Inhibitory Effect of Cortisol on the Degranulation of Eosinophilic Granular Cells in Tilapia

Tomomasa Matsuyama¹, Junko Kurogi¹ and Takaji Iida^{2*}

¹United Graduate School of Agricultural Sciences, Kagoshima University, Kourimoto 1-21-24, Kagoshima 890-0065, Japan

²Faculty of Agriculture, Miyazaki University, Gakuen Kibanadai-Nishi 1-1, Miyazaki 889-2192, Japan

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ABSTRACT — We examined the effect of cortisol on the degranulation and number of eosinophilic granular cells (EGCs) in tilapia, *Oreochromis niloticus*. One hour after injection of stimulants (killed *Escherichia coli* or substance P, a neurotransmitter) into the swim bladder, the percent of the degranulated EGCs in the swim bladder membrane was determined. Peritoneal implantation of coconut oil containing cortisol (50 mg/kg body weight) 24 h before injection of stimulants significantly reduced the percent of the degranulated EGCs, compared with the control fish administered with coconut oil without cortisol. Cortisol also suppressed the *in vitro* degranulation of EGCs stimulated with substance P or with tilapia normal sera containing zymosan. To investigate the effect of cortisol on the abundance of EGCs, the number of EGCs in the swim bladder membrane was counted. Density of EGCs was decreased after implantation of cortisol, compared with that of the control group. These results suggest that cortisol suppresses the degranulating activity of EGCs and decreases the density of EGCs in the tissue.

Key words: eosinophilic granular cell, cortisol, tilapia, degranulation, stress

Fish are stressed in intensive fish culture systems. It is well documented that stress increases the susceptibility to infectious diseases (Wedemeyer, 1997). Fish respond to stress by the release of stress hormones including cortisol (Sumpter, 1997). Whereas the anti-inflammatory effects of cortisol have been widely recognized in mammals, limited information is available for interaction between cortisol and inflammation in fish. Cooper *et al.* (1989) has recorded granulocytosis caused by social stress in tilapia. Kurogi and Iida (1999) observed neutrophilia with reduced numbers of neutrophils that migrated to the inflammatory site as a result of social stress in tilapia. They speculated then that neutrophil chemotactic factor(s) could not be generated in the inflammatory site in stressed tilapia.

We have revealed that degranulation of eosinophilic granular cells (EGCs) precedes migration of neutrophils (Matsuyama and Iida, 1999), and EGCs release neutrophil migrating factor(s) with the degranulation (unpublished data). It is probable that depressed neutrophil migration by stress is involved in the EGC activity.

EGCs are considered as mammalian mast cell analogues from their histological characteristics and response to noxious agents (Reite, 1998; Matsuyama and Iida, 1999). A potent inhibitory effect of glucocorticoid on the mammalian mast cell activity has been reported by some investigators (Daeron *et al.*, 1982; King *et al.*, 1985). Cortisol implantation in fish reduced immunological responses (Maule *et al.*, 1987) and increased the susceptibility to diseases (Pickering and Duston, 1983), as did stress (Wedemeyer, 1997). From these results, in the present study, cortisol implantation was applied to produce the physiological response to the stress in tilapia. The aim of this study is to investigate the effect of stress on EGCs in tilapia.

Materials and Methods

Fish

Tilapia (*Oreochromis niloticus*) with body weights from 30 to 52 g (36.4 ± 5.6 g) were maintained at 25°C in 180 L tanks with a re-circulating system and fed daily with dry pellets (Maruha, Japan).

* Corresponding author
E-mail: a2107u@cc.miyazaki-u.ac.jp

Plasma collection

Fish were carefully removed from the tank and quickly anesthetized with 10% eugenol (FA100, Tanabe, Japan). Blood was withdrawn from the caudal vessel with a heparinized syringe, and centrifuged at 600×g for 5 min. The supernatant plasma was stored at -30°C for later analysis.

Cortisol and glucose measurement

Plasma cortisol and glucose concentrations were measured by using commercial kits (Enzaplata cortisol, Ciba Corning Diagnostics Inc. for cortisol; Glucose B-test Wako, Wako Life Science for glucose) according to the manufacturer's protocol.

Administration of cortisol

Cortisol stock solution was prepared by dissolving an appropriate quantity of cortisol in 99% ethanol, allowing the ethanol to completely evaporate (Tripp *et al.*, 1987), and resuspending the residual cortisol in Hanks' balanced salt solution (calcium, magnesium and phenol red-free; HBSS). The concentration of cortisol solution was measured using the kit as described above.

Cortisol implantation was done according to Pickering and Duston (1983). Implants were prepared by suspending cortisol (10 mg/mL) in liquid coconut oil at 50°C and injecting the warm liquid into the peritoneal cavity of the fish (5 mL/kg body weight). Control fish were implanted with liquid coconut oil.

Determination of the degranulation of EGCs in vivo

Formalin-killed *Escherichia coli* IAM 1239 (1 mg/fish), or substance P (100 µg/fish, Wako, Japan) was used as a stimulant for degranulating EGCs. Twenty-four hours after the implantation of cortisol or coconut oil, the stimulant (0.2 mL/fish) was injected into the swim bladder as described by Endo *et al.* (1997). The plasma and swim bladder were sampled 1 h after injection of the stimulant. A stretched swim bladder preparation was made and the percent of the degranulated EGCs was determined according to Matsuyama and Iida (1999).

Preparation of EGCs

Fish were killed by anesthesia with 10% eugenol and the swim bladder was removed. The swim bladder was macerated and washed in HBSS to remove the EGCs. Tissue clumps were removed by filtering the cells through 40 µm nylon mesh. The filtered cells were centrifuged at 180 × g for 5 min at 4°C and erythrocytes were lysed with distilled water. Residual cells were washed twice gently by centrifugation at 180 × g for 5 min. The total number of the EGCs was counted using a hemocytometer and the EGCs were identified on the basis of their size, granularity and non-adhesion to glass surfaces.

Determination of the degranulation of EGCs in vitro

Substance P or tilapia normal sera containing zymosan was ascertained to induce degranulation of EGCs in a preliminary experiment (unpublished data). EGCs prepared above were suspended in 0.5 mL of HBSS or 50% tilapia normal sera at a concentration of 1.0×10^4 cells/mL, and a degranulation test was then started by adding 0.5 mL of cortisol solution containing substance P (200 µg/mL) or zymosan (0.2 mg/mL). EGCs were incubated at 25°C on a microtube rotator (MTR-103, Iuchi, Japan) at 8 rpm, and smeared by centrifugation 30 min after incubation. Degranulation was observed microscopically after staining with May-Grünwald Giemsa.

Tissue distribution of EGCs

A stock of 64 fishes was divided equally into two tanks and left to acclimate for 1 week. One group was given the cortisol implants as described above. The other group was injected with liquid coconut oil for a control. Fishes were sampled from each group at 1, 2, 3, 5, 10, 15, 20, 30 days post-implantation. At each sampling time, 4 fishes were taken from each tank, and plasma samples and the swim bladders were taken out. Throughout the experiment the fish were given a single and daily meal. Stretched preparation of swim bladder was made according to Matsuyama and Iida (1999). The number of EGCs in the swim bladder was counted in 100 randomly selected 0.25×0.25 mm squares under a microscope using micrometer eyepiece at 400× magnification. The density of EGCs in the swim bladder membrane was expressed as the number of EGCs per square centimeter.

Statistics

The results are expressed as mean (±S.E.). Statistical analysis was performed using Student's *t*-test; *P*<0.05 was considered significant.

Results

Effect of cortisol on the degranulation of EGCs in vivo

Cortisol implantation significantly reduced the degranulation of EGCs after stimulation with substance P or killed *E. coli* (Table 1). The plasma cortisol concentration of the cortisol-implanted fish was significantly

Table 1. Effect of cortisol on the degranulation of EGCs *in vivo*

Stimulant	Degranulation (%)		
	No implantation	Coconut oil	Cortisol
Substance P	60.7 ± 3.8	60.2 ± 5.2	41.3 ± 4.5*
Killed <i>E. coli</i>	57.9 ± 2.4	61.0 ± 7.8	27.8 ± 5.7*

*: *P*<0.05 when compared with the value of the non implanted control. n = 7

Table 2. Plasma cortisol and glucose concentrations 1 hour after injection with stimulants.

	Stimulants				
	Substance P			Killed <i>E. coli</i>	
	No implantation	Coconut oil	Cortisol	Coconut oil	Cortisol
Cortisol (ng/mL)	Under detection	3.9 ± 1.7	53.3 ± 18.7*	12.3 ± 5.5	118.6 ± 7.0*
Glucose (mg/dL)	22.5 ± 13.8	69.2 ± 22.7	91.0 ± 15.9†	55.3 ± 10.3	76.2 ± 19.3†

*: $P < 0.05$ when compared with the value of the coconut oil implanted control.†: $P < 0.05$ when compared with the value of the non implanted control. $n = 7$ **Table 3.** Effect of cortisol on the degranulation of EGCs *in vitro*

Stimulants	Degranulation (%)			
	Cortisol concentration (ng/mL)			
	0	10	100	1000
Substance P	42.1 ± 1.7	35.7 ± 3.9	18.6 ± 3.2*	15.8 ± 1.3*
Sera + zymosan	32.6 ± 3.7	32.3 ± 1.8	20.9 ± 1.1*	18.0 ± 3.3*

*: $P < 0.05$ when compared with the value of the 0 ng/mL cortisol concentration. $n = 7$

higher than that of coconut oil-implanted or non-treated fish. No significant difference in plasma glucose concentration was observed between the coconut oil- and cortisol-implanted group, although glucose level is significantly higher in cortisol-implanted groups than in non-treated group (Table 2).

Effect of cortisol on the degranulation of EGCs *in vitro*

Cells isolated from the swim bladder membrane contained EGCs ($22.4 \pm 2.8\%$), monocytes ($35.0 \pm 3.5\%$), neutrophils ($13.5 \pm 1.6\%$), lymphocytes or thrombocytes ($25.6 \pm 1.2\%$) and un identified cells ($3.5 \pm 0.4\%$) as determined by May-Grünwald Giemsa, peroxidase and non-specific esterase stain.

Medium containing either 100 or 1000 ng/mL cortisol significantly suppressed the degranulation of EGCs stimulated by substance P or by tilapia normal sera containing zymosan. At concentration of 0 and 10 ng/mL, cortisol had no effect on the degranulation of EGCs by the stimulants (Table 3).

Tissue distribution of EGCs

Implantation of coconut oil alone had no effect on the density of EGCs in swim bladder in any of the experiments. On the other hand, the number of EGCs in the swim bladder of cortisol-implanted tilapia decreased markedly at 2 d post-implantation, and remained lower than that of the control until the end of experiment (Fig. 1). In the cortisol-implanted group, the plasma cortisol concentration was elevated rapidly and it was significantly higher than that of coconut oil-implanted control group until 4 d post-implantation. Cortisol concentration in the cortisol-implanted group decreased then, and 5 days after implantation it was no different from that in the coconut oil-implanted group. However, the plasma glucose levels were significantly

Fig. 1. Density of the EGCs in the swim bladder membrane after implantation with cortisol (■) or coconut oil (●).

elevated by cortisol implantation until 20 d post-implantation (data not shown).

Discussion

In the present study, we demonstrated that intraperitoneal implantation of cortisol suppressed the degranulation of EGCs after injection of the stimulant. We also showed that *in vitro* degranulation of EGCs induced by substance P or by tilapia normal sera containing zymosan (in which anaphilatoxin was expected to be produced) was also suppressed by cortisol. These results indicate a cortisol-mediated direct inactivation of EGCs in fish as has been reported in mammalian mast cells (King *et al.*, 1985). Kurogi and Iida (1999) reported that stress suppresses the defense activity of neutrophils, and number of neutrophils that migrated to the site of

inflammation was reduced by stress. We have shown that degranulation of EGCs precedes the migration of neutrophils to the inflammatory site (Matsuyama and Iida, 1999) and that EGCs release neutrophil-migrating factors with the degranulation (unpublished data). Suppression of the degranulation of EGCs by cortisol appears to be one of the reasons why migration of neutrophils to the inflamed site is suppressed by stress (Kurogi and Iida, 1999).

The plasma cortisol and glucose concentrations were elevated by intraperitoneal implantation of cortisol. The cortisol concentration decreased rapidly after it reached the peak. Cortisol might be eliminated effectively through accumulation in the bile and activation of the hepatic system (Pottinger *et al.*, 1992). The time it took for the cortisol to fall to normal levels in tilapia was shorter than that for brown trout. Pickering and Duston (1983) reported that intraperitoneal implantation of cortisol in brown trout resulted in a significantly higher elevation of blood cortisol levels and these high levels were maintained for more than 5 weeks. Some difference was also observed in the response of the EGCs to the cortisol treatment between tilapia and brown trout. EGCs in the swim bladder membrane were not degranulated by the intraperitoneal implantation of cortisol or coconut oil in the present study. Conversely, Reite (1997) reported that intraperitoneal injection of hydrocortisone in brown trout degranulated EGCs in the swim bladder membrane, following accumulation of fluid and leukocytes within the bladder. However, it is difficult to compare these results because of differences in the fish species, body weight, water temperature, and the method and dose of cortisol administration.

The density of EGCs in the swim bladder membrane was reduced by cortisol implantation. A similar reduction in EGCs by cortisol was reported to occur in mammalian mast cells (King *et al.*, 1985). The mast cells proliferate in response to some cytokines (IL-3, IL-4, Nerve growth factor, etc), which are produced by T lymphocyte or fibroblast (Nechushtan and Razin, 1996). Cortisol suppresses the lymphocyte activity in fish as well (Pickering, 1984; Tripp *et al.*, 1987; Cooper *et al.*, 1989). Further work is needed to determine whether the decrease in the density of EGCs results from the primary action of cortisol on other cell types, such as lymphocytes. In contrast to the present results, Holland and Rowley (1998) reported that the number of EGC in gill explants increased after net handling stress. Mild stress may enhance the immune response as reported by Kurogi and Iida (1999).

Reduced number of blood lymphocytes following administration of cortisol will be recovered within a few days after elimination of plasma cortisol (Pickering, 1984). Interestingly, in spite of the decreased plasma cortisol concentration, the density of EGCs in the swim bladder membrane did not recover to the normal level

even at 30 days after the cortisol implantation. Thus, it is clear that even after stressors are removed, tilapia EGCs need a considerable amount of time to completely recover from a stressed condition. Some endogenous or exogenous stimulation(s) might be necessary for the proliferation of EGCs and recovery of their abundance.

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