General and Comparative Endocrinology 157 (2008) 35–40

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na^+,K^+-ATPase activity and isoform mRNA levels in Atlantic salmon

Stephen D. McCormick [a,*], Amy Regish [a], Michael F. O'Dea [a], J. Mark Shrimpton [b]

[a] USGS, Conte Anadromous Fish Research Center, One Migratory Way, PO Box 796, Turners Falls, MA 01376, USA
[b] Ecosystem Science & Management (Biology) Program, University of Northern British Columbia, Prince George, BC, Canada

ARTICLE INFO

Article history:
Received 29 February 2008
Revised 20 March 2008
Accepted 26 March 2008
Available online 31 March 2008

Keywords:
Cortisol
Osmoregulation
Gill Na,K-ATPase
Deoxycorticosterone
Aldosterone
Salinity tolerance
Ion regulation

ABSTRACT

It has long been held that cortisol, acting through a single receptor, carries out both glucocorticoid and mineralocorticoid actions in teleost fish. The recent finding that fish express a gene with high sequence similarity to the mammalian mineralocorticoid receptor (MR) suggests the possibility that a hormone other than cortisol carries out some mineralocorticoid functions in fish. To test for this possibility, we examined the effect of *in vivo* cortisol, 11-deoxycorticosterone (DOC) and aldosterone on salinity tolerance, gill Na^+,K^+-ATPase (NKA) activity and mRNA levels of NKA α1a and α1b in Atlantic salmon. Cortisol treatment for 6–14 days resulted in increased, physiological levels of cortisol, increased gill NKA activity and improved salinity tolerance (lower plasma chloride after a 24 h seawater challenge), whereas DOC and aldosterone had no effect on either NKA activity or salinity tolerance. NKA α1a and α1b mRNA levels, which increase in response to fresh water and seawater acclimation, respectively, were both upregulated by cortisol, whereas DOC and aldosterone were without effect. Cortisol, DOC and aldosterone had no effect on gill glucocorticoid receptor GR1, GR2 and MR mRNA levels, although there was some indication of possible upregulation of GR1 by cortisol ($p = 0.07$). The putative GR blocker RU486 inhibited cortisol-induced increases in salinity tolerance, NKA activity and NKA α1a and α1b transcription, whereas the putative MR blocker spironolactone had no effect. The results provide support that cortisol, and not DOC or aldosterone, is involved in regulating the mineralocorticoid functions of ion uptake and salt secretion in teleost fish.

Published by Elsevier Inc.

1. Introduction

Corticosteroids have two major functions in vertebrates: a glucocorticoid function affecting metabolism and growth, and a mineralocorticoid function, regulating the movement of ions and water. In many vertebrates these functions are served by two distinct hormones, cortisol (or in some species corticosterone) and aldosterone that each have a specific receptor, the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), respectively. In teleost fish it has generally been thought that a single hormone, cortisol, carries out both glucocorticoid and mineralocorticoid actions (Wendelaar Bonga, 1997; Mommsen et al., 1999), and that this hormone works through a single class of receptors (Chakraborti et al., 1987).

Recent molecular data provide evidence that teleost fish express an MR-like gene that shares more sequence similarity with the tetrapod MR than with the fish GR (Colombe et al., 2000; Greenwood et al., 2003; Bridgham et al., 2006). Expression studies of the rainbow trout MR and GR in a mammalian cell line indicate that transactivation of the MR is more sensitive to aldosterone and deoxycorticosterone, whereas the GR is more sensitive to cortisol (Sturm et al., 2005). Similar studies with the cichlid MR and GR found no strong difference in transactivation affinity between aldosterone and cortisol (Greenwood et al., 2003). MR mRNA is found in a wide variety of tissues including those involved in ion regulation (Greenwood et al., 2003; Sturm et al., 2005). Use of the MR antagonist spironolactone has been shown to affect chloride cell density in rainbow trout following transfer to ion-deficient fresh water, implicating a function of the MR in ion uptake in fish (Sloman et al., 2001). While these studies suggest that a MR-like receptor is expressed in fish, its biochemical characterization, localization and function have yet to be established.

Aldosterone is present only in extremely low levels in teleost fish; lower than are likely to have actions on either the GR or MR (Prunet et al., 2006). However, deoxycorticosterone (DOC) is present in some teleosts in concentrations ranging from 0.5 to 10 nM. Although these levels are much lower than circulating levels of cortisol, they are near the range that could interact with a receptor that has affinities ascribed to the trout MR (Sturm et al., 2005).

* Corresponding author. Fax: +1 413 863 9810.
E-mail address: mccormick@umext.umass.edu (S.D. McCormick).

0016-6480/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.ygcen.2008.03.024

Thus, Prunet et al. (2006) suggested that DOC may have biological actions in fish, possibly acting through the MR and resulting in physiologically relevant effects on osmoregulation.

The present studies were conducted to determine whether we were missing a mineralocorticosteroid in fish, and to determine the receptor pathway through which corticosteroids are acting to affect ion regulation. We used *in vivo* administration of cortisol, DOC and aldosterone to see if these steroids could carry out a well-known corticosteroid function in fish, the development of seawater tolerance. We examined whether these hormones could also regulate the levels of putative fresh water and seawater isoforms of the Na^+,K^+-ATPase (NKA), an ion transport enzyme involved in both ion uptake and salt secretion. Finally, we examined the impact of the GR inhibitor RU486 and MR inhibitor spironolactone on corticosteroid-induced regulation of these osmoregulatory parameters.

2. Materials and methods

Juvenile Atlantic salmon (*Salmo salar*) were obtained from the White River National Fish Hatchery (Bethel, VT, USA) and brought to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in autumn. Fish were reared in 1.7 m diameter tanks supplied with ambient river water at a flow rate of 4 L min^{-1} and provided supplemental aeration. They were maintained under natural photoperiod conditions and fed to satiation (Zeigler Bros., Gardners, PA, USA) using automatic feeders.

In vivo treatment with corticosteroids and receptor blockers followed the methods outlined in McCormick (1996). Feed was withheld for 24 h prior to injection, and fish were anesthetized with 100 mg L^{-1} MS-222 neutralized to pH 7.0. Hormones, receptor blockers or both were suspended in 1:1 vegetable oil:shortening as outlined in Specker et al. (1994), warmed to 35 °C and injected intraperitoneally at 5 μl per g wet weight of fish. DOC has a molecular weight lower than cortsiol and aldosterone, so approximately 10% lower doses of DOC were used to achieve approximately equimolar treatments. Fish were placed in 1 m diameter tanks filled with dechlorinated tap water at 10–12 °C with particle and charcoal filtration and continuous aeration. Fifty percent water change occurred twice weekly. Seawater challenge tanks were identical to fresh water tanks but contained 25 ppt seawater (Instant Ocean). All experiments were conducted with 0+ parr (10–30 g) between October and January. Food was withheld during the entire treatment period. In experiments measuring physiological responses, fish were treated for 14 days, which included a gill biopsy at 6 and 12 days and transfer to 25 ppt seawater on day 13 followed by terminal sampling 24 h later. Aldosterone and DOC exposures were done in separate experiment, with separate vehicle controls in each. In experiments measuring changes in mRNA, treatments occurred for 6 days (terminal sampling) in order to best capture the changes in mRNA levels which are likely to precede biochemical and physiological responses and can become reduced over time.

For sampling, fish were anesthetized as above and fork length to the nearest mm and weight to the nearest 0.1 g were recorded. Gill biopsies consisted of 4–6 gill filaments that were severed above the septum (approximate half of total length), placed in 100 μl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80 °C within 30 min. At terminal sampling, blood was drawn from the caudal vein into a 1 ml ammonium heparinized syringe and spun at 3200 g for 5 min at 4 °C. Plasma was aliquoted and stored at −80 °C. The second gill arch was removed, the gill filaments trimmed from ceratobranchials and placed in an autoclaved 1.5 ml microcentrifuge tube and frozen immediately at −80 °C for later extraction of total RNA.

Na^+,K^+-ATPase activity was determined with a kinetic assay run in 96-well microplates at 25 °C and read at a wavelength of 340 nm for 10 min as described in McCormick (1993). Gill tissue was homogenized in 125 μl of SEID (SEI buffer and 0.1% deoxycholic acid) and centrifuged at 5000*g* for 30 s. Ten microliter samples were run in two sets of duplicates; one set containing assay mixture and the other assay mixture and 0.5 mM ouabain. The resulting ouabain-sensitive ATPase activity measurement is expressed as μmoles ADP/mg protein/h. Protein concentrations were determined using BCA (bicinchoninic acid) Protein Assay (Pierce, Rockford, Il, USA). Both assays were run on a THERMOmax microplate reader using SOFTmax software (Molecular Devices, Menlo Park, CA, USA).

Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay as outlined in Carey and McCormick (1998). Sensitivity as defined by the dose–response curve was 1–400 ng ml^{-1}. The lower detection limit was 0.3 ng ml^{-1}. Using a pooled plasma sample, the average intra-assay variation was 5.5% (n = 10) and the average inter-assay variation was 8.8% (n = 10). Plasma aldosterone levels in aldosterone-treated fish were measured using a competitive enzyme immunoassay (Immuno-Biological Laboratories, Minneapolis, MN, USA) with plasma dilutions of 1:100. The lower detection limit at this dilution was 0.1 ng ml^{-1}.

Total RNA was purified by the TRIzol procedure (Invitrogen, Carlsbad, CA, USA) using 1 ml TRIzol/100 mg gill tissue according to manufacturer's recommendation. First strand cDNA was synthesized from 1 μg of total RNA using oligo(dT_{15}) primer. The expression of Na^+,K^+-ATPase α1a and α1b isoforms, GR1, GR2 and MR was estimated using quantitative real-time PCR (qRT-PCR; ABI Prism 7300 sequence analysis system; Applied Biosystems Inc., Foster City, CA, USA). PCRs contained 1 μL of cDNA, 4 pmoles of each gene specific primer and Universal SYBR green master mix (Applied Biosystems Inc., Streetville, ON, Canada) in a total volume of 20 μL. Primers designed for rainbow trout Na^+,K^+-ATPase α1a, Na^+,K^+-ATPase α1b and elongation factor 1α were used (Richards et al., 2003). Primers designed for rainbow trout GR1 were used (Vijayan et al., 2003). We designed primers for GR2 and MR using rainbow trout sequences (GenBank Accession Nos.: AY495372 and AY495584, respectively) to amplify ~100 bp product; GR2 forward primer 5′-CAT GGC AGA CCA GTG TGA AC-3′, GR2 reverse primer 5′-AGC AGC AGC AGA ACC TTC AT-3′, MR forward primer 5′- CTT CTT CCA GCT CAC CAA GC-3′ and MR reverse primer 5′-CCA CCT TCA GAG CCT GAG AC-3′. All qRT-PCRs were performed as follows: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Melt curve analysis was performed following each reaction to confirm the presence of only a single product of the reaction. Negative control reactions were performed for a selection of samples using RNA that had not been reverse transcribed to control for the possible presence of genomic contamination. Gene expression levels measured in qRT-PCR assays are often normalized to the mRNA level of an invariant reference gene. Normalization with a reference gene corrects for variation in reverse transcription (RT) efficiency and it is expected that variation in RT efficiency will affect target and reference genes equally. Richards et al. (2003) demonstrated that elongation factor 1α did not vary in response to salinity challenges in rainbow trout. Relative expression of the target genes was normalized to a reference gene by use of the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). mRNA amounts are expressed relative to the gill samples collected from vehicle-treated fish.

For each parameter a one-way analysis of variance (ANOVA) followed by Student–Neuman–Keuls test was used to test the significance of treatment with corticosteroids and/or receptor blockers (RU486 or spironolactone). All statistics were run using the Statistica (StatSoft Inc., Tulsa, OK) software package.

3. Results

Treatment with cortisol resulted in dose-dependent elevations of plasma cortisol (Table 1), while DOC and aldosterone had no effect (Tables 1 and 2). Cortisol treatment (50 μg/g) resulted in significant increases in gill Na^+,K^+-ATPase (NKA) activity after 6 and 12 days of treatment (Fig. 1), with the relative effect increasing over time (40% and 84% increases at 6 and 12 days, respectively). Although there were increases in gill NKA activity at 10 μg/g cortisol (10% and 18% after 6 and 12 days), these differences were not statistically significant (p = 0.46 and 0.31, respectively). Salinity tolerance as indicated by plasma chloride after 24 h in 25 ppt seawater was significantly increased by both 10 and 50 μg/g cortisol. Neither DOC at 9 and 45 μg/g (Fig. 1) nor aldosterone at 10 and 50 μg/g (Table 1) had a significant effect on gill Na^+,K^+-ATPase activity or salinity tolerance. Treatment of fish with aldosterone resulted in a dose-dependent increase in circulating aldosterone levels (Table 2).

Cortisol treatment for 6 days at 50 μg/g resulted in significant elevations in NKA α1a and α1b mRNA levels (90% and 2.8-fold, respectively; Fig. 2). DOC and aldosterone had no significant effect on NKA α1a and α1b mRNA levels. Cortisol, DOC and aldosterone did not significantly affect gill GR1, GR2 and MR mRNA levels (Table 3). However, gill GR1 mRNA was increased approximately 42% (p = 0.068) by cortisol treatment. Gill MR mRNA was also increased

Table 1
Plasma cortisol after treatment with corticosteroids for 13 days in fresh water followed by exposure to seawater (25 ppt) for 24 h

Treatments	Plasma cortisol (ng ml^{-1})
Vehicle	29.2 ± 14.6
Cortisol 10 μg/g	51.3 ± 16.5
Cortisol 50 μg/g	202.2* ± 58.9
DOC 9 μg/g	51.6 ± 25.6
DOC 45 μg/g	63.2 ± 36.7

Values are mean ± standard error (n = 10 fish per group). Asterisk indicates significant difference between the treated and vehicle group (p < 0.05, Student-Neuman-Keuls test).