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Cold-Stress Induced the Modulation of Catecholamines, Cortisol, Immunoglobulin M, and Leukocyte Phagocytosis in Tilapia

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The concentrations of norepinephrine in hypothalamus and norepinephrine and epinephrine in head kidney were significantly decreased in treated tilapia (*Oreochromis aureus*) during the time course of cold exposure (12°) as compared to the control (25°). The elevation of norepinephrine and epinephrine in plasma was detected earlier than that of cortisol in cold-treated tilapia. Phagocytic activity of leukocytes and the levels of plasma immunoglobulin M (IgM) were depressed in cold-treated tilapia as compared to the control group. Handling stress in the control (25°) also resulted in increased plasma cortisol and decreased plasma IgM levels but not phagocytic activity. *In vitro* cortisol suppressed leukocyte phagocytosis in a dose (10^{-12} to 10^{-4} M)-dependent manner. Adrenergic agonist (phenylephrine and isoproterenol) had a significant suppression of phagocytosis only at the highest dose (10^{-4} M). No effect on phagocytosis was detected in the treatment with norepinephrine and epinephrine. A combination of cortisol and isoproterenol (0.1 mM) had an additive effect in the suppression of phagocytosis. It is concluded that the cold stress modulated the changes of catecholamines and cortisol and further depressed phagocytic activity and antibody levels in tilapia. Cortisol could play a main and important role in the down-regulation of phagocytic activity. Adrenergic agonists also could interact with cortisol to further suppress immunity in tilapia.

Key Words: catecholamines; cold stress; cortisol; immunity; immunoglobulin M; leukocyte phagocytosis; tilapia.

INTRODUCTION

The stress of cold temperatures may activate the neuroendocrine pathway, including the hypothalamic–pituitary–interrenal axis, the hypothalamic–pituitary–thyroid axis, or the hypothalamus–autonomic nerves–chromaffin axis, to cause the release of cortisol, thyroid hormone, or catecholamines in vertebrates (Mazeaud *et al.*, 1977; Barton and Peter, 1982; Fregly, 1989; Wendelaar Bonga, 1997; Reid *et al.*, 1998; Stouthart *et al.*, 1998; Tanack *et al.*, 2000). Physiological stress correlates with increased susceptibility to diseases (Fletcher, 1986; Wendelaar Bonga, 1997; Weyts *et al.*, 1999). Cold stress suppressed nonspecific immunity against *Escherichia coli* in chickens (Matsumoto and Huang, 2000), altered antibody titer (Firouzi and Motamedifar, 1999), and modulated cell immunity in mice (Banerjee *et al.*, 1999). It is not clear how the mechanism of immunomodulation is induced by cold stress in fish. Information about the relationship

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among the changes of catecholamines and cortisol in tissues or plasma and nonspecific (phagocytosis) and humoral (immunoglobulin M, IgM) immunity is less available but is important to understand the mechanism of stress effects.

Catecholamines could affect the cardiovascular system (Randall and Perry, 1992), respiratory capacity (Nikinmaa, 1992; Soldatov, 1996), energy metabolism (van Raaij *et al.,* 1995), and immunity (Wendelaar Bonga, 1997). The interaction of neuroendocrine and immune systems is an important regulatory process in vertebrates (Blalock, 1994). α- and β-Adrenoceptor-mediated effects on the activity and cytokine production of leukocytes have been shown in humans and other animals (Ader *et al.,* 1995; Rothwell and Hopkins, 1995; Weyts *et al.,* 1999). Catecholamine production is not required for normal development of the immune system in mice, but plays an important role in the modulation of T cell-mediated immunity to infection and immunization (Alaniz *et al.,* 1999). Catecholamines selectively modulate interleukins and therefore may suppress cellular immunity but favor humoral immune responses in humans (Elenkov *et al.,* 1996). Much less information is available on the effects of catecholamines on immunity in fish. Exogenous epinephrine reduced leukocyte levels in killifish, *Fundulus heteroclitus* (Pickford *et al.,* 1971). Adrenergic agonists, phenylephrine and isoproterenol, depressed macrophage phagocytosis in the head kidney and spleen of rainbow trout (Narnaware *et al.,* 1994). Lesioning of the adrenergic nerves innervating the spleen stimulated antibody secretion in rainbow trout (Flory, 1989). Antibody response to lipopolysaccharide was stimulated by the α-andrenergic agonist (clonidine) but suppressed by the β-adrenergic agonist isoproterenol (Flory and Bayne, 1991). Therefore, catecholamines seem to have both inhibitory and stimulatory actions on immune functions in fish. It is indicated that whether the catecholamine effects are immunosuppressive or immunostimulatory depends on the cell type involved and the dose of modulator used (Homo-Delarche and Durant, 1994).

Suppression of the immune system by glucocorticoids has been shown in mammals (Goulding and Flower, 1997) and fish (Balm, 1997). Cortisol even has a permissive, suppressive or stimulatory action in various animals (Sapolsky *et al.,* 2000). Cortisol could inhibit the leukocyte function in specific and/or nonspecific immunity (T and B lymphocytes, natural killer cells, macrophages, and neutrophils) in mammals (Goulding and Flower, 1997). Cortisol selectively suppressed leukocyte phagocytosis in two teleosts, with strong effects in tilapia but weak effects in common carp (Law *et al.,* 2001). Cortisol caused a decreased number of lymphocytes and increased susceptibility to infection in brown trout, *Salmo trutta in vivo* (Pickering and Duston, 1983; Pickering, 1984). However, suppression of plasma IgM levels was only observed after a long-term treatment with a high dose of cortisol in masu salmon, *Oncorhynchus masou* (Nagae *et al.,* 1994a), and rainbow trout, *Oncorhynchus mykiss* (Hou *et al.,* 1999).

The objectives of this study were to investigate the responses of neuroendocrine and immune systems (phagocytosis and antibody titer) in tilapia exposed to the stress of cold temperatures (12°). The potential roles of cortisol and catecholamines on the phagocytic activity of peripheral blood leukocytes are also compared.

MATERIALS AND METHODS

Fish

Tilapia (*Oreochromis aureus,* average body weight 205 ± 25 g, body length 18 ± 3 cm) were purchased from the fish market. The fish were kept in a glass tank ($200 \times 50 \times 50$ cm) of a tapwater (freshwater) system (at 25°) for more than 1 month before transferring to small glass tanks ($60 \times 30 \times 30$ cm) for the experiment (each tank in a separate system). The fish were in a heathy condition and fed with a commercial tilapia feed (Fwu Sou Feed Co., Taichung, Taiwan) under a natural photoperiod.

Neuroendocrine Responses in Tilapia Exposed to Acute Cold Temperatures (Exp. I)

Tilapia ($n = 30$) were divided into five groups ($n = 6$ per group), control (25°), cold treated-1 (water temperatures changed from 25 to 19° within 15 min), cold treated-II (changed from 25 to 12° within 30 min), cold treated-III (changed from 25 to 12° within 30 min and maintained at 12° for 1 h), and cold treated-IV (changed from 25 to 12° within 30 min and maintained at 12° for 2 h). Each group had two tanks ($60 \times 30 \times 30$ cm) and 3 fish were kept in a glass tank for at least 2 weeks before and during the experiment. Different treatments of fish

were conducted at the various beginning times according to the experimental schedule; therefore, the fish were sacrificed at the same time at the end of the cold treatment. Fish in each group were anesthetized by directly adding 2-phenoxyethanol (0.5 ml/L) to the tank; blood was collected from the caudal vessel using a 1-ml syringe fitted with a 23-G needle rinsed with Na_2–EDTA as anticoagulant. Fish were killed, and tissues (hypothalamus, telencephalon, and head kidney) were collected. The sampling times in all the fish ($n = 6$) per group were within 3–5 min by six people. Tissues were frozen and stored in liquid nitrogen. Blood (0.5 ml) was added to the tubes containing 30 μl of 30% $Na_2S_2O_5$ at 4°, centrifuged (800*g* for 10 min), and then stored at −75° for further analysis of catecholamines. Another 0.5 ml of blood was centrifuged to collect plasma for cortisol analysis by a radioimmunoassay and for IgM assay by an enzyme-linked immunosorbent assay with a specific rabbit antibody raised against tilapia IgM. No tank effect in the data was found.

Effects of Cold Stress on Leukocyte Phagocytosis, Plasma Cortisol, and IgM (Exp. II)

Tilapia ($n = 8$) were divided into two groups, control and cold-stressed. Four fish from each group were kept in a glass tank (60 × 30 × 30 cm) for more than 2 weeks before conducting the experiment. Tilapia were exposed to acute cold temperatures on day 0. Water temperatures were changed from 25 to 12° at the rate of 4°/h and water temperatures were further maintained at 12° for 2 h on day 0. After the treatment of cold stress, cold-stressed fish were returned to 25° and allowed to recover. Similar handlings (sham operation) were also applied to the control (25°) tilapia. Blood was collected on days 1, 3, 5, and 15 from the caudal vasculature, for the measurement of leukocyte phagocytosis, IgM, and cortisol. Handling and bleeding procedures were the same as those of Exp. I.

Analysis of Catecholamines in Plasma and Tissue by a High-Performance Liquid Chromatography-Electrochemical Detector (HPLC-ECD)

Plasma (200 μl) was added to Al_2O_3 (10 mg), 2 M Tris/EDTA (400 μl, pH 8.6), 30% sodium disulfite (50 μl), and 3,4-dihydroxybenzylamine (DHBA; as an internal marker, 50 μl, 40 ng/ml; Sigma, St. Louis, MO). The solution was shaken at 4° for 20 min and then centrifuged at 10,000*g* for 1 min (4°). The Al_2O_3 pellet was washed three times with 1 ml of cold 0.2 M Tris/EDTA (pH 8.1) and centrifuged. The pellet was added to 125 μl of a cold solution (0.01% acetic acid, 0.005% sodium disulfide, and 0.0025% Na_2–EDTA) and shaken at 4° for 20 min to elute catecholamines. The mixture was centrifuged (10,000*g* for 1 min) and the supernatant was filtered with a 0.22-μm filter for the measurement of catecholamines.

Tissues (brain and head kidney) were homogenized with a sonicator in an antioxidant solution (0.05% Na_2–EDTA, 0.05% $Na_2S_2O_5$, 0.1% l-cystein, 0.2 M $HClO_4$; 1/4–1/6 g (tissue)/ml (solution), 4°) containing DHBA (40 ng/ml) as an internal marker. The mixture was centrifuged at 3500*g* for 10 min and the supernatant was filtered with a 0.22-μm filter for the measurement of catecholamines.

An HPLC-ECD system (Bioanalytical System, Inc., West Lafayette, IN) with a phase-II ODS column (100 × 3.2 mm; Bioanalytical System, Inc.) was used for analysis of catecholamines [mobile phase, 0.075 M monochloroacetic acid (pH 3.0; Merck, Darmstakt, Germany), 0.5 mM Na_2–EDTA, 1 mM octyl sodium sulfate (Sigma), and 1.5% acetonitrile (Mallickrodt, Paris, KY); flow rate, 0.7 ml/min]. Norepinephrine and epinephrine (Sigma) were dissolved in an antioxidant medium for use as standards (0.125–2.5 ng/50 μl). The volume of sample (plasma or tissue) or standard applied to HPLC-ECD was 50 μl. The recovery of catecholamines on the basis of DHBA was 95–99 and 50–75% in tissue and plasma, respectively. The value of catecholamines was calibrated on the basis of its own recovery in each sample.

Preparation of Peripheral Blood Leukocytes and the Analysis of Leukocyte Phagocytosis by a Microtiter Plate Assay

Fish were anesthetized in 0.02% benzocane solution (Sigma) and blood was collected from the caudal vasculature with heparin (Sigma). The preparation of blood leukocytes followed the methods of Law *et al.* (2001). Blood was mixed with AL medium (AIM-V medium and Leibovitz's L 15 medium; GIBCO BRL, Gaithersburg, MD). Lymphoprep (Nycomed Pharma As, Norway) was added to the mixed blood solution and then centrifuged. The leukocytes were obtained from the interface and washed with AL medium. By these procedures, we could obtain mainly leukocytes (>98%) for experiment.

The methods used to measure leukocyte phagocytosis followed the procedures of Law *et al.* (2001). About 1.5×10^5 leukocytes in 50 μl AL medium with 5.5 mM glucose were added to a microfluor black plate (Nunc-Immuno Modules, Roskilde, Denmark) in the presence or absence of test compounds and then incubated for 4 h at 25° in air. After incubation, 50 μl of FITC–latex beads (Sigma) and 50 μl of trypan blue quenching buffer (Sigma) were added. The fluorescent intensity was measured with a MFX Microtiter Plate Fluorometer (Dynex, USA). The phagocytosis index was 100% in the control and the relative phagocytosis index in the treated group was calculated. The tested compounds (Sigma) were as follows: cortisol, norepinephrine, epinephrine, phenylephrine (α-adrenergic agonist), and isoproterenol (β-adrenergic agonist). Each treatment had four replicates.

Cortisol Radioimmunoassay

Cortisol was measured with a validated radioimmunoassay with a specific antiserum as described in a previous study (Sun *et al.,* 1994).

The Measurement of Immunoglobulin M by an Enzyme-Linked Immunosorbent Assay

IgM was purified from plasma of tilapia immunized against bovine serum albumin (BSA; 100 μg) by ammonium sulfate precipitation and a column (10 × 2.5 cm) packed with DEAE–cellulose (Sigma). The IgM was incubated with alkaline phosphatase conjugated protein A (Sigma) for 1 h at 37° and then reacted with *p*-nitrophenylphosphate (Boehringer Mannheim GmbH, Mannheim, Germany) in 10 mM diethanolamine (Merck) with 0.5 mM $MgCl_2$ (pH 9.8; Sigma) as a substrate solution. Purified tilapia IgM (300 μg) was mixed with complete Freund's adjuvant (Sigma) and injected into the spleen of the rabbit to induce the anti-IgM serum.

IgM in fish plasma was measured with an enzyme-linked immunosorbent assay modified from the procedures of Chang *et al.* (1996). The experimental tilapia did not preimmunize against BSA. The plasma sample (100 μl) or standard IgM in 0.01 M phosphate-buffered saline (pH 7.0) was incubated in a 96-well plate for 16 h at 4°. The wells were blocked with a blocking buffer (0.05% BSA in a 0.01 M phosphate-buffered saline; pH 7.0) for 1 h at 37°. Antiserum against tilapia IgM was added to the well and incubated for 1 h at 37°. Then, alkaline phosphatase conjugated goat anti-serum against rabbit IgG (second antibody, 1/3000 in a 0.01 M phosphate-buffered saline; pH 7.0) was added for 1 h at 37°. The color reaction was proceeded with *p*-nitrophenol phosphate disodium salt (1 μg/μl; Pierce, Rockford, IL) in 10 mM diethanolamine with 5 mM $MgCl_2$ as the substrate solution. NaOH (5 M, 50 μl) was added to stop the reaction and OD at 405 nm was measured.

Statistical Analysis

One-way analysis of variance followed by Duncan's multiple range test were conducted to test the significance of differences between treatments at a single time and in the effect of specific treatment through time ($P < 0.05$). Results are given as a mean ± standard error of the mean (SE).

RESULTS

Decreases of Catecholamines in Hypothalamus, Telencephalon, and Head Kidney of Cold-Treated Tilapia (Exp. I)

Epinephrine was very low and could not be detected in hypothalamus and telencephalon. Norepinephrine concentration in the hypothalamus was significantly lower in the cold-treated tilapia than in the control (Fig. 1a). Decreased norepinephrine levels were significantly detected in the treatments of 19 and 12° (0–2 h) (Fig. 1a). Norepinephrine concentrations in telencephalon were only significantly lower in cold-treated tilapia for 2 h at 12° but not in other treated groups compared to the control (Fig. 1b). Norepinephrine and epinephrine levels in head kidney were also significantly decreased in tilapia exposed to cold temperatures (19° and then 12° from 0–2 h) (Figs. 2a and 2b). Norepinephrine levels in head kidney were higher (2.5-fold) than epinephrine (Figs. 2a and 2b).

Increases of Plasma Norepinephrine, Epinephrine, and Cortisol Concentrations but No Change of Plasma IgM Levels during Exposure to Cold Temperatures (Exp. I)

Plasma norepinephrine and epinephrine levels were significantly elevated in tilapia after exposure to cold

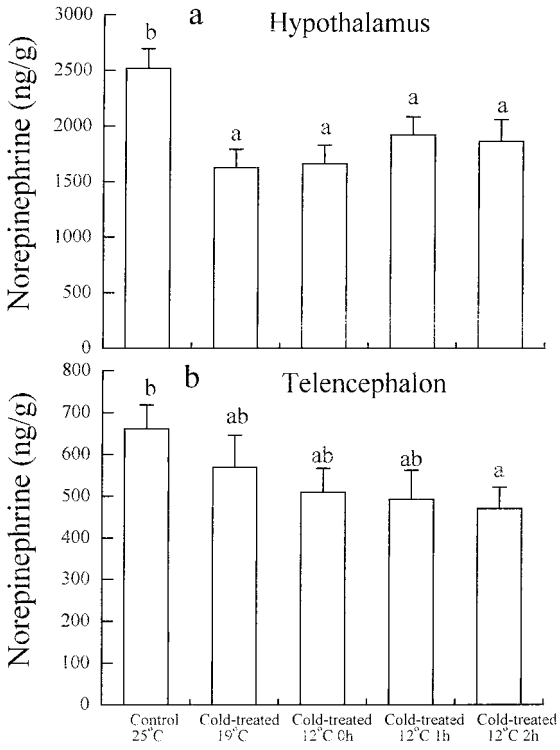

FIG. 1. The concentrations (mean ± SE, $n = 6$) of norepinephrine in hypothalamus (a) and telencephalon (b) in tilapia during time course of cold stress. Values with different letters indicate significant differences ($P < 0.05$).

temperatures (19°; 12° at 0 h) (Figs. 3a and 3b). Then, plasma norepinephrine and epinephrine levels were returned to the control value in tilapia exposed to 12° for 1- and 2-h intervals (Figs. 3a and 3b). Plasma cortisol levels were not different between cold-treated (19°) and control tilapia (Fig. 4a). Increased levels of plasma cortisol were observed in cold-treated tilapia when water temperature reached 12° at 0, 1, and 2 h. (Fig. 4a). No change of plasma IgM was observed in tilapia during the exposure to cold temperatures (Fig. 4b).

Depressed Leukocyte Phagocytosis, Decreased Plasma IgM, and Increased Cortisol in Stressed Tilapia after Cold Exposure (Exp. II)

Cold stress significantly elevated plasma cortisol levels on days 1, 3, and 15 as compared to the respective control group (25°) (Fig. 5a). Repeated bleeding during days 1 to 5 also resulted in increased levels of plasma cortisol in the control (Fig. 5a). Phagocytosis index was not significantly changed in the control on days 1, 3, 5, and 15 (Fig. 5b). In contrast, phagocytosis was significantly suppressed in the cold-treated tilapia after 3 and 5 days of cold exposure (Fig. 5b). The maximum effect on the suppression of the phagocytosis was 3 days after cold exposure (down to 60% of phagocytosis index). The phagocytosis on day 15 had recovered to the control value (Fig. 5b).

Decreased levels of plasma IgM occurred in the control on days 1, 3, and 5 (Fig. 5c). Plasma IgM levels were further depressed by cold exposure on days 1, 3, and 5 as compared to the respective date in the control (25°) (Fig. 5c). Plasma IgM levels were decreased in both cold-treated and control tilapia, and no difference was observed in plasma IgM levels on day 15 in the control and cold-treated tilapia (Fig. 5c).

Phagocytosis in Tilapia Leukocytes: Cortisol Dose Dependency of the Suppressive Effect

Various final concentrations (0 and 1 pM–0.1 mM) of cortisol were incubated with tilapia leukocytes for

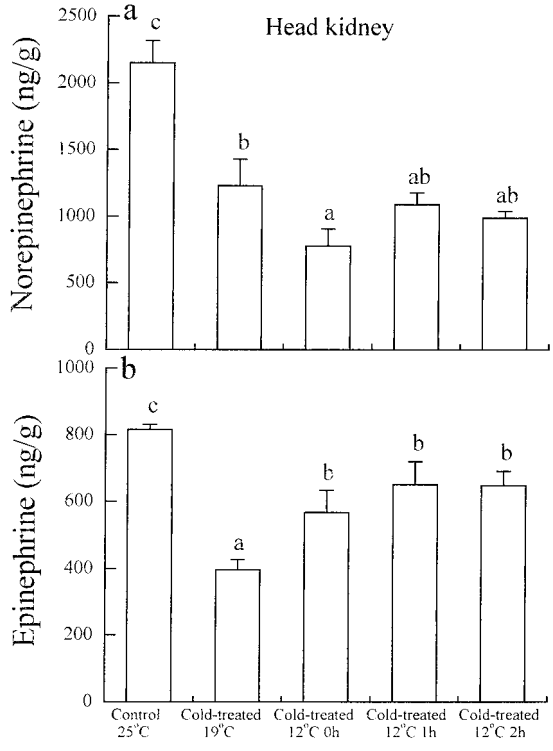

FIG. 2. The concentrations (mean ± SE, $n = 6$) of norepinephrine (a) and epinephrine (b) in head kidney in tilapia during the time course of cold stress. Values with different letters indicate significant differences ($P < 0.05$).

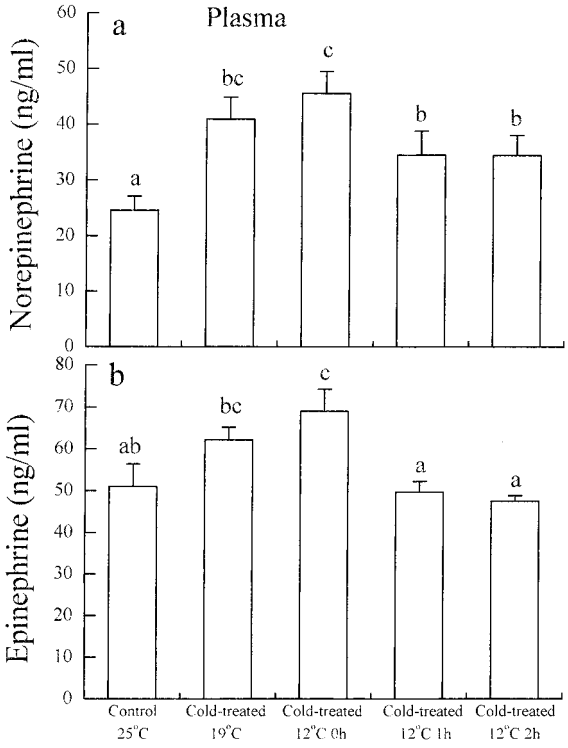

FIG. 3. The concentrations (mean ± SE, $n = 6$) of plasma norepinephrine (a) and epinephrine (b) in tilapia during the time course of cold stress. Values with different letters indicate significant differences ($P < 0.05$).

4 h. The dose-dependent response in the phagocytosis effects of cortisol is shown in the Fig. 6. Incubation with a dose of 10 nM or higher concentrations of cortisol significantly suppressed phagocytosis in a dose-dependent manner (Fig. 6).

Comparative Effects of Various Catecholamines and Agonists on Phagocytosis in Tilapia

Various final concentrations (1 pM–0.1 mM) of both norepinephrine and epinephrine had no significant effects on the suppression of phagocytosis (Fig. 6). Adrenergic agonists, phenylephrine and isoproterenol, had a significant effect on the suppression of phagocytosis only at the highest dose (0.1 mM) (Fig. 6).

Effects of Combined Treatment with Cortisol and Catecholamine Agonists

The effects of the combination of different concentrations of isoproterenol and cortisol are shown in the Fig. 7. In the presence of an inactive dose of isoproterenol (10 nM), which is inactive alone, the suppressive action of cortisol on phagocytosis was not enhanced. The maximal inhibitory effect corresponding to 70% is similar to cortisol alone (0.1 M) or the combined group (10 nM isoproterenol and 0.1 mM cortisol) (Fig. 7). The suppressive curve of the phagocytosis index in the combined group (0.1 mM isoproterenol + cortisol) was parallel to but lower than that of cortisol alone (Fig. 7). Furthermore, in the presence of the high dose of isoproterenol (0.1 mM), the effective dose of cortisol was reduced (1 pM), and its maximal inhibitory effect was enhanced (down to 64% versus 72% of the phagocytosis index) (Fig. 7).

DISCUSSION

The elevated catecholamines and cortisol in the plasma of cold-treated tilapia reflect the stress status

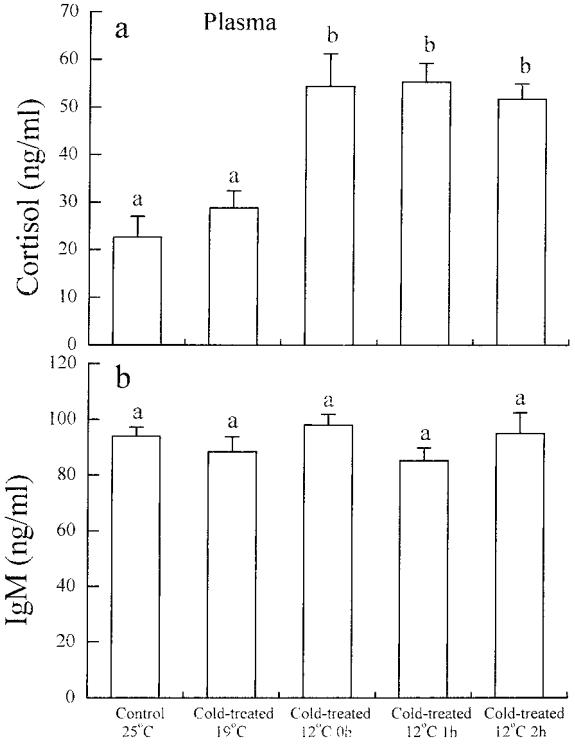

FIG. 4. The concentrations (mean ± SE, $n = 6$) of plasma cortisol (a) and immunoglobulin M (IgM) (b) in tilapia during the time course of cold stress. Values with different letters indicate significant differences ($P < 0.05$).

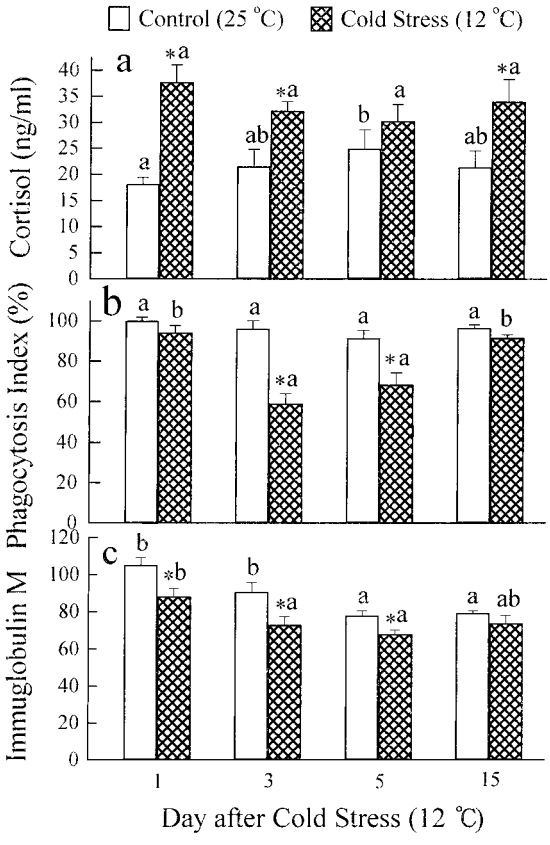

FIG. 5. Plasma cortisol (a), leukocyte phagocytosis index (b), and plasma immunoglobulin M (c) on days 1, 3, 5, and 15 in tilapia after cold stress (12°) on day 0. Value represents mean ± SE ($n = 4$). Symbol "*" indicates the significant difference from the respective control (25°) at the same time ($P < 0.05$). Values with different letters indicate significant differences in the same group ($P < 0.05$).

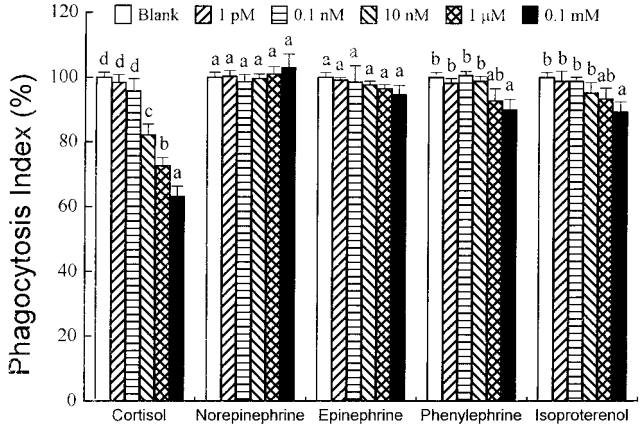

FIG. 6. *In vitro* effects of cortisol, norepinephrine, epinephrine, phenylephrine, and isoproterenol (0–0.1 mM for each chemical) on the phagocytosis index (%) in tilapia leukocytes. Value represents mean ± SE ($n = 4$ replicates). Values with different letters indicate significant differences in the same group ($P < 0.05$).

of the experimental fish during acute stress as demonstrated by Wendelaar Bonga (1997). The elevation of catecholamine levels to cold-stress occurred earlier than that of cortisol. The duration of the elevation of plasma catecholamines is shorter than that of cortisol. The present data also demonstrated the significant decrease of catecholamine levels in the head kidney of cold-stressed tilapia. These data supported the notion that released catecholamine in circulation was mainly from chromaffin cells of the head kidney in teleosts (Perry *et al.*, 1991; Wendelaar Bonga, 1997). The rapid decrease of norepinephrine in hypothalamus but not telencephalon was observed in cold-treated tilapia. Hypothalamic norepinephrine was also significantly decreased in tilapia (*O. mossambicus*) acclimated to warmer temperatures (26° vs 29–32°) (Tsai and Wang, 1997). The metabolic enzymes and products of monoamines in brain were not further detected in these current experiments. Therefore, it is too early to conclude the importance of the changes in brain catecholamines in cold-stress fish.

Phagocytes have a major role in the cell immune responses in fish, especially in the fight against bacteria. The factors in fish that are involved in the regulation of phagocytosis remain to be established. The present data clearly demonstrated that acute cold stress significantly depressed leukocyte phagocytosis in tilapia. The depression of the phagocytic activity did not occur 1 day after cold stress; it occurred 3 days

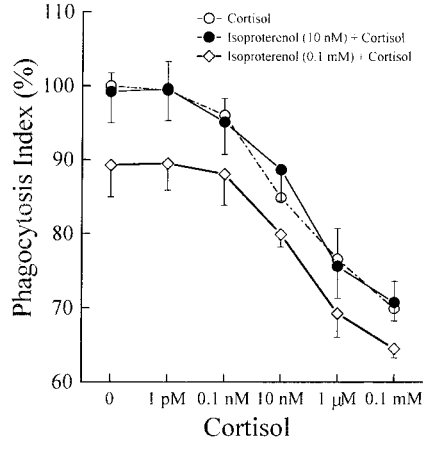

FIG. 7. *In vitro* interaction of cortisol (0–0.1 mM) with isoproterenol (10 nM and 0.1 mM) on the phagocytosis index (%) in tilapia leukocytes. Value represents mean ± SE ($n = 4$ replicates).

later. At least 2 weeks after cold stress is long enough for the fish to recover in phagocytic activity. Repeated handling stress in Exp. II (handling stress on day 0 and bleeding stress from days 1 to 5 at 2-day intervals) had no significant effect on leukocyte phagocytosis in control (25°). However, repeated handling and bleeding in Exp. II probably caused the decrease of IgM and increase of cortisol in the control group. Handling stress with daily injection over a 6-day period caused a suppression of kidney macrophage phagocytosis in rainbow trout, *O. mykiss* (Narnaware *et al.*, 1994), and reduced the number of plaque-forming cells in Atlantic salmon, *Salmo salar* (Mazur and Iwama, 1993). In contrast, enhancement of macrophage phagocytosis was observed in common carp (*Cyprinus carpio*) exposed to mildly low temperature (12°) for 28 days compared to the control (20°) (Le Morvan *et al.*, 1997).

In addition to the suppression of phagocytosis, cold stress also caused decreased plasma IgM levels in the present studies. Low temperatures from 23 to 11° over a 24-h period suppressed both B and T cell functions in channel catfish, *Ictalurus punctatus* (Bly and Clem, 1991). Plasma IgM levels were not significantly affected in the beginning of cold stress in Exp. I (during the 2-h period of cold stress at 12°). However, IgM (but not phagocytosis) was significantly depressed from days 1 to 5 after cold stress in Exp. II. Repeated handling stress also significantly depressed the levels of plasma IgM but not phagocytic activity in the control (25°) in Exp. II. Suppressive effect in plasma IgM levels by cortisol was not necessarily detected, especially in a short-term treatment in masu salmon, *O. masou* (Nagae *et al.*, 1994a), and rainbow trout, *O. mykiss* (Hou *et al.*, 1999). No correlation between plasma cortisol and IgM levels was observed during smoltification in masu salmon, *O. masou* (Nagae *et al.*, 1994b).

The possible mechanism of the depressed phagocytic activity and IgM in tilapia exposed to cold stress is important in stress physiology. The increased releases of cortisol (as demonstrated in Exps. I and II) and catecholamines (as demonstrated in Exp. I) are the possible endocrine factors which may be involved in the modulation of immune activity. In our recent experiment, *in vivo* injection of cortisol also significantly suppressed leukocyte phagocytosis in tilapia in a dose-dependent manner (unpublished data by W-H. Chen *et al.*)

The consistent data (Exp. II) for the high plasma cortisol levels and depressed phagocytic index support the important role of cortisol in the down-regulation of leukocytosis. The present data further demonstrated that *in vitro* cortisol significantly inhibited leukocyte phagocytosis in tilapia. Plasma cortisol levels were significantly elevated and could be 10^{-7} M when animals were exposed to stressed environments, and the elevated levels could last for several hours to days in this study and other experiments (Strange *et al.*, 1977; Leach and Taylor, 1980; Barton and Peter, 1982; Sun *et al.* 1994; Tanack *et al.*, 2000). Cortisol levels in the experiment on leukocyte phagocytosis in the present study were from physiological (10^{-8} to 10^{-7} M) to pharmacological (10^{-6} and 10^{-4} M) doses. Inhibitory effects on phagocytosis were consistently observed at various doses of cortisol in this experiment. Cortisol also reduced phagocytic ability in striped bass, *Morone saxatilis* (Stave and Robertson, 1985), common carp, *C. carpio* (Weyts *et al.*, 1998a; Law *et al.*, 2001), coho salmon, *Oncorhynchus kisutch* (Tripp *et al.*, 1987), Atlantic salmon, *S. salar* (Espelid *et al.*, 1996), and tilapia, *O. niloticus* (Law *et al.*, 2001). In contrast, cortisol at a low dose enhanced phagocytosis in humans (Kay and Czop, 1994) and bovine (Fox and Heald, 1981). No effect of cortisol on phagocytosis was found in anterior kidney and spleen of rainbow trout, *O. mykiss* (Narnaware *et al.*, 1994), and even protection against the effects of stress on circulating leukocytes by treatment with cortisol was observed in rainbow trout, *O. mykiss* (Narnaware and Baker, 1996).

The consistent data (Exp. II) for the high plasma cortisol levels and suppressed IgM levels (days 1, 3, and 5) also support the important role of cortisol on the decreased IgM levels. However, IgM levels on day 15 in the control (25°) were low but not different from those of the cold-stress fish on day 15 (Exp. II), although plasma levels of cortisol on day 15 in the control (25°) were significantly lower than those of the cold-stressed fish. The data showed that IgM levels were still low on day 15 (after 10 days of nonstressfully cultured condition) in Exp. II. The data indicate that the change of IgM is more susceptible to the stress than leukocyte phagocytosis in tilapia. The regulatory mechanism of the output of IgM and phagocytic activity is apparently different in tilapia. Apparently, repeated bleeding had effects on IgM levels (but not phagocytosis) in this experiment. These data provide interesting findings on the differential modulation in

phagocytosis and IgM levels by cold and handling stress. *In vitro* cortisol does not affect phagocytosis or respiratory burst activity in rainbow trout, *O. mykiss* (Narnaware *et al.*, 1994), and common carp, *C. carpio* (Weyts *et al.*, 1998a), unless high nonphysiological doses (micromolar) are used as in several flatfish species (Pulsford *et al.*, 1995).

There are a few studies on catecholamine effects on immunity in animals. In contrast to the suppressive effect of *in vitro* cortisol on phagocytosis in tilapia, catecholamines only had very weak effects. In the present studies, catecholamine pharmacological agonists but not catecholamines significantly suppressed leukocyte phagocytosis in tilapia but only at the highest dose (10^{-4} M). The suppressive effects on phagocytosis were also lower in the treatment with catecholamine agonists than in that with cortisol. Adrenergic agonists also depressed macrophage *in vitro* phagocytic activity in rainbow trout, *O. mykiss* (Narnaware *et al.*, 1994). Further, adrenergic blocker (phentolamine) prevented the depression of phagocytic activity in rainbow trout, *O. mykiss* (Narnaware and Baker, 1996).

The present study further demonstrated that *in vitro* cortisol and isoproterenol had an additive effect on the suppression of phagocytosis in tilapia. The strong inhibitory effect on phagocytosis of the combination of cortisol and isoproterenol, as demonstrated in this study, may provide an endocrine basis for the understanding of the immune function after exposure to stress. In the physiological system, cortisol possibly plays a more important role in the regulation of leukocyte phagocytosis than catecholamines in tilapia. α-2 Adrenergic receptor agonist enhanced stimulation of antibody-secreting cells from spleen, but β-adrenergic receptor agonists suppressed this response in rainbow trout, *O. mykiss* (Flory, 1990). Sympathetic nervous system had a tonic regulation of immune function in mice (Kruszewska *et al.*, 1995).

The affinity of glucocorticoid receptors is in the nanomolar range in the liver (K_d of ~0.5 nM) of rainbow trout, *O. mykiss* (Pottinger, 1990), leukocytes (K_d of ~1.0 nm) of coho salmon, *O. kisutch* (Maule and Schreck, 1990), and peripheral blood leukocytes (K_d of 3.8 nM) of common carp (Weyts *et al.*, 1998b). Phagocytosis of tilapia peripheral leukocytes is sensitive to cortisol concentrations as low as 10 nM (after 4-h incubation) in the present experiment, which is in the range of the binding affinity of glucocorticoid receptors. Catecholamine action through cyclic AMP or another messenger to regulate immune function is suggested (Cookmills *et al.*, 1995). Adrenoceptors have also been shown in hepatocytes and leukocytes, with the binding affinity from the nanomolar to the micromolar range in catfish, *Ictalurus punctatus* (Garcia-Sáine *et al.*, 1995), and other animals (Fabbri *et al.*, 1997; Friedman and Irwin, 1997), which is much lower than the effective dose to suppress phagocytosis in this experiment.

It is concluded that cold-stress modulated the changes of catecholamines and cortisol and further depressed antibody titer and phagocytic activity in tilapia. The time course to detect the changes of these parameters is as follows: first catecholamine, cortisol, and IgM and then phagocytosis. Both cortisol and catecholamines depressed *in vitro* leukocyte phagocytosis, while cortisol plays a main and important role in the down-regulation of phagocytic activity. Adrenergic agonists also could interact with cortisol to further suppress nonspecific immunity. Cortisol and catecholamines probably associate with the immunosuppression in fish exposed to cold stress. Our findings provide valuable information for the understanding of the interaction of endocrine and immune systems in fish. However, larger sample sizes, experimental replicates, and nonrepeated bleeding should be further considered for future experiments to obtain a better picture.

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