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Effect of cortisol on permeability and tight junction protein transcript abundance in primary cultured gill epithelia from stenohaline goldfish and euryhaline trout

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ABSTRACT

Primary cultured gill epithelia from goldfish and rainbow trout were used to investigate a role for cortisol in the regulation of paracellular permeability and tight junction (TJ) protein transcript abundance in representative stenohaline versus euryhaline freshwater (FW) fish gills. Glucocorticoid and mineralocorticoid receptors are expressed in cultured goldfish gill preparations and cortisol treatment (100, 500 and 1000 ng/mL) dose-dependently elevated transepithelial resistance (TER) and reduced paracellular [^{3}H]PEG-4000 flux across cultured goldfish gill epithelia. Despite these dose-dependent 'tightening' effects of cortisol, the response of goldfish TJ protein transcripts (i.e. occludin, claudin b, c, d, e, h, 7, 8d and 12, and ZO-1) were surprisingly small, with only claudin c and h, and ZO-1 transcript levels significantly decreasing at a dose of 1000 ng/mL. Extending the duration of cortisol exposure from 24 to 48 or 96 h (at 500 ng/mL) did little to alter this phenomenon. By comparison, exposing primary cultured trout gill epithelia (i.e. a euryhaline fish gill model) to 500 ng/mL cortisol resulted in a qualitatively similar, but quantitatively stronger epithelial 'tightening' response. Furthermore, transcript abundance of orthologous trout TJ proteins (i.e. occludin, and claudin 30, 28b, 3a, 7, 8d and 12) significantly elevated as would be expected in a 'tighter' epithelium. Taken together, data suggest a conservative role for cortisol in the endocrine regulation of paracellular permeability across the goldfish gill that may relate to stenohalinity.

1. Introduction

Cortisol is the main corticosteroid in fishes, functioning as both a glucocorticoid and mineralocorticoid hormone (reviewed by [35]). Although cortisol is involved in the stress response, growth and reproduction, this remarkably versatile hormone also has a well-established role in the endocrine control of osmoregulation [35,33]. In this regard, cortisol appears to possess a dual function, as it has long been associated with salt secretion across the gills of fishes under hyperosmotic conditions (i.e. seawater, SW) and has more recently been linked to ion uptake across the gill epithelium in a hypoosmotic setting (i.e. freshwater, FW) [14,33]. These observations have been generated largely by studies that have focused on the role of cortisol in altering elements of the transcellular transport pathway in gill tissue [14]. However, there is also evidence to suggest that cortisol may play an important role in regulating the physiological properties of the paracellular pathway in gill epithelia [7,23,24] and that tight junction (TJ) proteins may be integrally involved in this endocrine mediated event [4,7,43].

TJs comprise transmembrane and cytoplasmic protein networks encircling the apical-most domain of vertebrate epithelial cells and form a semi-permeable seal that limits the movement of water and solutes across the paracellular pathway. While transmembrane TJ proteins, such as occludin and claudins, form the physical paracellular barrier that selectively restricts the passage of ions and solutes between epithelial cells, the cytoplasmic adaptor or 'scaffolding' TJ proteins, such as ZO-1, tether occludin and claudins to actin filaments within the cytoskeleton. This link between the 'sealing' transmembrane TJ proteins and the actin cytoskeleton allows signals from the external environment to be transmitted to the inside of the cell in order to influence transcriptional pathways that regulate TJ permeability and thus the 'tightness' or 'leakiness' of the epithelium (reviewed by [19]).

Corticosteroids have a well documented ability to alter the permeability characteristics of cultured vertebrate epithelia and endothelia (e.g. [17,41,48]), including the gill epithelia of euryhaline fishes such as the rainbow trout [23] and tilapia [24]. These latter observations are in line with a growing body of evidence that suggests an important role for TJ proteins in the regulation and maintenance of hydromineral balance in fishes, particularly during conditions of altered environmental salinity. In this regard, significant changes in occludin and claudin transcript and/or protein abundance in the teleost gill have been demonstrated following

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acclimation to ion-poor water [6,13], SW [2,3,13,42] as well as hypersaline SW [2]. However, the majority of the aforementioned studies and all *in vitro* studies that have examined the effects of cortisol on gill permeability have been conducted using either euryhaline fishes or gill epithelial models derived from euryhaline fishes. To the best of our knowledge, no studies have examined the effects of cortisol on the permeability characteristics and TJ components of a stenohaline FW fish gill, or compared these with a euryhaline fish gill.

Therefore, the first objective of the present study was to utilize a recently developed cultured gill epithelium derived from the goldfish (see [8]) to investigate a role for cortisol in the endocrine regulation of gill permeability in a representative stenohaline FW fish. A second objective was to compare results obtained in the goldfish gill model to those observed in a cultured euryhaline fish gill epithelium derived from rainbow trout (see [22]). Since corticosteroid-induced alterations in the permeability characteristics of vertebrate epithelia have been linked to alterations in the transcriptional and post-translational abundance of TJ proteins (e.g. [7,16,17,41]), a third objective was to identify and examine the transcript abundance of select TJ proteins in goldfish and rainbow trout gill preparations that are associated with changes in epithelial permeability in other vertebrates. Where possible, these TJ components were selected on the basis that they have already been associated with changes in the hydromineral status of fishes, such as occludin (see [5–7]), claudin h (which is a claudin 3a ortholog; see [2,4,13]), claudin 8d (see [3,4,13]), and claudin b and e (orthologs of claudin 30 and 28b, respectively; see [42,43]). It is our view that in order to gain broader insight into the various mechanisms that regulate gill permeability in fishes, it is important to consider the physiology and molecular components of a stenohaline model in addition to the traditional euryhaline archetype.

2. Materials and methods

2.1. Animals

Goldfish (*Carassius auratus*, 18–30 g) and rainbow trout (*Oncorhynchus mykiss*, ~125 g) were obtained from local suppliers (Aleongs International, Mississauga, ON, Canada; Humber Springs Trout Club and Hatchery, Orangeville, ON, Canada) and held in either 200-L (goldfish) or 600-L (trout) opaque polyethylene tanks. Tanks were supplied with flow-through dechlorinated FW (approximate composition in mM: $[Na^+]$ 0.59, $[Cl^-]$ 0.92, $[Ca^{2+}]$ 0.76, $[K^+]$ 0.43, pH 7.35) at 25 ± 1 °C for goldfish and 10 ± 2 °C for rainbow trout. All fish were held under the above described conditions for at least 3 weeks prior to use and fed *ad libitum* once daily with commercial pellets (Martin Profishent, Elmira, ON, Canada).

2.2. Preparation of cultured gill epithelia

Procedures for goldfish gill cell isolation and the culture of goldfish gill epithelia (composed of pavement cells only) were conducted according to previously described methods (see [8]). Methods for rainbow trout gill cell isolation and the preparation of rainbow trout gill epithelia (composed of pavement cells only) were conducted according to procedures originally developed by [45] and described in detail by [22]. In brief, isolated goldfish or rainbow trout gill cells were initially cultured in flasks with Leibovitz's L-15 culture medium supplemented with 2 mM L-glutamine (L15) and 6% fetal bovine serum (FBS). At confluence (~2 days for goldfish; ~4–5 days for rainbow trout), cells were harvested from flasks by trypsination and seeded into cell culture inserts (polyethylene terephthalate filters, 0.9 cm^2 growth area, 0.4 μm pore size, 1.6×10^6 pore/cm^2 pore density; BD Falcon™, BD Biosciences, Mississauga, ON, Canada). Epithelia were allowed to develop under symmetrical culture conditions (i.e. with FBS-supplemented L15 culture medium bathing both apical and basolateral surfaces of the epithelial preparations) and were maintained in an air atmosphere at either 27 or 18 °C for cultured goldfish and rainbow trout gill epithelia, respectively. All experimental procedures conformed to the guidelines of the Canadian Council on Animal Care and were approved by the York University Animal Care Committee.

2.3. Transepithelial resistance (TER) and [^{3}H]PEG-4000 flux measurements

Measurements of TER were conducted using chopstick electrodes (STX-2) connected to a custom-modified EVOM epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA). All TER measurements are expressed as kΩ cm^2 and background-corrected for TER measured across 'vacant' culture inserts containing appropriate media. Paracellular permeability across cultured epithelia was determined using the paracellular marker, [^{3}H] polyethylene glycol (molecular mass 4000 Da; PEG-4000; PerkinElmer, Woodbridge, ON, Canada) according to previously described methods and calculations [46]. Briefly, the appearance of [^{3}H]PEG-4000 in the apical compartment was monitored as a function of time and epithelial surface area after the addition of 1 μCi of [^{3}H]PEG-4000 to basolateral culture media.

2.4. Cortisol treatment

Single-use aliquots of a stock cortisol solution were prepared by dissolving cortisol (hydrocortisone 21-hemisuccinate sodium salt; Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) in sterile phosphate-buffered saline (PBS; pH 7.7). Aliquots were stored at −30 °C until use. Cortisol treatment of cultured goldfish and rainbow trout gill epithelia commenced at ~24 h after seeding cells in culture inserts when TER measurements were ~100–200 Ω cm^2 above background levels (see Section 2.3). Inserts were randomly assigned to either a control group or a cortisol-treated group. In the cortisol-treated group, basolateral culture media were supplemented with an appropriate amount of thawed stock cortisol in order to achieve the desired concentration of hormone. Control media contained no cortisol supplement.

2.4.1. Series 1

To investigate the dose-dependent effects of cortisol on cultured goldfish gill epithelia, basolateral media were supplemented with three concentrations of cortisol (100, 500 and 1000 ng/mL), the lower two of which are within the physiological range for goldfish [37]. Once control and hormone-treated preparations developed a stable plateau in TER (~21 h after the addition of cortisol), [^{3}H]PEG-4000 permeability was measured over a 3 h flux period and then epithelia were collected for RNA extraction (see Section 2.5). Therefore, in these experiments, epithelia were treated with cortisol for a total of 24 h.

2.4.2. Series 2

To examine the time-course effects of cortisol on cultured goldfish gill epithelia, basolateral media were supplemented with 500 ng/mL cortisol, and control and cortisol-treated preparations were collected for RNA extraction at 48 and 96 h after the addition of cortisol. To confirm that cortisol was having the desired effect, the TER across epithelia was monitored periodically throughout the incubation period. A single dose of 500 ng/mL cortisol was used in this series based on physiological relevance and on observations made in *series 1*. Control and cortisol-supplemented media were renewed once at 48 h for epithelia collected at 96 h.