferred to indoor 240-L flow-through tanks (50 × 160 × 30 cm) and supplied with spring water at 1.5 L/min at 10 °C. The density of the fish was between 7.5 and 15 g body weight/L. They were maintained under a lighting regime of 14 h light: 10 h dark for at least 4 weeks before use and fed at 2% of body weight once per day. Feeding was stopped 24 h prior to the experiment.

2.2. Experiments and sampling

To assess the effects of stress, the water depth in the flow-through tanks with fish was lowered from 20 to 3 cm. The fish density was between 50 and 100 g body weight/L. Fish were sampled before initiation of stress as the initial control, and 30 min and 24 h after the water depth was lowered. All fish in each tank were caught at the same time and were anesthetized with ethyl 4-aminobenzoate (Benzocaine, 0.05 g/L, Wako, Osaka). Blood was collected from caudal vessels using a heparinized syringe in less than 5 min after onset of anesthetization. Blood was separated into two aliquots. One aliquot was centrifuged at $5000 \times g$ for 5 min, and the plasma was stored at -80 °C until the cortisol, lysozyme, and IgM levels were measured. The other aliquot was diluted to 1:2 in minimum essential medium (MEM) with Eagle's salt (Sigma, MO) containing 0.2% heparin sodium and buffered with 7.5% $NaHCO_3$ (pH 7.6). The mixture was placed on a 54% Percoll

(Amersham Biosciences, NJ) cushion and centrifuged at $400 \times g$ for 25 min. The leucocyte band was harvested, twice washed with phosphate-buffered saline (PBS, pH 7.6), and centrifuged at $500 \times g$ for 5 min. Contamination with erythrocytes was less than 1%. The isolated PBL fraction was counted by a hemocytometer (Bio Medical Science, Tokyo), placed in RNA later® (Ambion, Austin, TX), and stored at -20 °C until mRNA was extracted.

2.3. Plasma analysis

Plasma cortisol concentrations were determined by an enzyme-linked immunosorbent assay (ELISA) (Oxford Biomedical Research, Oxford, MI). Plasma levels of lysozyme activity were measured by a turbidimetric method based on lysis of freeze-dried particles of *Micrococcus lysodeikticus* (Sigma) using hen egg white lysozyme (Sigma) as a standard (Takemura and Takano, 1995). Plasma levels of IgM were estimated using ELISA as described by Nagae et al. (1993). Absorbance was measured using a microplate spectrophotometer (SpectraMax 190, Nihon Molecular Devices, Osaka).

2.4. RNA extraction and real-time PCR

Total RNA from PBL was extracted using ISOGEN® (Nippon Gene, Toyama) according to the manufacturer's instruction, and RNA concentrations were determined using a microplate spectrophotometer. Total RNA was then treated with RNase-free DNase I (Takara, Shiga). After inactivation of DNase, reverse transcription was carried out using a PrimeScript® RT Reagent kit (Takara).

Real-time PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Quantification of trout β-actin, IL1-β, GR-1, GR-2, or MR was performed as described, previously (Yada et al., 2005, 2007; Yada, 2012). As the standard for insulin-like growth factor (IGF)-I and caspase-6, the cDNA fragments were amplified from rainbow trout liver cDNA with AmpliTaq Gold® DNA polymerase (Applied Biosystems) and primers that were designed based on Shamblott and Chen (1992) and Laing et al. (2001) (Table 1). The amplification regime was 30 cycles consisting of 94 °C for 1 min, 56 °C for 30 s, and 72 °C for 1 min. PCR products were separated by agarose gel electrophoresis, and purified using GENECLEAN® (QBIOgene, CA). The PCR mixture (20 µl) contained Power SYBR Green PCR Master Mix (Applied Biosystems), 300 nM each of forward and reverse primers and either a standard (6×10^2–6×10^7 copies/reaction) or a sample that was a product of reverse-transcribed RNA (0.04–40 ng/reaction). After denaturation at 95 °C for 10 min, amplification was carried out with 45 cycles at 95 °C for 15 s and at 60 °C for 1 min. mRNA levels were standardized as mol/g RNA in each sample and expressed as the relative expression to the mean of the initial sampling period or of the unstressed group.

2.5. Proliferation and apoptosis

The proliferative activity of PBL from the unstressed or stressed fish was estimated as an in-vitro incorporation of 5-bromo-2′deoxy-uridine (BrdU) using commercial reagents (BrdU labeling and detection kit III, Roche Diagnostics, Mannheim). Leucocytes were isolated as described above and incubated overnight with a medium containing BrdU. Then,

Table 1
Design of primers for cloning of standard cDNAs and real-time PCR for trout IGF-I and caspase-6.

		Forward primer	Reverse primer
IGF-I	Standard	5′-TGGAGAGAGAGGCTTTTATTTCAGTAA-3′	5′-TCCTACGCTCTGTGCCTCTGT-3′
	Real-time PCR	5′-AATGTACTGTGCCCCTGTCAAGT-3′	5′-GGTGTTCTTGGCATGTCTGTGT-3′
Caspase-6	Standard	5′-GACAACAAAACATCCCAGACGA-3′	5′-TGCCCACCAGGCTCTTACAC-3′
	Real-time PCR	5′-GGAGCACTTCTTCTGGCATCTG-3′	5′-ATTGGAGCGGTCAGCATTG-3′

the incorporation of BrdU was estimated by enzyme immunoassay using an anti-BrdU-serum. Absorbance was measured at 370 nm by a microplate reader. Other leucocyte samples were used for the flow cytometric measurements. The percentages of fragmented leucocytes at the final stages of apoptosis were estimated by a hand-held automated cell counter (Scepter 2.0, Merck Millipore, MA) with Scepter Sensors-40 μm (Merch Millipore) as in the manufacture's manual.

2.6. Culture

PBL were isolated from the unstressed fish reared in indoor 240-L flow-through tanks or the stressed fish kept in shallow water for 24 h as described above. The cells were adjusted to 1×10^7 cells/ml MEM with 0.5% trout serum; 100 μl were seeded into 96-well microplates (Iwaki, Tokyo), and were incubated with LPS and/or cortisol at 15 °C under an atmosphere of 95% air/5% CO_2 for 48 h. Cortisol and LPS were purchased from Sigma. Cortisol was dissolved in ethanol at 1 μg/μl and diluted with MEM. After the culture, total RNA was extracted, treated with RNase-free DNase I, and reverse transcription of RNA was carried out as described above.

2.7. Statistical analyses

Significance of difference for in-vivo experiments between two groups was analyzed by the Mann-Whitney U test. Calculations were performed using Nonparametric Statistics by VBA macro-programs for Windows (Publication Department of Medical Books, Shinko Trading Company, Tokyo).

3. Results

Plasma cortisol levels significantly increased 30 min after the onset of confinement and crowding stress in shallow water, and the high levels were sustained for 24 h (Fig. 1). In contrast, plasma lysozyme levels had decreased after fish were in shallow water for 24 h when compared with the initial unstressed fish. Stress in shallow water also suppressed plasma IgM levels significantly. In PBL isolated from stressed fish, there was no significant change in expression levels of β-actin. However, we found significant increases in mRNA levels of the mitotic and apoptotic genes, IGF-I and caspase-6. Stress in shallow water produced a significant decrease in mRNA levels of the pro-inflammatory cytokine, IL-1 β (Fig. 2). Among CR genes, GR-2 mRNA levels showed a three-times transient elevation 30 min after the start of treatment. Then, confinement and crowding stress for 24 h resulted in one-third, and fifteenth decreases, in GR-2 and MR mRNA levels, respectively. There was no significant change in mRNA levels of GR-1 after stress.

After exposure to stress in shallow water for 24 h, proliferative activity as incorporation of BrdU and apoptotic cell death as fragmentation were compared between PBL from the unstressed and stressed fish (Table 2). There was no significant difference in the cell yield and ratio of fragmented cells in PBL from the unstressed and stressed fish. In contrast, BrdU incorporation in PBL from the stressed fish was significantly lower than that from the unstressed fish.

In PBL isolated from the unstressed trout, IL-1 β mRNA levels increased significantly after in-vitro administration of LPS, but cortisol levels did not (Fig. 3). MR, GR-1, and -2 mRNA levels were stimulated by administration of both cortisol and LPS. Simultaneous administration of cortisol and LPS resulted in significant increases in mRNA levels of IL-1 β and three CRs. In PBL from the stressed trout, IL-1 β mRNA response to cortisol and LPS was essentially similar to that from the unstressed fish (Fig. 3). In contrast, CR mRNA levels in PBL from the stressed fish did not respond to cortisol and/or LPS administration.

Proliferative activity was quantified in PBL isolated from the unstressed and stressed trout (Fig. 4). Cortisol showed a significant inhibitory effect on the proliferation of both PBL from the unstressed and stressed trout. Proliferation of PBL from the unstressed fish was

stimulated by LPS; however, there was no significant effect of LPS on PBL from the stressed fish. Simultaneous administration of cortisol and LPS showed no influence on BrdU incorporation in PBL in either the unstressed or stressed groups.

Apoptotic cell death of PBL from the unstressed and stressed trout was estimated as the percentage of fragmented cells (Fig. 4). The stimulatory effects of cortisol on the ratios of fragmented cells were shown with or without LPS in PBL both from the unstressed and stressed trout. The difference between the unstressed and stressed fish was observed in the response of cell fragmentation against LPS administration. A significant effect of LPS was shown in PBL from the stressed fish, but not in the cells from the unstressed group.

4. Discussion

The present study demonstrated that stress from confinement and crowding in shallow water ablates the response of CR gene expression in trout leucocytes to in-vitro administration of cortisol and LPS. Significant down-regulation of CR genes in trout leucocytes by chronic stress for 24 h agreed with the results of previous studies of acute stress and salinity acclimation (Yada et al., 2007, 2008). In accordance with the in-vivo decrease in CR mRNA levels, the suppressive effect of stress on the fish immune system seems to be modulated by the expression of CRs, at least in mRNA levels. Using radioligand binding assay, Maule and Schreck (1991) revealed that chronic stress in coho salmon (O. kisutch) in shallow water lowers the affinity of CR in leucocytes isolated from the spleen and anterior kidney. The results in the present study show that stress influences, at least, the transcriptional processing of CR expression in the fish immune system.

However, effects of cortisol on PBL, and the decrease in proliferation and increase in cell death, were unchanged by the stress in shallow water. On the contrary, mitotic effects of LPS were attenuated in the stressed fish. Stress possibly alters the translational processing of CR, then ablates the response in proliferation to cortisol. In the brain of rainbow trout, the CR protein content detected with the antibody raised against recombinant trout CR were decreased by treatment with an endocrine disruptor, salicylate (Gravel and Vijayan, 2006). Furthermore, in coho salmon leucocytes, the affinity and number of CR differ in their response to different stressors as well as in their distribution in immune tissues (Maule and Schreck, 1991). In this study, the proliferative activity of PBL estimated by BrdU incorporation was lowered after shallow-water stress, implying cortisol-mediated immunosuppression. Differences in proliferative response and cell fragmentation against in-vitro LPS administration between the unstressed and stressed fish suggest a modification of leucocyte-responsiveness against immunostimulation by stress-related endocrine change. Translational regulation of CR in the fish immune system should be detailed in future studies of the mechanism for desensitization of the stress response.

IL-1 β is an important cytokine for initiating inflammatory responses and mediating regulation of other cytokines (Delves et al., 2017). In the present study, the responsiveness of IL-1 β to administration of an endotoxin, LPS, in PBL of the stressed trout was similar to the unstressed fish. In Atlantic salmon (Salmo salar), the effect of daily repeated stress by handling influenced LPS-stimulated IL-1 β expression in phagocytic leucocytes. However, the effect seemed to depend on the duration of stress; that is, it was stimulatory for week 1, then inhibitory for weeks 2 and 3, and it had no effect from week 4 (Fast et al., 2008). It should be noted that the duration of the stress in that experiment might have been too short to have affected LPS-stimulated IL-1 β expression, because in the present study, the stimulatory effect of LPS on CR was ablated. The results of the study by Fast et al. (2008) reflect differences in the regulation between the immune and endocrine systems in trout blood leucocytes responding to stress. As in mammals, LPS is thought to activate pathogen recognition receptors in fish leucocytes, including the toll-like receptor family, and stimulate transcription of IL-1 β and other
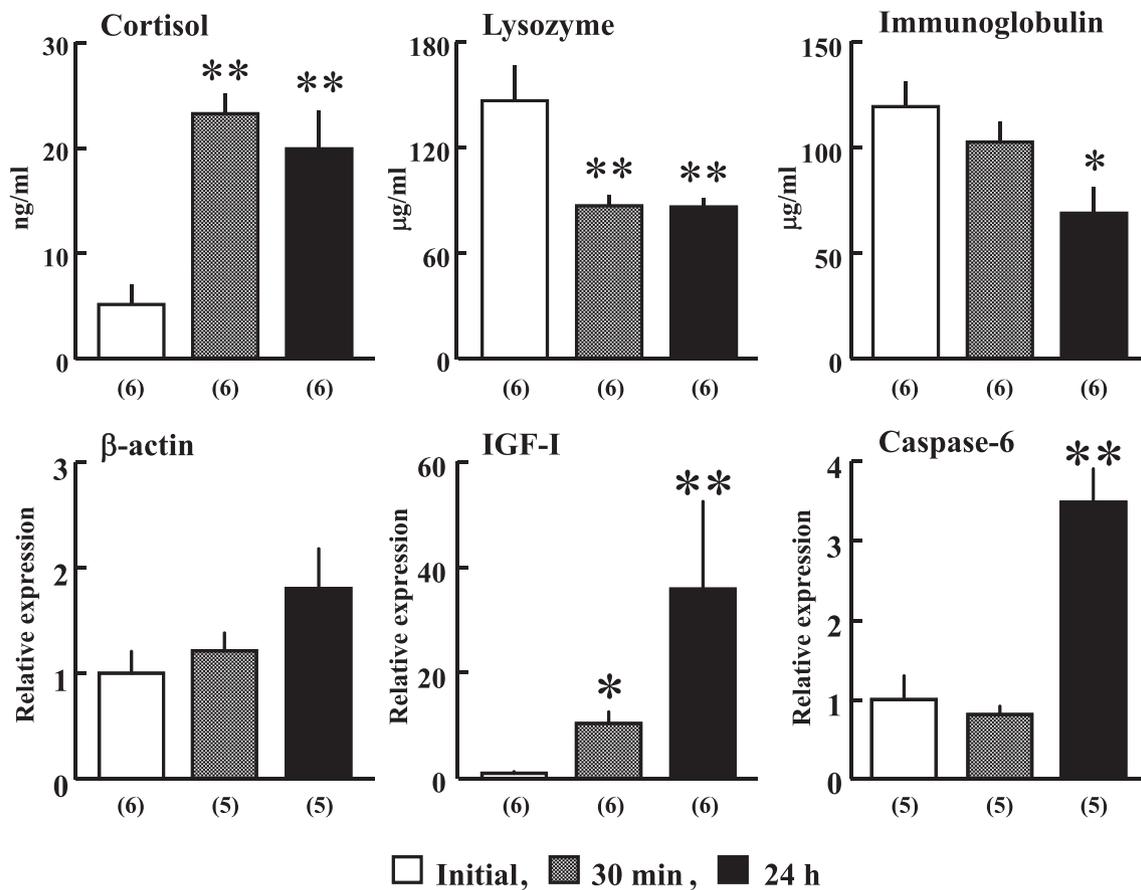
Fig. 1. Changes in plasma levels of cortisol, lysozyme, and immunoglobulin, and mRNA levels of β -actin, IGF-I, and caspase-6 in initial (open column) or stressed fish in shallow water for 30 min (shaded column) or 24 h (closed column). Data are expressed as means + standard error. Numbers are presented in parentheses. [*,**]Significantly different from the initial at $P < 0.05$ and 0.01, respectively.

inflammatory cytokine genes (Iliev et al., 2005; Swain et al., 2008). Modulation of the fish endocrine system by LPS treatment has been investigated, especially regarding the release of pituitary hormones (Balm et al., 1993, 1995). The effects of LPS on CR genes expressed in the immune system have also been examined in several other fish species. Gilthead seabream (*Sparus aurata*) possess at least one type of GR, and LPS injection stimulated mRNA levels of GR and IL-1 β expressed in the spleen (Acerete et al., 2007). In-vitro administration of LPS to carp (*Cyprinus carpio*) phagocytic leucocytes stimulated IL-1 β and GR-1 mRNA levels; however, there was a significant decrease in GR-2 to 50% of the level of the control (Stolte et al., 2008). In the present study, ablation of the LPS-response of CR mRNA levels in stressed trout suggested differential regulation of endocrine response against pathogens between species, genes, and the type and duration of stress.

There was no significant effect of in-vitro administration of cortisol at 200 nM (about 72 ng/ml) on IL-1 β mRNA levels in the PBL of both unstressed and stressed trout. That result coincided with previous observations in carp phagocytic leucocytes treated with cortisol around 100 nM (Engelsma et al., 2001; Stolte et al., 2008). However, high doses of cortisol at 320 and 400 ng/ml suppressed IL-1 β expression in both trout and carp, respectively (Zou et al., 2000; Engelsma et al., 2002). In the present study, 200 nM of cortisol was enough to stimulate CR mRNA levels in trout leucocytes. Expression of specific CR genes is more sensitive to cortisol stimulation than those of cytokine genes, at least regarding IL-1 β. However, a stress related elevation in IL-1 β mRNA levels is observed in the head kidney of carp and kidney leucocytes of Atlantic salmon (Metz et al., 2006; Fast et al., 2008). Although there was no significant change in PBL, chronic stress also caused increases in IL-1 β mRNA levels in the head kidney, spleen, and gills of trout (data

not shown). The lack of significant change in IL-1 β expression in PBL of the stressed trout could be due to differences in the tissues, species, stressors, or mitotic phase of leucocytes.

In the stressed trout, an elevation in plasma cortisol was followed by immunosuppression, such as depression of plasma lysozyme and IgM levels, as observed in the previous studies (Schreck, 1996; Mommsen et al., 1999; Wendelaar Bonga, 1997; Saurabh and Sahoo, 2008). An increase in mRNA levels of caspase-6, which is required for the signal transduction of apoptosis, was also observed in leucocytes of stressed fish. That result corresponded well to a previous observation of kidney leucocytes from stressed trout (Laing et al., 2001). Laing et al (2001) also indicated that cortisol-induced activation of caspase-6 in trout leucocytes was mediated through glucocorticoid receptor. Taken together, these results suggest that elevation of cortisol secretion in shallow water stress is followed by an activation of apoptosis in trout leucocytes, which finally results in immunosuppression.

Another possible influence of stress in shallow water on PBL is the alteration of mitotic status. Shallow water stress provoked almost a 40-times increase in IGF-I mRNA levels in PBL. That result seems inconsistent with well-established findings on stimulatory actions of IGF-I on body growth and cell proliferation for vertebrates including fish species (Duan, 1998; Wood et al., 2005; Hakuno and Takahashi, 2018). IGF-I also is a stimulatory factor in the immune system in general (Venters et al., 2001). In fish, in-vivo and in-vitro administration of IGF-I results in the enhancement of several immune functions in trout (Yada, 2009). Beside immune functions, an activation of IGF-I expression after cellular damage has been observed in muscular and ventricular systems in mammals (Reiss et al., 1994; Cheng and Du, 2007; McKay et al., 2008). Although details remain unclear, there seems to be a possible relationship between cellular damage and IGF-I expression in the fish
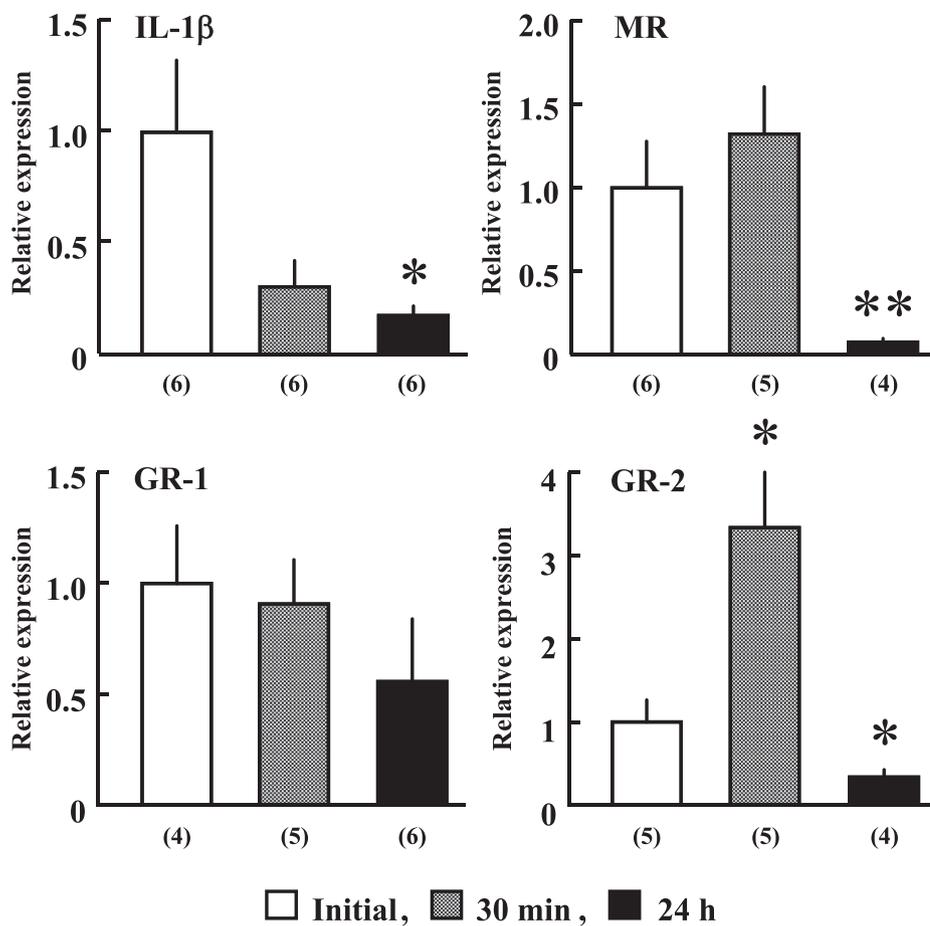
Fig. 2. Changes in mRNA levels of IL-1 β, MR, GR-1, and -2 in PBL from initial (open column) or stressed fish in shallow water for 30 min (shaded column) or 24 h (closed column). Data are expressed as mean + standard error. Numbers are presented in parentheses. [*],[**]Significantly different from the initial at $P < 0.05$ and 0.01, respectively.

Table 2

BrdU incorporation and cell fragmentation in PBL isolated from the intact and stressed fish.

	Unstressed	Stressed
n	8	8
Isolation yield (10^6 cell/ml blood)	10.10 ± 0.34[a]	8.59 ± 0.84
BrdU incorporation (absorbance)	0.55 ± 0.03	0.41 ± 0.02[b]
Cell fragmentation (percent)	1.71 ± 0.16	1.85 ± 0.21

[a] Values are expressed as means ± SE.

[b] Significantly different from values of intact at $P < 0.05$ by Mann-Whitney *U* test.

immune system.

Desensitization or tolerance after repeated and prolonged stress in relation to the regulatory system of cortisol secretion in both tetrapods and fish species has been thoroughly investigated (Wendelaar Bonga, 1997; Kusnecov et al., 2001; Barton, 2002). Hypothalamic and pituitary regulations of cortisol release in fish are influenced by the experience of stress (Vijayan and Leatherland, 1990; Rotllant et al., 2000). Cortisol does not seem to suppress all fish immune functions but acts as the regulator of leucocyte redistribution. In carp, cortisol induces the apoptosis in B-lymphocytes, whereas apoptosis of neutrophils is reduced by cortisol (Weyts et al., 1998a,b). This study did not detail subtypes of leucocytes, and there is a possibility that the ablation of responses to cortisol in CR mRNA levels or proliferative activity in PBL of the stressed trout arises from opposite responses that different types of leucocytes exhibit to stress and cortisol.

Down-regulation of CR mRNA levels was observed in leucocytes not only from peripheral blood and from the head kidney of seawater-acclimated trout (Yada et al., 2008); there were also increases in mRNA levels of CR genes in the gill and body kidney during the seawater acclimation of trout. This indicated differential regulation of CRs between the osmoregulatory and immune systems (Yada et al., 2007). A change in salinity with a disturbance of osmoregulation is an important environmental stressor in fish, which activates the hypothalamus-pituitary-interrenal axis (Wendelaar Bonga, 1997; McDonald and Milligan, 1997; Verburg-van Kemenade et al., 2009; Yada and Tort, 2016). In addition, in euryhaline fishes including the smolt of salmonids, a salinity change seems to be tolerated by osmoregulatory adaptability and does not suppress immune functions (Yada et al., 2008; Yada and Tort, 2016). Several innate immune functions are particularly enhanced in euryhaline fishes during acclimation to seawater (Yada and Tort, 2016). Thus, the regulatory system of stress response in the fish immune system should be investigated during desensitization to stressors as well as during adaptation to environmental changes.
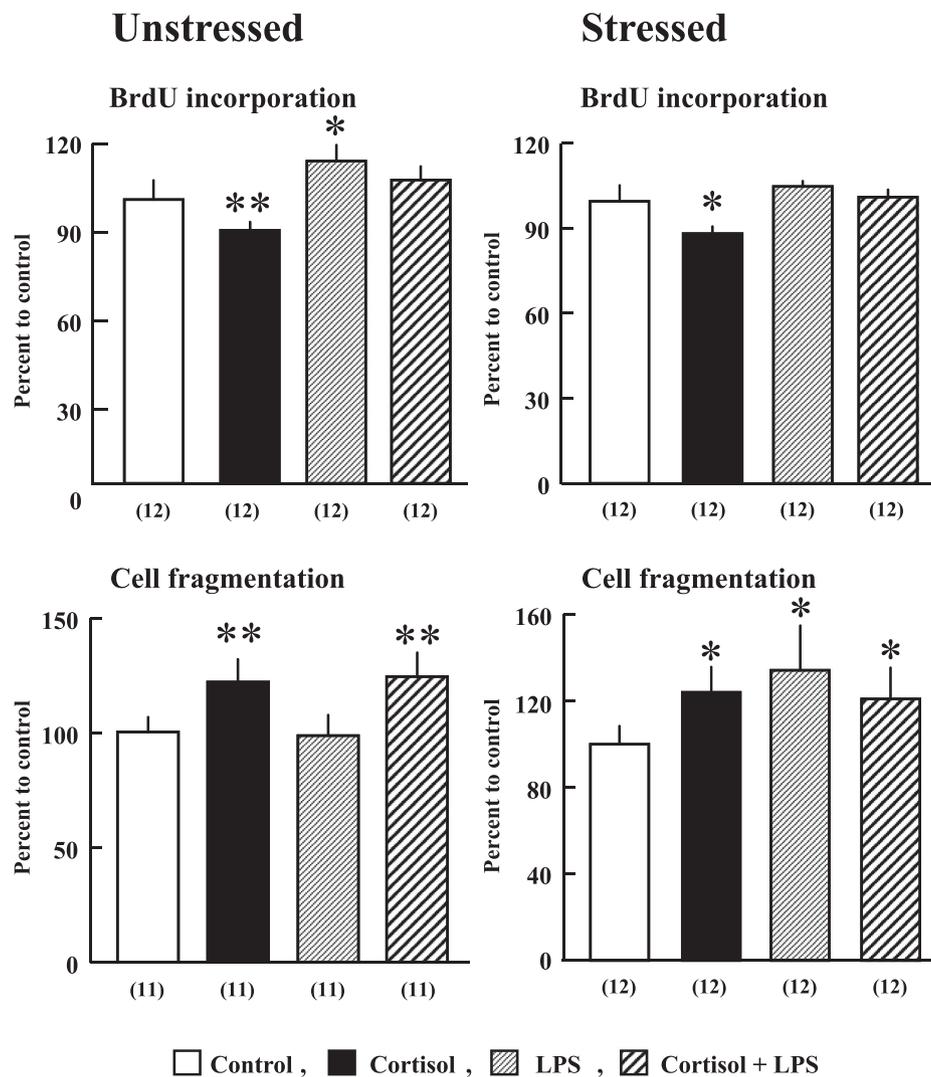
In summary, we revealed that down-regulation of CR expression in the PBL of stressed trout coincided with desensitization to but not with disappearance of immunosuppression by cortisol administration. Conversely, immunostimulatory effects of LPS were attenuated in the stressed fish. These findings imply a possibility that stress in fish modifies both endocrine effect on immunity and immune effect on endocrine regulation. Receptor-mediating action of cortisol in fish immunity during stress response might be involved in a loop interaction between immune and endocrine systems.

Acknowledgments
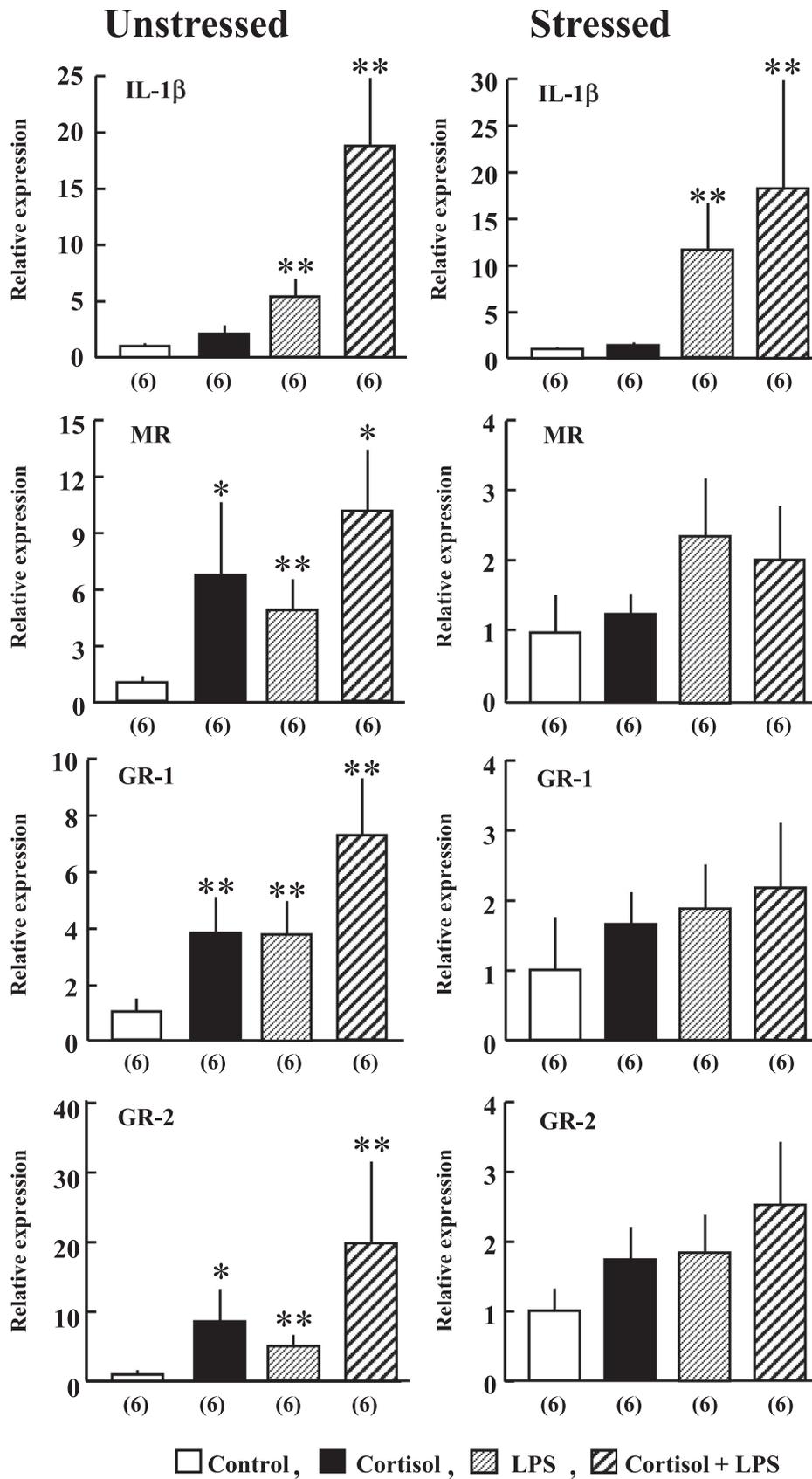
Fig. 3. Effects of cortisol (200 nM, closed column), LPS (10 μg/ml, shaded column), and cortisol + LPS (hatched column) on mRNA levels of IL-1β, MR, GR-1, and -2 expressed in PBL isolated from unstressed and stressed fish. Data are expressed as mean + standard error. Numbers are presented in parentheses. *,**Significantly different from the control (open column) at *P* < 0.05 and 0.01, respectively.

Unstressed

BrdU incorporation

Stressed

BrdU incorporation

Cell fragmentation

Cell fragmentation

□ Control , ■ Cortisol , ▨ LPS , ▨ Cortisol + LPS

Fig. 4. Effects of cortisol (200 nM, closed column), LPS (10 µg/ml, shaded column), and cortisol + LPS (hatched column) on BrdU incorporation and cell fragmentation in PBL isolated from unstressed or stressed fish. Data are expressed as percent to control and mean + standard error. Numbers are presented in parentheses. *,**Significantly different from the control (open column) at $P < 0.05$ and 0.01, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.04.011.

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