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Apolipoprotein AI could be a significant determinant of epithelial integrity in rainbow trout gill cell cultures: A study in functional proteomics

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Abstract

The freshwater fish gill forms a barrier against an external hypotonic environment. By culturing rainbow trout gill cells on permeable supports, as intact epithelia, this study investigates barrier property mechanisms. Under symmetrical conditions the apical and basolateral epithelial surfaces contact cell culture media. Replacing apical media with water, to generate asymmetrical conditions (i.e. the situation encountered by the freshwater gill), rapidly increases transepithelial resistance (TER). Proteomic analysis revealed that this is associated with enhanced expression of pre-apolipoprotein AI (pre-apoAI). To test the physiological relevance, gill cells were treated with a dose of 50 μg ml^{-1} human apolipoprotein (apoAI). This was found to elevate TER in those epithelia which displayed a lower TER prior to apoAI treatment. These results demonstrate the action of apoAI and provide evidence that the rainbow trout gill may be a site of apoAI synthesis. TER does not differentiate between the trans-cellular (via the cell membrane) and para-cellular (via intercellular tight junctions) pathways. However, despite the apoAI-induced changes in TER, para-cellular permeability (measured by polyethylene glycol efflux) remained unaltered suggesting apoAI specifically reduces trans-cellular permeability. This investigation combines proteomics with functional measurements to show how a proteome change may be associated with freshwater gill function.
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1. Introduction

Aside from its role in osmo- and ionic regulation the freshwater fish gill also maintains a physical barrier against a hypo-osmotic environment. The precise mechanisms behind the barrier properties are not well understood but one method of investigation could be to employ rainbow trout (*Oncorhynchus mykiss*) gill cells cultured as intact epithelia on permeable supports [1].

Three variants of this model exist, based on the original flask culture methods for gill cells [2]: the Single Seeded Insert (SSI) [3] consists of pavement epithelial cells only, whilst the Double Seeded Insert (DSI) [4] and Single Direct Seeded Insert (SDSI) [5] include both pavement cells and mitochondria rich "chloride cells". Under symmetrical conditions cell culture media is present in both the apical and basolateral compartments (i.e. above and below the intact epithelia, respectively). The medium in the apical compartment can then be replaced with freshwater to generate an asymmetrical culture [3] and thus reproduce the physical situation encountered by the freshwater fish gill in vivo (i.e. simultaneous contact with an apical hypotonic and basolateral isotonic solution). In all three variations of this in vitro system the addition of water to the apical compartment results in a rapid increase in TER [3,5,6]. Therefore comparisons between corresponding homologous symmetrical and asymmetrical epithelia could potentially

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identify any precise biochemical or physiological processes associated with effective barrier properties.

As an electrophysiological measurement TER combines the resistances of both the trans- and para-cellular pathways (i.e. across the cell membrane and between inter-joining cells, respectively) and can be used to measure overall epithelial integrity, and TER values in excess of ten thousand Ω cm^2 have been recorded in both SSI and DSI cultures ([7,8], respectively). This compares with TER values of 'tens' of Ω cm^2 in the kidney proximal tubule and 'thousands' of Ω cm^2 in the bladder (for review, refer to Ref. [9]), and thus confirms the branchial epithelium to be one of the most impermeable epithelia [10].

TER appears to be independent of total cellular protein in both SSI and DSI cultures ([7,11], respectively), whereas in SDSI cultures there is thought to be a weak positive relationship between protein content and TER [5]. In SSI cultures the inhibition of protein synthesis, by cycloheximide, results in a decline in TER [7]. On the other hand TER is increased in SSI cultures treated with 100 and 1000 ng ml^{-1} cortisol [6]. Similar observations have been reported for DSI cultures treated with 500 ng ml^{-1} of cortisol or a combination of 500 ng ml^{-1} of cortisol plus 50 ng ml^{-1} of prolactin [8]. SSI TER is also initially increased by 10 and 100 ng ml^{-1} of 3,5′ ,3′ -triiodo-L-thyronine (T_3), but exposure for more than 12 h leads to a decline [11]. Cycloheximide is a general inhibitor of protein synthesis and cortisol, prolactin and T_3 are hormones (cortisol being a steroid). Therefore the data obtained with cycloheximide suggest that branchial integrity is dependent on the continued production of most or all of the proteins present in gill cells whilst data derived using cortisol, prolactin and T_3 suggest that gill epithelial integrity is under some degree of exogenous control. However none of these studies determined if any specific proteins present in gill cells is instrumental in regulating gill epithelial barrier properties.

The aim of this investigation was to address this issue. Therefore this study is divided into two aspects. The analysis of the cultured gill epithelia proteome was employed to make an initial search for any protein(s) specifically associated with the increase in TER resulting from asymmetrical culture conditions (i.e. with apical freshwater). Having found a candidate protein, we performed experimental manipulations to determine if it genuinely exerted an effect on branchial integrity.

2. Materials and methods

2.1. Cultured gill epithelia

All gill cells were collected under sterile conditions and cultured, as intact epithelia, on polyethylene terephthalate membrane supports (Becton Dickinson), using the DSI method [4]. Each DSI gill cell preparation comprised gill cells from 2 fish: cells from the first seeded on day 1 and the second on day 2. Cultured epithelia were allowed to develop to a plateau TER value at days 7–9 [4]. Representative epithelia, from each preparation, were then randomly designated for either symmetrical or asymmetrical culture conditions. In symmetrical cultures L15 culture media + 5% FBS (Gibco) was present in both the apical (upper) and basolateral (lower) compartments. In the asymmetrical cultures L15 + 5% FBS was retained in the basolateral compartment but the media in the apical compartments was replaced with sterile tap water.

Recently the use of 5% freshly collected rainbow trout plasma has been found to result in an identical TER development in comparison to 5% FBS [5]. The potential advantage of using rainbow trout plasma as a media supplement, particularly for proteomic research, is the elimination of foreign (i.e. bovine) proteins [5]. Therefore symmetrical and asymmetrical epithelia were also cultured using rainbow trout plasma as a media supplement. Rainbow trout plasma was collected by cannulation [5,12] of 4 rainbow trout (approximately 200–300 g), pooled and added to the culture media at a final concentration of 5% (v/v).

2.2. 2D electrophoresis, proteome analysis and protein identification

Twenty four hours after the induction of asymmetrical culture conditions (or an equivalent time period for symmetrical cultures) the water and media were aspirated from the apical and basolateral compartments and the epithelia were thoroughly washed in phosphate buffered saline (PBS) to remove any extra-cellular material. The intact epithelia were then frozen and stored at −70 °C. For two-dimensional (2D) electrophoresis the cells were lysed in ice cold lysis buffer: 8 M urea containing 10% (v/v) 0.5 M Tris–HCl (pH 7.4), 0.02 M EDTA, 0.05 M dithiothreitol (DTT), 10% (v/v) glycerol, 6% (v/v) ampholytes (Resolyte, pH 3.5–10; Merck-BDH), 2% (v/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2 mg ml^{-1} RNase and 0.2 mg ml^{-1} DNase. The lysate was clarified by centrifugation at $10{,}000 \times g$, for 5 min at 4 °C and the supernatant stored at −70 °C for proteomic analysis.

The total sample protein loading for the 2D gels was determined by performing a preliminary 1-dimensional electrophoresis gel and staining with colloidal Coomassie blue; the gels were prepared and processed as described previously [13]. The gill cell preparations were analysed by 2D electrophoresis, using the method described previously [14]. Briefly, the soluble protein extracts from cell lysates were mixed with re-swelling buffer: 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.3% (w/v) DTT to give a final volume of 140 μl. One hundred and twenty-five microliters of this mixture was then used to re-hydrate a 7 cm, pH 4–7 immobilised pH gradient (IPG) strip (Amersham Biosciences). The gel re-hydration was carried out overnight at room temperature. Isoelectric focusing was performed on a Multiphor II (Amersham Biosciences) in three stages with

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