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In vivo effects of thyroid hormone, corticosteroids and prolactin on cell proliferation and apoptosis in the anterior intestine of the euryhaline mudskipper (*Periophthalmus modestus*)

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Abstract

We have previously shown that anterior intestinal epithelium of the euryhaline mudskipper (*Periophthalmus modestus*) undergoes apoptosis during seawater (SW) acclimation, whereas elevated cell proliferation was observed in freshwater (FW)-acclimated fish. To understand the possible endocrine regulation of the gastrointestinal cell turnover during salinity acclimation, we examined the ratios of apoptotic and proliferating cells in the anterior intestine of one-third SW-acclimated mudskipper treated with triiodothyronine (T_3), cortisol, 11-deoxycorticosterone (DOC, the putative teleostean mineralocorticoid), or prolactin (PRL). *In situ* nick end labeling of genomic DNA (TUNEL) and immunohistochemistry of proliferating cells nuclear antigen (PCNA) were used as indicators of apoptosis and cell proliferations, respectively. Cortisol significantly elevated apoptosis ($P<0.05$) in the epithelia and connective tissues and also stimulated the epithelial cell proliferation ($P<0.05$). PRL induced epithelial cell proliferation ($P<0.05$), but did not affect apoptotic status of the intestinal epithelium. Neither T_3 nor DOC had any impact on cell proliferation or apoptosis. Together, our results suggest a role for cortisol and PRL in the regulation of anterior intestinal epithelial turnover during salinity acclimation in this species.

Keywords: Prolactin; Cortisol; Apoptosis; Cell proliferation; Fish; Mineralocorticoid; Osmoregulation

Introduction

In order to replace the diffusive loss of water to the surrounding environment, seawater (SW)-acclimated euryhaline fishes have a gastrointestinal tract with higher ion/water flux in concert with greater permeability compared to freshwater (FW)-acclimated fish (Smith, 1930). These differences in permeability correlate with morphological modifications in the epithelium. Specifically, esophageal epithelium is simple and columnar in SW fish, whereas it is stratified in FW-acclimated fish (Hirano and Mayer-Gostan, 1976; Yamamoto and Hirano, 1978). We have recently suggested that changes in epithelial cell proliferation and apoptosis may be responsible for the morphological differences in the gastrointestinal tract seen in FW versus SW fish (McCormick, 2001; Sakamoto and McCormick, 2006). Using the anterior intestine of the euryhaline mudskipper (Perciformes; Gobioidei), comparable to the esophagus since the mudskipper does not have a stomach and the esophagus is connected directly to the proximal intestinal swelling, as a model system we observed elevated apoptosis in the anterior intestine during SW acclimation, while increased cell proliferation was seen with FW acclimation (Takahashi et al., 2006a). These cellular modifications are thought to be important for this species to tightly regulate their plasma sodium levels after transfer to different salinities (Sakamoto et al., 2005a). However, we have no information at present concerning the endocrine regulation of cell turnover in gastrointestinal tract during acclimation to different salinities.

Thyroid hormone is thought to play a role in the intestinal cell proliferation and apoptosis during amphibian metamorphosis when the larval-type epithelium transforms to the adult-type (Ishizuya-Oka and Shimozawa, 1991). However, there is conflicting evidence

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regarding the actions of thyroid hormone on teleost osmoregulatory organs, including the gastrointestinal tract (Peter et al., 2000; McCormick, 2001), although changes in plasma levels of thyroid hormone suggest that this hormone plays a role in the osmoregulation of the mudskipper (Lee and Ip, 1987).

In gastrointestinal tract of euryhaline fish, prolactin (PRL), which facilitates FW adaptation, and cortisol, the major corticosteroid, are known to play central roles in differentiating the osmoregulatory function (Sakamoto and McCormick, 2006). PRL decreases NaCl and water absorption by reducing the epithelial permeability (Hirano, 1986). In amphibians, PRL is known for its anti-metamorphic effects, where it prevents thyroid-hormone-induced apoptosis during resorption of the anuran tadpole tail (Tata, 1993). On the other hand, cortisol, considered as a key hormone of SW acclimation, increases ion and water absorption across the intestinal mucosa of SW teleosts (Veillette et al., 1995). This is achieved by an increase in ion and water permeability, as well as by an increase in the active uptake of ions, which increases the osmotic uptake of water (Loretz, 1995; Karnaky, 1998). Also, cortisol has been reported to be important in acclimation to FW (Flik and Perry, 1989; McCormick, 2001). Recently some studies suggested that cortisol expedite the differentiation of ion-uptake chloride cell (FW-form) in branchial epithelia possibly *via* the mineralocorticoid receptor (MR) with PRL (Sakamoto et al., 2001; Sloman et al., 2001; Marshall et al., 2005; Scott et al., 2005). However, the molecular basis of cortisol's actions is unclear particularly in the gastrointestinal tract. Furthermore, 11-deoxycorticosterone (DOC), precursor of aldosterone, has been identified very recently as a more potent endogenous agonist for the teleost MR rather than cortisol (Sturm et al., 2005). Circulating DOC levels in teleosts appear to be similar to those of cortisol (Prunet et al., 2006) and may be important in hydro-mineral balance (Johnson, 1992).

Against this background, we evaluated the regulation of cell turnover in the anterior intestine after treatments of the euryhaline mudskipper with triiodothyronine (T_3), cortisol, DOC or PRL, and we show the novel effects of PRL and cortisol on intestinal cell proliferation and/or apoptosis.

Materials and methods

Animals

Adult mudskippers (*Periophthalmus modestus*) of both sexes weighing 4–6 g were collected from the estuary of the Fujii River that flows into the Inland Sea of Seto; they are usually exposed to river FW and hypertonic SW. The hormonal status and plasma ion of this species under varying environmental salinities were described in our previous reports (Sakamoto et al., 2002, 2005a). This fish from brackish water were acclimated in laboratory tanks (3 L) maintained at isotonic one-third SW (10 ppt, 149 mM Na, 176 mM Cl^-, 3.8 mM Ca, 346 mOsml/kg) at 22–25 °C for more than one-week. A small plate was placed in each tank to allow animals the opportunity to climb on them. To avoid stress, fish were anaesthetized before handling with 0.01% tricaine methane sulfonate (Sigma, Tokyo) neutralized with sodium bicarbonate. All fish were handled, maintained, and used in accordance with Guidelines for Animal Experimentation established by Okayama University.

Experimental protocol

The fish were maintained in one-third SW with or without T_3 (3, 5, 3′-triiodo-L-thyronine, sodium salt 10 ng/ml), cortisol (hydrocortisone sodium succinate, 10 μg/ml) or DOC (21-hydroxypregn-4-ene-3, 20-dione, 10 μg/ml) for one week without food.No effects of fasting on the intestinal cell turnover, hormonal status or plasma ion were observed (Sakamoto et al., 2002, 2005a; Takahashi et al., 2006a). To maintain a fresh supply of these hormones the solution was changed daily. For the effect of PRL, fish were anesthetized and injected intramuscularly every second day with vehicle (0.01% Triton X-305 in saline) or vehicle plus chum salmon PRL (at dose of 1 μg/5 μl/g body mass) for a total of four injections. The chum salmon PRL (Yasuda et al., 1986) exhibits approximately 70% amino-acid identity to the mudskipper PRL (Sakamoto et al., 2005a) and, specifically, binds to fish PRL receptors, unlike mammalian PRLs, which also binds to growth hormone receptors (Prunet and Aupdrin, 1994). We chose these doses/treatments based on our preliminary studies on dose-dependency, published effective physiological doses and plasma hormone concentrations (Sakamoto et al., 1997, 2001, 2002; Harada et al., 2003). After 1 week hormonal treatments, the anterior intestine (approximately 10–15 mm) was immediately removed and fixed in 4% paraformaldehyde in phosphate–buffered saline (PBS) at 4 °C overnight. Our previous studies with this anterior intestine show consistent changes in cell proliferation and apoptosis during acclimation of mudskipper to different salinities (Takahashi et al., 2006a). The intestine was dehydrated through graded alcohol concentrations and embedded in paraplast. Sections were cut at 5 μm and attached to 3-ammino-propyltriethoxysilane-coated slides. Four to seven mudskippers were examined for each group. Each set of intestines were processed at the same time to avoid procedural variability among groups.

In situ 3′ end labeling of DNA (TUNEL)

DNA fragmentation associated with apoptosis was detected according to the TUNEL method of Gavrieli et al. (1992) using an *in situ* cell death detection kit (Roche, Tokyo; Takahashi et al., 2006a). The TUNEL procedure produces similar results to those we showed with internucleosomal DNA fragmentation analyzed by gel electrophoresis, and appears to discriminate apoptotic cells from necrotic cells in this mudskipper anterior intestine (Takahashi et al., 2006a). The slides were treated with 20 μg/ml proteinase K (Roche, Tokyo) at 20 °C for 30 min, washed in PBS for 15 min, and immersed in 0.3% H_2O_2 in methanol at 20 °C for 30 min to inactivate endogenous peroxidase activity. After being washed in PBS, the sections were incubated with TdT and fluorescein-labeled dUTP at 37 °C for 60 min in a moist chamber, the reaction was terminated by transferring the slides to PBS for 15 min. The sections were incubated with peroxidase-labeled anti-fluorescein antibody at 37 °C for 30 min and then for 5 min with DAB substrate solution (Roche Tokyo). As a positive control, some fixed