Inhibitory Effect of Cortisol on the Defense Activities of Tilapia Neutrophils *in Vitro*

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ABSTRACT—On the premise that fish neutrophil activities are depressed under stressed conditions, this study investigated the direct effect of cortisol on the *in vitro* activity of neutrophils collected from tilapia *Oreochromis niloticus*. Neutrophils were obtained from swim bladders and exposed to different levels of cortisol (0–1000 ng/mL), and their chemotactic, phagocytic and respiratory burst activities were assessed. This treatment suppressed neutrophil activities as seen in the reduction of chemotactic, phagocytic and respiratory burst activities. Neutrophil activities appeared to be suppressed in a dose-dependent manner leading to a considerable reduction in neutrophil activities with high concentrations of cortisol. Nevertheless, we showed that the respiratory burst activity was able to recover from the observed effect after cortisol was removed. The results in this study strongly suggest that cortisol has a directly adverse effect on fish neutrophil defense activities, but that the effect is reversible.

Key words: cortisol, neutrophil activity, *Oreochromis niloticus*, tilapia, stress

Stress has been well documented for its immuno-suppressive actions. In teleost fish, immunological changes associated with stress have been studied for almost twenty years (Ellis, 1981). Corticosteroids, produced by the activation of HPI (hypothalamus-pituitary-interrenal) axis which stress stimulates, are considered to be important mediators of the immune response (Balm, 1997; Sumpter, 1997). Cortisol is released from interrenal tissue after the stimulation of higher brain centers in fish (Balm, 1997) and is widely recognized as an immuno-suppressive agent both in mammals (Oda and Katori, 1992) and in fish.

Kurogi and Iida (1999) demonstrated that social hierarchy in tilapia induced suppression of neutrophil defense activities in subordinate fish and that cortisol concentrations in blood plasma of the subordinate fish were significantly higher than dominant fish. Much attention has been paid to the interactions between cortisol and fish lymphocytes (Tripp *et al.*, 1987). However, little is known whether cortisol influences neutrophil activity, even though neutrophils play an important role in the first stages of infection. In this study, we aimed to reveal

the effect of cortisol on neutrophil defense activities *in vitro* by means of analysing their chemotactic, phagocytic and respiratory burst activities.

Materials and Methods

Fish

Tilapia (*Oreochromis niloticus*) of 76 g mean weight (hatched and raised in our laboratory at Miyazaki University) were used in this study. The fish were maintained for at least 3 wk in 150 L plastic tanks containing constantly aerated water at 25°C. They were fed daily with a commercial diet.

Preparation of cortisol solution

Cortisol (hydrocortisone; Wako) stock solution was prepared according to the method described by Tripp *et al.* (1987). Briefly, the stock solution was prepared by dissolving an appropriate quantity of cortisol in 95% ethanol, allowing the ethanol to completely evaporate, and re-suspending the residual cortisol in Hanks' balanced salt solution without phenol red (HBSS). The solution was filter-sterilized and the final concentration was measured using a commercial kit (Enzaplata cortisol; Chiba Corning Diagnostics Inc.) prior to use.

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Neutrophil preparation

Neutrophils used in this experiment were collected from the tilapia swim bladders. The collecting procedure was in accordance with the method described previously by Endo *et al.* (1997). Collected neutrophils were washed twice by centrifugation at $200\times g$ for 5 min and re-suspended in HBSS.

Neutrophil activities

Chemotactic activity was estimated by using a modified Boyden chamber, for 3 h, according to Nagamura and Wakabayashi (1985). Zymosan-treated normal tilapia sera were used as a chemotactic factor. The neutrophils in the filter were stained with hematoxylin, and the number of cells migrating more than $80\text{ }\mu\text{m}$ were counted under a microscope.

Phagocytic activity for 1 h against opsonized zymosan, calculated as index of phagocytosis (Le Morvan *et al.*, 1997), and respiratory burst activity for 10 min, measured as CLA-enhanced chemiluminescent response, were assessed as previously reported (Kurogi and Iida, 1999).

Cortisol treatment

The neutrophil density was adjusted to 1×10^6 cells/mL with HBSS containing different concentrations of cortisol (0, 1, 10, 100, 1000 ng/mL) and incubated at 25°C for 1 h (Stave and Robertson, 1985). After the incubation, the defense activities of the neutrophils were estimated. Because cortisol was contained in the neutrophil suspensions during the measurements of the activities, total cortisol treatments were for 4 h, 2 h or 70 min for the chemotactic, phagocytic or respiratory burst activities, respectively. These assessments were repeated 5 times each.

Restoration of neutrophil respiratory burst activity from the effect of cortisol was determined by removal of cortisol, 1000 ng/mL, from the mixture. The cortisol solution was removed from the mixtures by centrifuging at $200\times g$ for 5 min twice after 1 h incubation. Washed neutrophils were then divided into 2 parts; half was replaced with cortisol solution (1000 ng/mL) and the other was put in HBSS. The activity was immediately assessed. The experiment was repeated 5 times.

Statistic analysis

All data were analysed by Student's *t*-test and significant differences were determined at $P<0.05$.

Results

Neutrophil activities

Chemotaxis of neutrophils was depressed by cortisol in a dose-dependent manner as seen in Fig. 1. Percentage of the neutrophils migrating more than $80\text{ }\mu\text{m}$ in cortisol concentrations of 100 and 1000 ng/mL were

Fig. 1. Effect of cortisol concentrations on chemotactic activity of tilapia neutrophils *in vitro*. Chemotaxis expressed as percentage of the neutrophils migrating more than $80\text{ }\mu\text{m}$. Bars with asterisks are significantly different from 0, control ($p<0.05$). $n=5$.

Fig. 2. Effect of cortisol concentrations on phagocytic activity of tilapia neutrophils *in vitro*. Phagocytosis calculated as index of phagocytosis. Bars with asterisks are significantly different from 0, control ($p<0.05$). $n=5$.

1.9 ± 1.8 and 0.7 ± 0.6 , respectively, with significant decreases compared with the control value ($6.8\pm 1.9\%$).

Reduced phagocytosis and respiratory burst of the neutrophils in cortisol solution were observed as seen in Figs. 2 and 3 respectively. These reductions by cortisol were also detected dose-dependently. At more than 10 ng/mL, phagocytosis was suppressed significantly from control, and at more than 100 ng/mL, respiratory burst was repressed significantly from control.

Fig. 3. Effect of cortisol concentrations on respiratory burst of tilapia neutrophils *in vitro*. Respiratory burst expressed as chemiluminescent response (counts/10 min). Bars with asterisks are significantly different from 0, control ($p < 0.05$). $n = 5$.

Recovery from the effect of cortisol

One hour after incubating neutrophils in HBSS, the respiratory burst activity of neutrophils slightly decreased, without significant difference from the activity before incubation. However, incubation for 1 h in cortisol solution (1000 ng/mL) significantly reduced the activity. On the other hand, remove of cortisol after 1 h incubation in the cortisol solution almost completely restored the activity (Table 1).

Table 1. Restoration of respiratory burst of tilapia neutrophils from adverse effect of cortisol *in vitro*

Culture solution	Respiratory burst (counts/10 min) ^{a)}	
	Incubation time (min) 0 ^{b)}	60
Control (HBSS) ^{c)}	297.4±70.5	241.2±18.9
Cortisol ^{d)}	198.8±57.3*	126.7±83.2*
Cortisol-HBSS ^{d)}	(198.8±57.3*)	216.3±95.0

^{a)} Respiratory burst was measured as chemiluminescence for 10 min (counts/10 min).

^{b)} The neutrophils were assessed their ability in HBSS or cortisol solution immediately after the collection.

^{c)} Control neutrophils were incubated with HBSS for 60 min.

^{d)} After neutrophils were incubated with cortisol solution for 60 min, neutrophils washed with HBSS by centrifugation were then divided into 2 parts; half was replaced with cortisol solution (Cortisol) and the other was put in HBSS (Cortisol-HBSS).

Asterisks indicate significant differences from control.

$n = 5$

Discussion

The immunological changes associated with the response of fish to stress have received considerable attention since the early 1980s (Ellis, 1981). The stimulation of higher brain center triggered by stress evokes HPI (hypothalamo-pituitary-interrenal) axis actions and produces many kinds of hormones such as corticotropin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), catecholamine and cortisol through the stimulation processes reviewed by Weyts *et al.* (1999).

Recently research has focused upon the effect of "stress hormones" on fish defense mechanisms. In channel catfish *Ictalurus punctatus*, Ellsaesser and Clem (1987) showed cortisol treatment *in vivo* led to lymphopenia and neutrophilia. Nagae *et al.* (1994) demonstrated that cortisol suppressed plasma immunoglobulin M in masu salmon *Oncorhynchus masou*. Reduced generation of antibody-producing cells in coho salmon *O. kisutch* has also been attributed to cortisol (Maule *et al.*, 1987). A considerable amount of evidence suggests that physiological consequences through the activation of the HPI are as a direct result of elevated cortisol concentrations that occur in response to stress. Moreover, previous *in vitro* studies showed the inhibition of chemiluminescence response of phagocytes in striped bass *Morone saxatilis* (Stave and Robertson, 1985), reduced B cell activation in coho salmon (Tripp *et al.*, 1987) and suppressed nitric oxide production in goldfish *Carassius auratus* macrophages by cortisol (Wang and Belosevic, 1995).

The results of this study show that cortisol has drastic dose-dependent effects on fish neutrophil defense activities *in vitro*, which can be seen as a clear reduction in their chemotactic, phagocytic and respiratory burst activities at high doses. Concentrations less than 1 ng/mL (1–1000 pg/mL) indicated neither an increase nor decrease in the activities compared to control of 0 ng/mL (data not shown). Our findings suggest that increased plasma cortisol in fish would depress neutrophil defense mechanisms. Previously, we identified impaired neutrophil defense activities in socially stressed fish (Kurogi and Iida, 1999), where the cortisol concentration of the stressed fish was about 20 ng/mL in plasma. The down-regulation of the activities in that study was most likely due to the direct effect of cortisol. In addition, we suggest that the neutrophil activity can be restored once cortisol is eliminated. This outcome is in accordance with a similar report in humans (Dandona *et al.*, 1999).

Some studies have indicated that glucocorticoids depressed porcine neutrophil activities such as migration and chemiluminescence *in vitro* (Salak *et al.*, 1993), and respiratory burst of human neutrophils were also depressed by cortisol (Umeki and Soejima, 1990). However, neutrophils are not always adversely affected by cortisol. In fact, *in vitro* chemotaxis of cattle neutro-

phils was increased by dexamethasone (Anderson *et al.*, 1999). In teleost, Weyts *et al.* (1998) have shown that respiratory burst activity of carp neutrophils was not influenced by cortisol *in vitro* or *in vivo*. They used cortisol at less than 10^{-6} M (3.6 ng/mL). In the present study, cortisol (more than 10 ng/mL) obviously suppressed the defense activities of tilapia neutrophils, but it is still not clear how cortisol affects the neutrophil activities. The results from some studies have shown inconsistency about the mechanisms involved (Lippman and Barr, 1977; Fantuzzi and Ghezzi, 1993; Weyts *et al.*, 1998). It is possible that cortisol can adversely affect neutrophils in several ways.

A few other studies have reported that fish with elevated plasma cortisol had a significantly higher susceptibility to infectious diseases (Pickering and Duston, 1983; Pickering and Pottinger, 1985). They showed that there was no association with reduced lymphocytes in causing diseases, meaning that causing diseases relates to cortisol rather than number of circulating lymphocytes. Cortisol and fish defense mechanisms still have complex inter-relationships. Therefore it remains for further *in vivo* study to reveal this relationship.

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