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Cortisol reduces paracellular permeability and increases occludin abundance in cultured trout gill epithelia

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ABSTRACT

A role for the tight junction (TJ) protein occludin in the regulation of gill paracellular permeability was investigated using primary cultured "reconstructed" freshwater (FW) rainbow trout gill epithelia composed solely of pavement cells. Cortisol treatment reduced epithelial permeability characteristics, measured as changes in transepithelial resistance (TER) and paracellular [^{3}H]PEG-4000 flux. Cortisol also reduced net Na^+ flux rates when epithelia were exposed to apical FW. cDNA encoding for the TJ protein occludin was cloned from rainbow trout and found to be particularly abundant in gill tissue. In cultured gill preparations, occludin immunolocalized to the TJ complex and transcript abundance dose-dependently increased in response to cortisol treatment in association with reduced paracellular permeability. Occludin protein abundance also increased in response to cortisol treatment. However, occludin mRNA levels did not change in response to apical FW exposure, and [^{3}H]PEG-4000 permeability did not decrease. These data support a role for occludin in the endocrine regulation of paracellular permeability across gill epithelia of fishes.

1. Introduction

The tight junction (TJ) complex plays an important role in the regulation of epithelial permeability in vertebrates. It is composed of a number of transmembrane and cortical proteins and the presence, as well as abundance, of different TJ proteins appears to be a key element in TJ heterogeneity between and within tissues. Occludin is a tetraspan transmembrane TJ protein that is broadly expressed in vertebrate epithelia (Feldman et al., 2005). Since the initial discovery of occludin (Furuse et al., 1993), numerous studies have suggested an important role for this TJ protein in the regulation of epithelial permeability (Feldman et al., 2005). More specifically, an increase in occludin abundance is most often associated with reductions in paracellular permeability across diverse epithelia and endothelia (Feldman et al., 2005). However, the majority of work conducted on the physiological function of occludin in vertebrate epithelia has been accomplished using mammalian models. Recently it has been proposed that occludin may contribute to the regulation of epithelial permeability in aquatic vertebrates under conditions of altered hydromineral status (Chasiotis and Kelly, 2008, 2009; Chasiotis et al., 2009). In this regard, occludin has been found to be abundant in epithelia that regulate salt and water balance in fishes, such as the gill, kidney and gastrointestinal (GI) tract (Chasiotis and Kelly, 2008; Chasiotis et al., 2009). In the freshwater (FW) goldfish kidney, a spatially distinct distribution pattern of occludin can be observed along the nephron (Chasiotis and Kelly, 2008). The "tight" distal tubules and collecting ducts of the nephron exhibit robust occludin immunoreactivity (occludin-ir), while the "leakier" proximal regions of the nephron exhibit little or no occludin-ir. Furthermore, in the gill tissue of goldfish, occludin protein abundance significantly increased when fish were acclimated to ion-poor water (Chasiotis et al., 2009). This has been proposed to contribute to a reduction in the permeability of the paracellular pathway across the gill (Chasiotis et al., 2009) and is consistent with the observations of Cuthbert and Maetz (1972) who reported that the gill epithelium of goldfish exposed to ion-poor conditions exhibits a considerable reduction in outwardly directed ion movement. This presumably results in a beneficial reduction in passive ion loss in an environment where limitations are set on active ion acquisition.

Despite the above observations, and to the best of our knowledge, there are no studies that have related alterations in the specific machinery of the TJ complex in fishes with measured changes in epithelial permeability. Primary cultured gill epithelial models that allow for the "reconstruction" of FW gill epithelia *in vitro* present an appropriate tool for such studies (see Wood and Pärt, 1997; Fletcher et al., 2000; Kelly et al., 2000; Kelly and Wood, 2002). These models exhibit passive transport and perme-

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ability characteristics that closely mimic the *in vivo* characteristics of the FW gill epithelium (Wood and Pärt, 1997; Fletcher et al., 2000; Kelly et al., 2000; Kelly and Wood, 2002). Furthermore, corticosteroid (cortisol) treatment of cultured gill epithelia results in a distinct epithelial tightening effect which is driven at least in part by reduced paracellular permeability properties (Kelly and Wood, 2001, 2002). This sensitivity to cortisol provides a simple means by which to manipulate transepithelial as well as paracellular permeability characteristics and these observations in fishes are in accord with the tightening effects of corticosteroids on other vertebrate epithelia and endothelia (Zettl et al., 1992; Stelwagen et al., 1999; Antonetti et al., 2002; Förster et al., 2005).

Based on this knowledge, the objectives of the current study were to examine cortisol-induced alterations in the permeability characteristics of a cultured gill epithelium prepared from FW rainbow trout and relate alterations in paracellular permeability to modifications in occludin abundance. We hypothesized that if occludin is involved in regulating the barrier properties of gill epithelia in fishes, occludin abundance should increase in association with reductions in paracellular permeability.

2. Materials and methods

2.1. Cultured rainbow trout gill epithelia

The preparation and culture of gill epithelia from FW rainbow trout was carried out in order to produce preparations composed of gill pavement cells only. Methods have been detailed by Kelly et al. (2000) and were originally developed by Wood and Pärt (1997). Briefly, cultured epithelia were prepared using stock rainbow trout (200–450 g) held in flow-through dechlorinated tap water (approximate composition in mmol l^{-1}: $[Na^+]$ = 0.55–0.59, $[Cl^-]$ = 0.70–0.92, $[Ca^{2+}]$ = 0.76–0.90, $[K^+]$ = 0.04–0.05, pH range 7.4–8.0) at 10–12 °C. Cells were initially cultured in 25 cm^2 flasks in Leibovitz's L-15 media supplemented with 2 mmol l^{-1} glutamine and 6% foetal bovine serum (L15). At confluence (~4–5 days), cells were harvested and seeded into cell culture inserts (0.9 cm^2 growth area, 0.4 μm pore size, 1.6×10^6 pores/cm^2 pore density; Falcon BD, Mississauga, ON, Canada). Culture inserts were housed in companion cell culture plates (Falcon BD) and after cell seeding (at a density of 700,000 cells/culture insert), epithelia were allowed to develop a stable transepithelial resistance (TER) (over ~5 days) with L15 culture media present on both apical and basolateral surfaces of the preparation (i.e. symmetrical culture conditions). The treatment of epithelia with cortisol was conducted according to methods previously outlined by Kelly and Wood (2001). Two physiologically relevant doses of cortisol were selected (50 and 500 ng/ml) based on the aforementioned study as well as observations made by Kelly and Wood (2002). Cortisol was added to culture media in flasks 24 h after first seeding and when epithelia were cultured in inserts, cortisol was added to the basolateral media only. Therefore, epithelia cultured in flasks and subsequently in inserts were exposed to cortisol for a total of 9–10 days. In a separate set of experiments which were conducted in order to determine whether alterations in occludin transcript abundance translated into alterations in occludin protein abundance, only flask-cultured epithelia were used to harvest tissue. The rationale for this was that inserts did not provide enough protein for Western blot analysis after conducting the extraction protocol used in these studies (see Section 2.6). Therefore, in these experiments cultured epithelial cells were exposed to a single dose of cortisol (500 ng/ml) for 5 days. This period of time and dose of cortisol is sufficient to elicit a significant increases in TER and accompanying decrease in [^{3}H]PEG-4000 permeability (see Section 2.2) in cultured epithelia (data not shown). Finally, in experiments where FW was added to the apical side of cultured preparations (i.e. asymmetrical culture conditions), temperature-equilibrated sterile dechlorinated FW (composition as detailed above) was used.

2.2. Electrophysiological, [^{3}H]PEG-4000 and net Na^+ flux measurements

Measurements of TER were conducted using chopstick electrodes (STX-2) fitted to a custom-modified voltohmmeter (World Precision Instruments, Sarasota, FL, USA). TER was recorded every 24 h after seeding cells onto culture inserts to monitor epithelial development. Under asymmetrical conditions, TER was monitored at 3 h intervals. All measurements of TER are reported as background-corrected values taking into account the resistance measured across a "vacant" culture insert containing appropriate solutions.

Paracellular permeability across cultured epithelia was examined using the paracellular permeability marker, [^{3}H]polyethylene glycol (molecular mass 4000 Da; 'PEG-4000'; NEN-Dupont, Mississauga, ON, Canada) according to previously detailed methods and calculations (Kelly and Wood, 2001). [^{3}H]PEG-4000 (1 μCi) was added to the basolateral compartment of culture preparations and its appearance in the apical compartment monitored as a function of time and epithelial surface area. Net Na^+ flux rates from basolateral to apical compartments under asymmetrical culture conditions were measured and calculated according to methods detailed by Kelly and Wood (2001).

2.3. Cloning and qRT-PCR analysis of rainbow trout occludin cDNA

Total RNA was isolated from trout gill tissue using TRIzol® Reagent (Invitrogen Canada Inc., Burlington, ON, Canada), according to manufacturer's instructions. Gill RNA was then treated with DNase I (Amplification Grade; Invitrogen Canada Inc.) and first-strand cDNA was synthesized using SuperScript™ III Reverse Transcriptase and Oligo(dT)$_{12-18}$ primers (Invitrogen Canada Inc.).

Using a ClustalX multiple sequence alignment of occludin coding sequences from 9 different species (human [NM_002538]; mouse [NM_008756]; rat [NM_031329]; cow [NM_001082433]; dog [NM_001003195]; platypus [XM_001510548]; opossum [XM_001380557]; frog [NM_001088474]; zebrafish [NM_212832]), degenerate primers were designed based on highly conserved regions. A partial rainbow trout cDNA fragment was amplified by reverse transcriptase PCR (RT-PCR) using occludin degenerate primers under the following reaction conditions: 1 cycle of denaturation (95 °C, 4 min), 40 cycles of denaturation (95 °C, 30 s), annealing (53 °C, 30 s) and extension (72 °C, 30 s), respectively, final single extension cycle (72 °C, 5 min) (0.2 μM dNTP, 2 μM forward and reverse primers, 1× Taq DNA polymerase buffer, 1.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase) (Invitrogen Canada Inc.). Gel electrophoresis (1% agarose for ~90 min at 5 V/cm) verified a PCR product at the predicted amplicon size of ~796 bp. The DNA fragment was excised from the gel and purified using a QIAquick Gel Extraction Kit (QIAGEN Inc., Mississauga, ON, Canada). The purified amplicon was sequenced in the York University Core Molecular Biology and DNA Sequencing Facility (Department of Biology, York University, Toronto, ON, Canada). A partial coding sequence (CDS) of trout occludin was confirmed using a Basic Local Alignment Search Tool (BLAST) search.

To obtain the complete rainbow trout occludin CDS, both 5′- and 3′-rapid amplification of cDNA ends (RACE) PCR was performed using a SMART™ RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA, USA) as per manufacturer's instructions. RACE-PCR products were resolved by electrophoresis, purified and sequenced as described above in order to complete the trout occludin CDS, GenBank accession number GQ476574.

2.4. Occludin expression profile and qRT-PCR analysis of occludin mRNA abundance in rainbow trout tissues

Quantitative real-time PCR (qRT-PCR) was used to examine occludin mRNA distribution and abundance in discrete rainbow trout tissues, as well as occludin transcript abundance in cultured epithelia from flasks and cell culture inserts. For expression profile studies, total RNA was extracted from the following tissues: brain, eye, gill, bulbus arteriosus, atrium, ventricle, esophagus, anterior and posterior stomach, pyloric ceca, anterior intestine, middle intestine and posterior intestine, liver, gallbladder, spleen, swimbladder, kidney, muscle, adipose tissue and blood. The extraction of RNA and synthesis of cDNA from all tissues was conducted as outlined in the previous section. Primers for trout occludin (forward: 5′ CAGCCCAGTTCCTCCAGTAG 3′ and reverse: 5′ GCTCATCCAGCTCTCTGTCC 3′, predicted amplicon size ~340 bp) were designed using the CDS generated by 5′- and 3′-RACE-PCR described above. β-Actin was used as an internal control (forward: 5′ GGACTTTGAGCAGGAGATGG 3′ and reverse: 5′ GACGGAGTATTTACGCTCTGG 3′, predicted amplicon size ~354 bp). β-Actin primers were designed based on GenBank accession number AF157514.

qRT-PCR analysis of occludin and β-actin was conducted using SYBR Green I Supermix (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and a Chromo4™ Detection System (CFB-3240, Bio-Rad Laboratories Canada Ltd.) under the following conditions: 1 cycle denaturation (95 °C, 4 min) followed by 40 cycles of denaturation (95 °C, 30 s), annealing (58 °C, 30 s) and extension (72 °C, 30 s), respectively. To ensure that no primer–dimers or other non-specific products were synthesized during reactions, a melting curve analysis was carried out after each qRT-PCR run.

2.5. Immunolocalization of rainbow trout occludin

Trout gill epithelia cultured in inserts were allowed to develop a stable TER under symmetrical culture conditions. Epithelia were briefly rinsed with phosphate-buffered saline (PBS, pH 7.7) and fixed for 20 min at room temperature (RT) with 3% paraformaldehyde. Fixed epithelia were then permeabilized with ice-cold methanol for 5 min at −20 °C, washed with 0.01% Triton X-100 in PBS for 10 min and blocked for 1 h at RT with antibody dilution buffer (ADB; 10% goat serum, 3% BSA and 0.05% Triton X-100 in PBS). Epithelia were incubated overnight at RT with a custom-synthesized polyclonal antibody raised in rabbit against a synthetic peptide (CHIKKMVGDYDRRA) corresponding to a 14-amino acid region of rainbow trout occludin (1:100 dilution in ADB; New England Peptide, LLC, Gardner, MA, USA). For a negative control, epithelia were also incubated overnight with ADB lacking primary antibody. Epithelia were then washed with PBS and incubated for 1 h at RT with TRITC-labeled goat anti-rabbit antibody (1:500 in ADB; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). After a final wash with PBS, epithelia were excised from the insert housings using a scalpel and mounted on glass microscopy slides with Molecular Probes ProLong Antifade (Invitrogen Canada