Sodium-retaining Effects of Cortisol, Prolactin, and Estradiol-17 β in Medaka *Oryzias latipes* Exposed to Acid Water

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(Received October 12, 1998)

To investigate endocrine control of osmoregulation in the fish under acidic environment, sodium-retaining effects of cortisol, prolactin and estradiol-17 β were examined in the acid-exposed medaka *Oryzias latipes*. Exposure from neutral fresh water (pH 7.0) to acidic fresh water (pH 3.8) for 24 h caused a significant decrease in plasma sodium levels in both sexes of the medaka. Single injection of cortisol prior to the exposure retained plasma sodium levels and stimulated gill Na⁺,K⁺-ATPase activity in acid water. Ovine prolactin also prevented a decrease in plasma sodium levels after acid exposure, whereas gill Na⁺,K⁺-ATPase activity was unaffected. In male fish, estradiol-17 β prevented a decrease in plasma sodium level without affecting gill Na⁺,K⁺-ATPase activity. On the other hand, estradiol-17 β had no effect on plasma sodium in female fish. There was no additive action of estradiol-17 β on the prolactin-stimulated retention of plasma sodium levels in male fish. These findings showed that cortisol and prolactin have sodium-retaining activity in acid-exposed medaka of both sexes, and estrogen seems to involve in the sex difference in acid tolerance.

Key words: acid water, osmoregulation, medaka, Na⁺,K⁺-ATPase, sodium, cortisol, prolactin, estradiol-17 β , sexual difference

Acidification of environmental water generally results in acute damage followed by death or causes a variety of chronic physiological problems in freshwater fish.^{1,2)} Physiological problems of fish exposed to acid water are thought to be brought about a chain of events starting with a large increase in net sodium loss mainly from the gills.^{1,3)} Involvement of gill Na⁺, K⁺-ATPase, which is mainly localized in gill chloride cells, in the excretion of excess ions is well known in seawater-acclimated fish.^{4,5)} On the other hand, gill Na⁺, K⁺-ATPase is also thought to contribute to the uptake of ions in fresh water.^{4,7)} An increased activity of gill Na⁺, K⁺-ATPase coincided with recovery of plasma sodium levels in the rainbow trout *Oncorhynchus mykiss* surviving after the exposure to acid water.⁸⁾ Cortisol is known to stimulate the activity of gill Na⁺, K⁺-ATPase.^{4,5)} Acid exposure leads to a rise in plasma cortisol levels in several teleosts.⁹⁾ After exposure to acid water, morphological changes in prolactin cells in the pituitary have been observed in the tilapia *Oreochromis mossambicus*, implying prolactin's involvement in sodium retention under acidic environment.^{10,11)} Prolactin is well established as an important osmoregulatory hormone in euryhaline fish, and its sodium-retaining effect has been reported in fish in neutral fresh water.^{7,12,13)} Cortisol and prolactin are the candidates of hormonal adjustment for ionic balance in the fish exposed to acid water.³⁾ However, the osmoregulatory actions of cortisol and prolactin in the fish under acidic environment are still unclear.

Our previous study revealed that, in the medaka *Oryzias latipes*, the female fish is more tolerant of acidic environment than the male.¹⁴⁾ The sexual difference in acid toler-

ance may involve with the reproductive failure which observed in the fish under acidic condition.^{1,2)} Sexual difference in osmoregulatory ability and inhibitory effects of sex steroid on seawater adaptation were observed in salmonids.⁷⁾ The sexual difference in acid tolerance in the medaka suggests the involvement of sex steroid with osmoregulation also in the acid-exposed fish.

The present study was undertaken to clarify the endocrine controls of osmoregulation in the fish under acidic environment. Effects of administration of exogenous cortisol, prolactin, and estradiol-17 β which is the main estrogen in teleostean fishes, prior to acid exposure on plasma sodium levels and gill Na⁺, K⁺-ATPase activity in acid water were examined in both sexes of the medaka in relation to the sexual difference in acid tolerance in this species.

Materials and Methods

The red variety of adult medaka *Oryzias latipes* were purchased from a local fish farm in Niigata. They were reared in outdoor freshwater ponds. Original pH of water used in the present study was 7.6. One month before experiments, the fish, weighing 0.4–0.8 g, were transferred to indoor 200 l rectangular tanks containing fresh water at 25°C, pH being adjusted to 7.0 with 0.1 N sulfuric acid by a pH controller (NPH-6800, Nissin, Tokyo) connected to a peristaltic pump (AC-2110, Atto, Tokyo) under a lighting regime of 14 h light and 10 h dark. They were fed commercially-reared mosquito larvae.

Cortisol (ICN, Aurora, Ohio) and estradiol-17 β (Wako,

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Osaka) were dissolved in ethanol (10 mg/ml) and diluted with saline (0.9% NaCl). Ovine prolactin (Sigma, St. Louis, Missouri) and ovine albumin (Sigma) were dissolved and diluted with saline. Doses of hormones injected were 0.1–1.0 $\mu\text{g/g}$ body weight for cortisol and estradiol-17 β and 5–50 $\mu\text{g/g}$ body weight for prolactin. The fish were anesthetized with 2-phenoxyethanol (1:2000) and a single injection of hormone or vehicle (saline or ethanol-containing saline) was given intraperitoneally through the dorsal musculature as described by Utida *et al.*¹⁵ The volume of fluid injected was about 10 μl . After the injection, the fish were kept in neutral fresh water for 30 min for recovery, and then transferred to tanks containing acidified fresh water at 25°C, pH being adjusted to 3.8 (vehicle- or hormone-injected) or 7.0 (vehicle-injected) as described above. Twenty four hours after transfer, about 2 μl of blood samples were collected directly from the caudal artery with a fine-tipped capillary tube as described previously.¹⁴ Plasma samples were immediately separated from the blood by centrifugation at 3,000 $\times g$ for 5 min, and kept frozen at -80°C until analyses. Immediately after bleeding, gill filaments were cut from ceratobranchials to measure Na^+ , K^+ -ATPase activity as described previously.¹⁴

Sodium concentrations in plasma were measured by flame atomic absorption spectrophotometry (AA-855, Nippon Jarrell-Ash, Kyoto). Gill Na^+ , K^+ -ATPase activity was measured as the ouabain-sensitive hydrolysis of ATP coupled to the oxidation of nicotinamide adenine dinucleotide (reduced form, NADH) using a microplate reader (MTP-120, Corona Electronics, Ibaragi) as described

previously.¹⁴ Protein concentrations were measured by Protein Assay Kit (Bio-Rad, California) using bovine serum albumin as a standard.

Differences in plasma sodium levels and gill Na^+ , K^+ -ATPase activity were analyzed by ANOVA followed by Duncan's multiple range test. In the case of plasma sodium levels in cortisol-injected females, there was significant difference in variance between groups, then Mann-Whitney *U*-test was applied. Calculations were performed using a computer program, STATISTICA (Design Technologies Incorporation, Tokyo).

Results

A single injection of cortisol prior to exposure to acid water showed significant sodium-retaining effect in both sexes at doses from 0.1 $\mu\text{g/g}$ (Fig. 1). Vehicle-injected male and female in acid water showed significant increase in gill Na^+ , K^+ -ATPase activity compared to those in neutral water. Injection of cortisol resulted in further significant activation of gill Na^+ , K^+ -ATPase at 1.0 $\mu\text{g/g}$ in both sexes.

As shown in Fig. 2, a single injection of ovine prolactin showed sodium-retaining effect from 5 $\mu\text{g/g}$ in male, and at 20 and 50 $\mu\text{g/g}$ in female. An injection of ovine albumin (50 $\mu\text{g/g}$) had no significant effect on plasma sodium levels in either sex (data not shown). Vehicle-injected fish in acid water showed higher activity in gill Na^+ , K^+ -ATPase than in neutral water, but no significant effect of prolactin as seen on gill Na^+ , K^+ -ATPase activity even at the highest dose (50 $\mu\text{g/g}$).

Fig. 1. Effects of cortisol on plasma sodium levels and Na^+ , K^+ -ATPase activity of male and female medaka 24 h after exposure to acid water at pH 3.8.

Data are expressed as means $\pm$ SEM. The sample size is indicated in parenthesis. **Significantly different from fish injected with vehicle and transferred to acid water at $P < 0.05$ and $P < 0.01$, respectively. +Significant difference between columns at $P < 0.05$ and $P < 0.01$, respectively.

Fig. 2. Effects of prolactin on plasma sodium levels and Na⁺,K⁺-ATPase activity of male and female medaka 24 h after exposure to acid water at pH 3.8.

Data are expressed as means $\pm$ SEM. The sample size is indicated in parenthesis. **Significantly different from fish injected with vehicle and transferred to acid water at $P < 0.05$ and $P < 0.01$, respectively. †† Significant difference between columns at $P < 0.05$ and $P < 0.01$, respectively.

Fig. 3. Effects of estradiol-17 β on plasma sodium levels and Na⁺,K⁺-ATPase activity of male and female medaka 24 h after exposure to acid water at pH 3.8.

Data are expressed as means $\pm$ SEM. The sample size is indicated in parenthesis. **Significantly different from fish injected with vehicle and transferred to acid water at $P < 0.01$. †† Significant difference between columns at $P < 0.05$ and $P < 0.01$, respectively.

Fig. 4. Effect of combined injection of prolactin and estradiol-17 β on plasma sodium levels on male medaka 24 h after exposure to acid water (pH 3.8).

Data are expressed as means $\pm$ SEM. The sample size is indicated in parenthesis. **Significantly different from fish injected with vehicle and transferred to acid water at $P < 0.01$. NS means no significant difference ($P > 0.05$).

There was a difference between sexes in the effect of estradiol-17 β on plasma sodium levels in acid water (Fig. 3). Males treated with estradiol-17 β showed restored plasma sodium levels at 0.5 and 1.0 $\mu\text{g/g}$, whereas no significant change was seen in females even at the highest dose (1.0 $\mu\text{g/g}$). Estradiol-17 β did not produce any significant change in gill Na^+ , K^+ -ATPase activity in either male or female.

Possible additive effect of estradiol-17 β and prolactin were then examined on plasma sodium levels in male (Fig. 4). A significant effect of prolactin was observed at 20 and 50 $\mu\text{g/g}$ as in the previous results. However, estradiol-17 β had no effect when injected simultaneously with prolactin (20 or 50 $\mu\text{g/g}$).

Discussion

The present study shows positive effects of cortisol, prolactin, and estradiol-17 β on sodium retention in acid-exposed medaka. Cortisol and prolactin are the two most likely candidates of hormonal adjustments for ionic balance in the acid-exposed fish, because of their responses in secretion to acid exposure.³⁾ Results in this study supports the hypothesis that the two hormones play important roles during adaptation to acid water in the fish surviving under acidic environment. On the other hand, negative effect of sex steroid on osmoregulatory ability is well known in seawater adaptation in salmonids.⁷⁾ In juvenile Atlantic salmon reared in fresh water, administration of estradiol-17 β results in decreased gill chloride cell density and Na^+ , K^+ -ATPase activity.¹⁶⁾ In contrast, in this study, administration of estradiol-17 β did not affect gill Na^+ , K^+ -ATPase activity in both sexes of acid-exposed medaka. The conflicting observations may stem from species difference, difference in condition of sexual maturation, or difference in environmental pH. Although estradiol-17 β may not act directly on osmoregulatory organs, the sodium-retaining effect observed in the male medaka strongly suggests an involvement of sex steroid in acid tolerance in fish.

Cortisol activates gill Na^+ , K^+ -ATPase activity in both

freshwater- and seawater-adapted fish, and stimulates ion excretion in seawater, while stimulating the uptake in fresh water.^{17,18)} In the present study, the activation of gill Na^+ , K^+ -ATPase by cortisol injection suggests a stimulation of sodium uptake by cortisol in acidic fresh water as in neutral fresh water. There was no information on the circulating level of cortisol in medaka, while the injected doses of cortisol were less than that promoting sodium influx in the rainbow trout in neutral fresh water (4 $\mu\text{g/g}$).¹⁷⁾ In the gill of chum salmon *Oncorhynchus keta*, the expression of cortisol receptor mRNA and the immunoreactivity of Na^+ , K^+ -ATPase are localized in the same types of chloride cells, and the cell size and the level of cortisol receptor mRNA in seawater fish are greater than those in freshwater fish.¹⁹⁾ Those facts imply that, in seawater-adapted fish, cortisol stimulates salt excretion via an activation of Na^+ , K^+ -ATPase localized in gill chloride cells. On the other hand, it is still unclear whether the same types of chloride cells are able to secrete and uptake ions under different ion conditions of environmental waters. The present study suggests an important role of cortisol in sodium retention under acidic environment via an activation of gill Na^+ , K^+ -ATPase. Further study on the role of cortisol in the acid-exposed fish may provide methods to examine the functional differentiation of chloride cells.

In the present study, ovine prolactin produced significant retention of plasma sodium levels without affecting gill Na^+ , K^+ -ATPase activity in the medaka, and the effective doses were essentially similar to those having freshwater-adapting effect in the hypophysectomized medaka.¹⁵⁾ In the freshwater-adapted killifish *Fundulus heteroclitus* and the rainbow trout, administration of prolactin reduced gill Na^+ , K^+ -ATPase activity.^{20,21)} In contrast, prolactin-treated Atlantic salmon *Salmo salar* show an activation of gill Na^+ , K^+ -ATPase.²²⁾ These conflicting observations seem to reflect species differences, or difference in physiological conditions.⁷⁾ On the other hand, one of the osmoregulatory effects of prolactin in freshwater teleosts is through reduction of sodium permeability of the gills.^{7,10,11)} Administration of prolactin in the acid-exposed medaka might cause an augmentation of the process observed in the fish in neutral fresh water.

The difference in the sodium-retaining effect of estradiol-17 β between male and female medaka suggests an involvement of estrogen in the sexual difference in acid tolerance which we reported previously.¹⁴⁾ In contrast to teleostean females, measurements by radioimmunoassay show only low levels of circulating estradiol-17 β in males.²³⁾ In female fish, the main effect of estradiol-17 β is the control of vitellogenesis probably by the mobilization of lipid and calcium.^{23,24)} The stimulatory action of estrogen on vitellogenesis is documented also in mature male fish.^{25,26)} In this study, a single injection of estradiol-17 β did not affect plasma calcium levels in male medaka (data not shown), and estradiol-17 β seems to stimulate the sodium retention independently of an activation of vitellogenesis. It is well known that estrogen stimulates prolactin secretion in mammals.²⁷⁾ Also in fish, prolactin secretion is activated by estradiol-17 β .^{28,29)} No additive effect of estradiol-17 β and prolactin on sodium retention in acid-exposed male medaka suggests that estradiol-17 β acts via a stimulation of prolactin secretion. Results of the present study sug-

gest an involvement of estrogen with a sexual difference in acid tolerance which may result in reproductive failure in acidic environment.¹⁴⁾ On the other hand, involvement of other sex steroids including androgen and maturation-inducing steroid is not well known. Further studies are required to clarify the possible effects of these steroids on adaptation to acid water.

Acknowledgments We are grateful to Professor Tetsuya Hirano, Hawaii Institute of Marine Biology, University of Hawaii, and Professor Howard A. Bern, University of California at Berkeley, for their encouragement during the course of this study and also critical reading of the manuscript. We thank Drs. Teruo Azuma and Kazumasa Ikuta, National Research Institute of Aquaculture, for their valuable comments. This study was supported in part by grants-in-aid from the Ministry of Agriculture, Forestry and Fisheries, and by the Global Environment Research Promotion Fund from Environment Agency, Japan.

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