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Research Paper

Effect of severe environmental thermal stress on redox state in salmon

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ABSTRACT

Fish are exposed to many kinds of environmental stressors and the chances of succumbing to infectious diseases may be increased a result. For example, an acute increase in temperature can induce numerous physiological changes in the body. In the present study, we examined the redox state in response to a severe acute stress resulting from heat shock in teleost coho salmon (*Oncorhynchus kisutch*). The plasma lipid peroxides levels in fish gradually increased after heat shock treatment. By 2.5 h post-heat stress, plasma glutathione (GSH) levels had decreased, but they had returned to basal levels by 17.5 h post-stress. Plasma superoxide dismutase activities in stressed fish were significantly increased compared with those in control fish at 17.5 h post-stress, but had returned to basal levels by 48 h post-stress. Expression levels of hepatic GSH and heat shock protein 70 gradually increased after heat shock treatment. These results concerning the changing patterns of multiple important redox-related biomarkers suggest that severe thermal stressors can affect the redox state and induce oxidative stress in ectothermal animals, such as fish, *in vivo*. Hence, manipulation of appropriate thermal treatment may possibly be useful to control fish fitness.

Introduction

Aquatic organisms are exposed to local and global environmental stressors, such as pollutants and acute changes in temperature [1–6]. Exposure of organisms to stressors may result in a series of biochemical and physiological changes. At the organismal level, these changes are mediated by the neuroendocrine system. In addition to this neuroendocrine stress response, there is a cellular stress response following exposure to stressful situations. These stress responses in organisms affect their general health, disease resistance, growth, and reproduction [3,5–7]. An acute increase in temperature is known as heat shock and can induce numerous changes in the body. The physiological states of fish depend on the environmental temperature. As a result, temperature is an important factor influencing their biological geographic distribution. Furthermore, daily and seasonal temperature changes have an impact during the lifetime of individual fish [8]. Unfortunately, studies on the heat shock response in fish have primarily focused on the expression and characterization of cellular molecular chaperons, heat shock proteins (HSPs) [1,3,8–10].

Recently, in the course of studies on the fish fitness in response to a stress, we found that mild stress caused by handling as an acute physiological stressor regulates the expression of growth-related genes, such as growth hormone receptor (*ghr*) and insulin-like growth factor-1 (*igf1*) genes, in fish [11]. The growth of fish is known to be genetically regulated and to be also influenced by cellular, endocrinological, and environmental factors. The responses of endocrine tissue are affected by the integration of external stimuli with internal signals according to the physiological state [1,3,5–7,12–16]. Fish growth can be enhanced by improved nutrition, husbandry conditions, elevated temperature, and changes in the endocrine system of the animal [5,15,17]. Accordingly, it is of interest to determine the effects of severe stressors on the expressions of important genes such as growth-related genes in fish. It is needed to reveal the features of and the resulting effects of stressors on fish fitness in order to improve their production and health. In addition, fish are thought to be an ideal and a convenient model to examine the effects of thermal and other complex stressors on the organism for both short and long periods. This is because fish are a typical ectothermic vertebrate. However, little is known about the effects of acute increases in temperature, which should be severe stressors for fish, on conditions such as the redox state in fish.

In the present study, we examined the redox state in response to a severe stress derived from heat shock in teleost coho salmon (*Oncorhynchus kisutch*). Coho salmon is one of the most valued

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species used in aquaculture worldwide and is known to be susceptible to increases in temperature [18]. Additionally, in Japan, coho salmon farming is one of the basic industries in the north-eastern (Tohoku) Pacific coastal area, where the great earthquake and massive tsunami occurred in 2011. We discuss the relationships between the thermal stress responses and the redox states in fish in the context of our findings.

Materials and methods

Animal experiment

All experimental procedures were approved by the Animal Care Committee at Tohoku University (Sendai, Japan). Coho salmon *O. kisutch* were purchased from a local hatchery (Miyagi, Japan). After acclimatization for 2 weeks at the Aquarium Facility of Tohoku University, fish were exposed to heat shock (+11 °C for 2 h) and sampled at 2.5, 17.5, and 48 h after stress.

The fish (approximate body weight, 144 g) were reared in 60-L flow-through tanks at 8 °C (light/dark=12 h/12 h). Healthy, mixed sex, fish were divided into 4 groups ($n=8$). The fish in the first group were undisturbed (prestressed) fish used as a control, maintained under quiet and suitable conditions, and sampled at 13:30. The fish in the second group were subjected to heat shock from 9:00 to 11:00 and sampled at 2.5 h post-stress (at 13:30). The fish in the third group were subjected to the stressor from 18:00 to 20:00 and sampled at 17.5 h post-stress (at 13:30). The fish in the fourth group were also subjected to the stressor from 11:30 to 13:30 and sampled at 48 h post-stress (at 13:30). Accordingly, all tissues and blood for analysis were sampled at the same time, so that the effects of several factors, such as diurnal rhythm and photoperiod, on the expressions of redox state-related factors could be minimized.

Food was withheld for over 48 h before each sampling period. At each sampling period (2.5, 17.5, and 48 h post-stress), fish were sacrificed by an overdose of buffered MS222 (m-aminobenzoic acid ethyl ester methanesulfonate). Blood was withdrawn and plasma was separated by centrifugation. Fish were gutted, and the tissues were quickly removed. All plasma and tissue samples were frozen at −80 °C until analysis.

Measurements

Plasma cortisol and glucose levels

Plasma cortisol levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit from Oxford Biomedical Research, UK [19]. Plasma glucose was measured using an enzymatic assay method with a Glucose CII-Test Wako kit from Wako Pure Chemical Industries, Ltd., Japan.

Lipid peroxides, glutathione, superoxide dismutase, and heat shock protein 70 levels

Lipid peroxides (LPO) were determined as thiobarbituric acid reactive substances (TBARS) by a HPLC-fluorescence method [20]. TBARS concentrations were determined from a standard curve established with TBA-malondialdehyde (MDA, 1,1,3,3-tetramethoxypropane) adducts.

Glutathione (GSH) levels were determined by a glutathione reductase-recycling method with a Total Glutathione Quantification kit from Dojindo Laboratories, Japan. This kit can measure the total amount of reduced GSH and oxidized form of GSH (GSSG).

Superoxide dismutase (SOD) activity was assayed by the formazan-WST method (Total SOD Assay kit, Dojindo Laboratories, Japan).

Levels of HSP70 protein were determined by immunoblotting as described by Basu et al. (2001) [19]. Anti-HSP70 and anti-β-actin antibodies were purchased from Sigma-Aldrich, St. Louis, MO. The expressions of HSP70 were normalized by those of β-actin.

Protein contents were measured by a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

Statistical analysis

All samples were run in duplicate and results were expressed as means ± SEM. All data were subjected to one-way analysis of variance (ANOVA). Means were compared with the Tukey–Kramer multiple comparison test. Differences were considered to be statistically significant at $p<0.05$.

Results

Plasma cortisol and glucose levels

At 2.5 h post-heat stress, plasma cortisol levels had increased compared with those in control fish, but had returned to basal levels at 17.5 h post-stress (Fig. 1). At 2.5 and 17.5 h post-stress, plasma glucose levels had increased as compared with those in control fish (the average plasma glucose concentration in control fish was 66.4 mg/dL). However, at 48 h post-stress, plasma glucose levels in stressed fish had decreased and were not significantly different from those in control fish (data not shown).

Plasma LPO, GSH, and SOD levels

As shown in Fig. 2A, the plasma LPO levels in stressed fish gradually increased after heat shock treatment, and had significantly increased compared with those in control fish at 17.5 and 48 h post-stress.

At 2.5 h post-heat stress, plasma GSH levels had decreased, but had returned to basal levels at 17.5 h post-stress (Fig. 2B). At 48 h post-stress, plasma GSH levels in stressed fish had increased significantly as compared with those in control fish.

Plasma SOD activities in stressed fish had increased significantly compared with those in control fish at 17.5 h post-stress, but had returned to basal levels at 48 h post-stress (Fig. 2C).

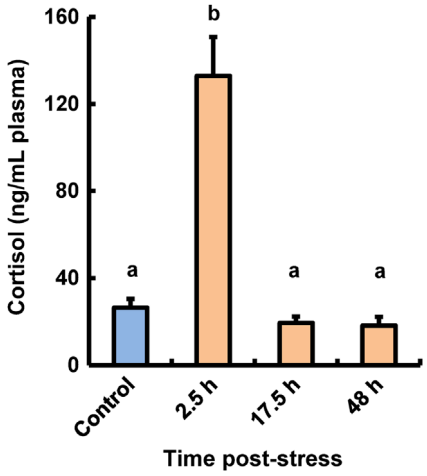

Fig. 1. Effect of thermal stressors on cortisol levels in plasma of coho salmon *O. kisutch*. Data represent means ± SEM ($n=8$). Statistical relationships between groups are indicated by letters where significant differences were detected ($p<0.05$).

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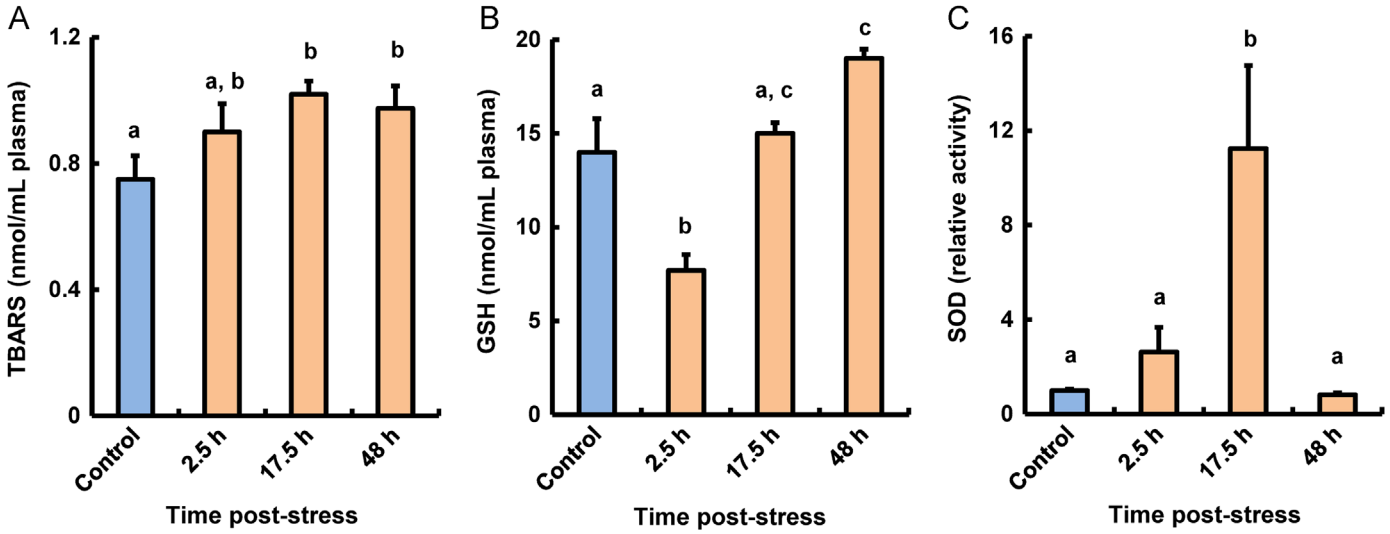

Fig. 2. Effect of thermal stressors on LPO (A), GSH (B), and SOD (C) levels in plasma of coho salmon *O. kisutch*. Data represent means ± SEM ($n=5$). Statistical relationships between groups are indicated by letters where significant differences were detected ($p<0.05$).

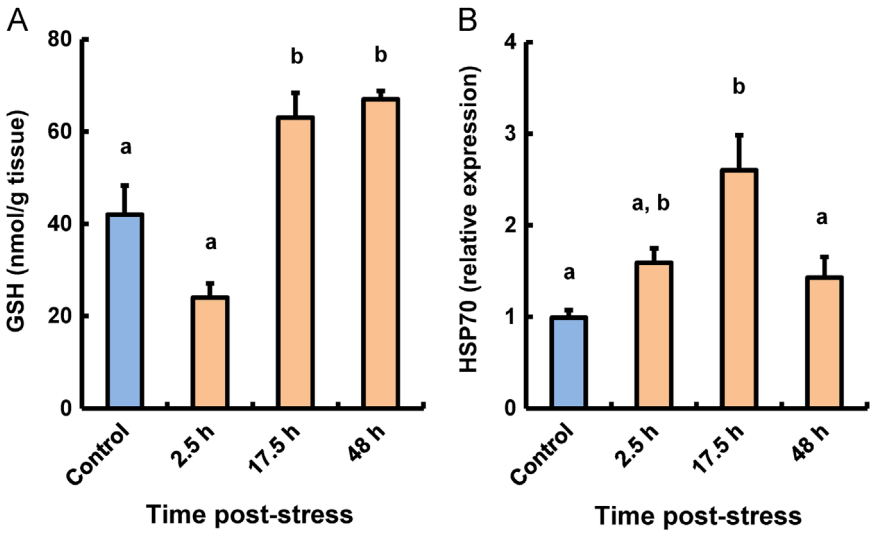

Fig. 3. Effect of thermal stressors on GSH (A) and HSP70 (B) levels in the liver of coho salmon *O. kisutch*. Data represent means ± SEM ($n=5$). Statistical relationships between groups are indicated by letters where significant differences were detected ($p<0.05$).

Hepatic GSH and HSP70 levels

Hepatic GSH levels in stressed fish at 17.5 and 48 h post-stress had significantly increased compared with those in control fish (Fig. 3A). The changing patterns of hepatic GSH levels in stressed fish were similar to those in the plasma of stressed fish (Figs. 2B and 3A).

Hepatic HSP70 expression levels are shown in Fig. 3B. Hepatic HSP70 expression levels gradually increased after heat shock treatment and reached their maximum values at 17.5 h post-stress.

Discussion

The environmental temperature can induce numerous physiological changes in the biological functions of organisms. In particular, increased temperature can result in serious damage to cold-blooded fish species and cause metabolic stress in the body. Increased environmental temperature results in increased oxygen consumption and stimulates various metabolic processes on the basis of known thermodynamic principles [21,22]. In the present study, it was shown that heat shock-induced severe thermal stressors can influence several redox-related biomarkers and the redox homeostasis in teleost coho salmon.

LPO levels in fish tissues change under various stressful conditions and are known to be a sensitive indicator of damage to various tissues under different environmental conditions [4,15,23–29]. In this study, the plasma LPO levels in fish exposed to heat shock were observed to increase. These LPO in stressed fish plasma are considered to be metabolites derived from various damaged tissues. Accordingly, severe stressors caused by heat shock should result in damage to various tissues of coho salmon.

Fish have both enzymatic and non-enzymatic antioxidative defense systems against reactive oxygen species (ROS)-related damage [4,21]. Reduced GSH, the major nonprotein cellular thiol, is a cysteine-containing tripeptide (γ-glutamylcysteinylglycine) with reducing and nucleophilic properties that is one of the major regulators of the intracellular redox state and plays an important role in the non-enzymatic defense system [4,21,30–32]. GSH is also known to be the substrate for glutathione peroxidase, an antioxidative enzyme that

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scavenges ROS and LPO generated within cells [4,21,33,34]. The changing patterns of GSH levels in both plasma and liver observed in this study were similar to those in the livers of fish that were administered with an oxidant [25,35,36]. In the muscle and gill, GSH levels increased in response to thermal stress (data not shown). At the initial post-heat stress stage, plasma GSH may be consumed to eliminate ROS generated in blood. After 17.5 and 48 h of recovery, GSH plasma levels gradually increased, which suggested enhanced synthesis and transport of GSH from the liver. The liver is known to be the major source of GSH in vertebrates [32,37].

Antioxidative enzymes, such as SOD, glutathione peroxidase, and catalase, can scavenge radicals and contribute to the body's enzymatic antioxidative defenses. The changes in the expression of antioxidative enzymes in fish have been observed with regard to stress [22,36,38,39]. In particular, SODs catalyze the reaction of dismutation of superoxide ($O_2^{\bullet}-$) and H_2O_2, and are considered to play key roles in the first step of the enzymatic antioxidative defense system [4,21,22,40-42]. In this study, plasma SOD activities in stressed fish showed a transient increase at 17.5 h post-stress. Hence, increased SOD expression might neutralize the harmful effects of superoxides for the initial period of stressful conditions in tissues.

Organisms maintain a balance between generation and neutralization of ROS under normal physiological conditions. However, heat exposure and enhanced oxygen consumption are considered to promote the generation of ROS, such as $O_2^{\bullet}-$, H_2O_2, hydroxyl radical ($^{\bullet}OH$), and peroxyl radical ($ROO^{\bullet}$). The resulting ROS exceed organismal scavenging capacity and attack cell components, such as nucleic acids, proteins, lipids, and membranes [4,21,22,36,40,43]. ROS production in cells, especially in the mitochondria, has been found to be increased in exercised mammalian muscle, heat-stressed bivalve gills, chicken muscles, lugworm, and cultured cells as compared with non-stressed control tissue [21,26,44–48]. The induction of various HSP families regarding environmental stressors, such as heat shock, bacterial pathogens, and pollutants, has been reported in cell lines and various tissues of fish [1,8–10,19]. In this study, HSP70 was also induced in stressed fish liver. HSP70 is known to assist the folding of ascent polypeptide and mediate the repair of denatured proteins, the breakdown and replacement of the proteins that are not repairable [1,8]. Therefore, the induction of hepatic HSP70, which was observed in stressed fish in this study, indicates high level of protein damage was induced by heat shock. Thus, the present results regarding the expression patterns of multiple redox-related biomarkers, such as LPO, GSH, SOD, and HSP, in response to thermal stressors suggest that severe thermal stress due to heat shock induces oxidative stress in coho salmon, which may enhance oxidation in the body and result in damage to tissues. Under oxidative stress conditions, the levels of antioxidative substances, such as GSH and SOD, may increase due to their *de novo* synthesis to protect tissues against oxidative damage. In addition, a redox state, such as antioxidative state, has already been reported to modulate the synthesis of HSP in mammalian tissue [49].

In fish, pituitary-secreted growth hormone (GH) - liver-derived insulin-like growth factor (IGF)-1 axis plays a critical role in the regulation of both growth and development. Secretion of GH is known to be under hypothalamic regulation by means of many modulators [12,14,16,50,51]. Additionally, the GH-IGF-1 axis might be influenced by stressors, such as temperature and oxygen levels. In practice, muscular GH receptors (GHR) protein expression in fish has been observed to increase after heat shock treatment [13]. We recently found that hepatic *igf1* expression rapidly increased at 1.5 h post-stress without a change in *ghr*, whereas both *ghr* and *igf1* levels decreased at 16 h after mild-stress treatment [11]. Accordingly, the expression of *igf1* gene seems to be independently affected by stress other than GH and its signal transduction through GHR. These results suggest that growth-related gene expressions could be affected differently by the types and strength of stress, and thermal and handling stress could have positive effects on the growth-related factor expressions in fish. Consequently, it is of interest to determine the transcriptomic features regarding growth-related gene expressions in fish in response to heat shock-induced oxidative stress.

Intercellular signaling is known to be often affected by ROS or a pro-oxidative shift in the redox state resulting in the up- or down-regulation of the expressions of several genes and proteins [3,21,22,26,30,36,43,46,48,52–54]. We have previously observed that an antioxidative supplement, such as astaxanthin, known as a non-enzymatic small molecular component of the antioxidative defense system *in vivo*, could dramatically reduce oxidative stress-induced damage in fish [2,3,27,55,56]. Antioxidative substances could play a critical role in the tolerance against oxidative stress by organisms. Thus, the possible beneficial effects of antioxidative supplements in oxidative stressed fish should be determined.

In conclusion, the results of this study provide information that may be useful for improving fish fitness. An oxidative stress recently became a common theme in relation to the impact of climate change, such as climate warming, on natural ecosystems [21]. It is known that severe oxidative stress due to ROS leads to oxidative damage *in vivo*. However, a moderate level of oxidative stress could modulate cellular functions and have positive effects on animal health [57–60]. Accordingly, manipulation of appropriate thermal treatment could be employed to control and improve the health and production of fish. Further studies are now in progress to reveal the relationships between the redox state, oxidative stress, growth-related factors, and the fitness in fish.

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