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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.

Keywords: Proteome; Apolipoprotein AI; Transepithelial resistance; Trans-cellular flux

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 × 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

a ramped voltage change (linear increase with time) during the first two steps: 0–200 V over 1 min, 200–3500 V over 90 min and 3500 V for 90 min. All the stages were at 2 mA and 5 W. The IPG strips were then equilibrated in a buffer (50 mM Tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; 2% (w/v) sodium dodecyl sulfate, SDS) containing 1% (w/v) DTT for 30 min followed by a further 30 min incubation in the same buffer containing 2.5% (w/v) iodoacetamide in place of the DTT. Each IPG strip was then laid onto a 10–15% gradient polyacrylamide slab gel (8 × 7 cm) for the second dimension electrophoresis and the gels run at 75 V for 75 volthours and then subsequently at 150 V for a total of 450 volthours [13,15]. For analytical gels the proteins were detected by silver staining using the Plus-One™ silver kit (Amersham Biosciences) according to the manufacturer's protocol.

The stained gels were scanned using a Molecular Dynamics Personal Densitometer (Amersham Biosciences) at 50 μm resolution to generate 8-bit images. These images were transferred to Phoretix 2D™ Analytical software, version 6.01c (Nonlinear Dynamics, Newcastle, UK). All image analysis and comparisons were carried out using this software.

Sufficient cultured epithelia were produced so that each treatment (i.e. symmetrical and asymmetrical conditions) was repeated 3 to 5 times on homologous epithelia from each initial gill cell preparation and separate 2D gels were run for each repeat. The proteomic comparison aspect of this study contains data from a total of 7 separate DSI gill cell preparations involving 34 separate cell cultures.

Selected spots, characteristic of asymmetrical culture conditions, were cut from stained gels and subjected to in-gel trypsin digestion. Briefly, excised spots were washed, reduced, *S*-alkylated, and digested within the gel using sequencing grade modified trypsin (Promega) as described elsewhere [16,17]. An aliquot of the peptide extract was passed through a GELoader tip containing POROS R2 sorbent (PerSeptive BioSystems, USA) [17]. The adsorbed peptides were washed extensively prior to elution in 0.5 μL of a saturated solution of α-cyanol-4-hydroxycinnamic acid in 50% acetonitrile, 5% formic acid. The mass spectra were obtained on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer operated in the reflection delayed extraction mode. The spectra were internally calibrated using the trypsin auto-digestion products. The tryptic peptide profiles were used to search the NCBI (National Centre for Biotechnology Information) nucleotide database using the MASCOT programme. The peptide masses were compared to the theoretical peptide masses of all available proteins and predicted proteins from DNA sequences. All peptide fragments that were obtained for each digest were submitted for searching. The search parameters were as follows: maximum allowed error of peptide mass 250 ppm, cysteine as *S*-carbamidomethyl-derivative and oxidation of methionine allowed.

2.3. Apolipoprotein treatment and TER

The protein spot found to be most characteristic of asymmetrical culture conditions proved to be rainbow trout apolipoprotein precursor (pre-apoAI) (refer to Results). Since rainbow trout apoAI is not commercially available, human apoAI was used to evaluate the effects of apoAI on branchial integrity. Human apoAI (Sigma) was supplied as 1 mg dissolved in 675 μl of 10 mM NH_4HCO_3. This solution was further diluted in L15 + 5% FBS so that 50 μg ml^{-1} was added to any apoAI already in the cell culture media. DSI gill cells cultures were prepared as previously described. The existing L15 media was then removed from both the apical and basolateral compartments. Sterile tap water was added to the apical compartments (as before) and, simultaneously, new L15 + FBS, or new L15 + FBS containing 50 μg ml^{-1} apoAI (and NH_4HCO_3), or new L15 + FBS containing an equivalent amount of NH_4HCO_3 only, was added to the basolateral compartment. TER was measured across these epithelia, using an Evom TM Epithelial Voltohmeter connected to STX-2 'chopstick' electrodes (World Precision Instruments) [3], at intervals (refer to Results) following the basolateral L15 changes/apical water addition.

2.4. Apolipoprotein AI treatment and [^{3}H]polyethylene glycol-4000 permeability

The para-cellular permeability of the apoA1 treated epithelia was measured using [^{3}H]polyethylene glycol-4000 (PEG) (New England Nuclear DuPont) as a para-cellular marker [18,19]. Three hours after the apical L15 was substituted with water and the basolateral L15 changes (described above) were made, 1.0 μCi of PEG was also added to the basolateral compartment of each gill cell culture. After a further 24 h (i.e. 27 h after the initial imposition of asymmetrical culture conditions) PEG activity was measured in the apical and basolateral compartments in order to calculate the basolateral to apical para-cellular efflux.

2.5. Calculations and statistics

The numbers (n) quoted in the Results refer to the number of separate DSI preparations involved. The data in Figs. 3 and 5 are shown as mean + standard deviation. [^{3}H]PEG permeability (P, cm s^{-1}) was calculated according to:

$$P\left(\text{cm s}^{-1}\right) = \frac{\left(\Delta[\text{PEG}^*]_{\text{AP}}\right) \times (\text{volume}_{\text{AP}})}{[\text{PEG}^*]_{\text{BL}} \times (\text{Time}) \times (3600) \times (\text{Area})}$$

...where $\Delta[\text{PEG}^*]_{\text{AP}}$ is the change in radioactivity in the apical compartment, $\text{volume}_{\text{AP}}$ is the volume of water in the apical compartment (1.5 ml), $[\text{PEG}^*]_{\text{BL}}$ is the mean radioactivity in the basolateral compartment, 3600 converts the

flux time (h) to seconds and Area refers to the surface area of the epithelial support (0.9 cm^2).

Student's *t*-tests were used to compare the mean normalised volume of selected spots from symmetrical and asymmetrical gill cell cultures. Analysis of variance was used to compare TER changes during the time course following the induction of asymmetrical culture conditions and the effects of the basolateral apoAI on TER at each time point, and also TER and PEG flux following apoAI treatment. TER data were also compared, at each time interval after the commencement of asymmetrical culture conditions and the basolateral apoAI addition, by non-parametric Wilcoxon analysis. The relationship between TER and PEG permeability was evaluated by the linear regression of Log_{10} transformed TER and PEG permeability data. In all statistical analysis a probability of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. Proteomics and apolipoprotein AI precursor (pre-apoAI) detection

Fig. 1 depicts a typical 2D gel from asymmetrical DSI gill cell cultures. Fig. 1 also indicates the 5 protein spots which were selected for identification whilst Fig. 2 depicts the enlargements of the areas of the gels containing the protein thought to be pre-apoAI (see below). The maximum number of protein spots resolved in a single asymmetrical cell culture was 494 (minimum = 380).

Protein spots thought to be associated with the increased TER brought about by asymmetrical culture conditions were selected using similar criteria to that employed to determine osmotic and thermal acclimation in the gill cells of the long-jawed mud sucker (*Gillichthyes milabilis*) [20]; i.e. the spot was present on all asymmetrical culture 2D gels and there was

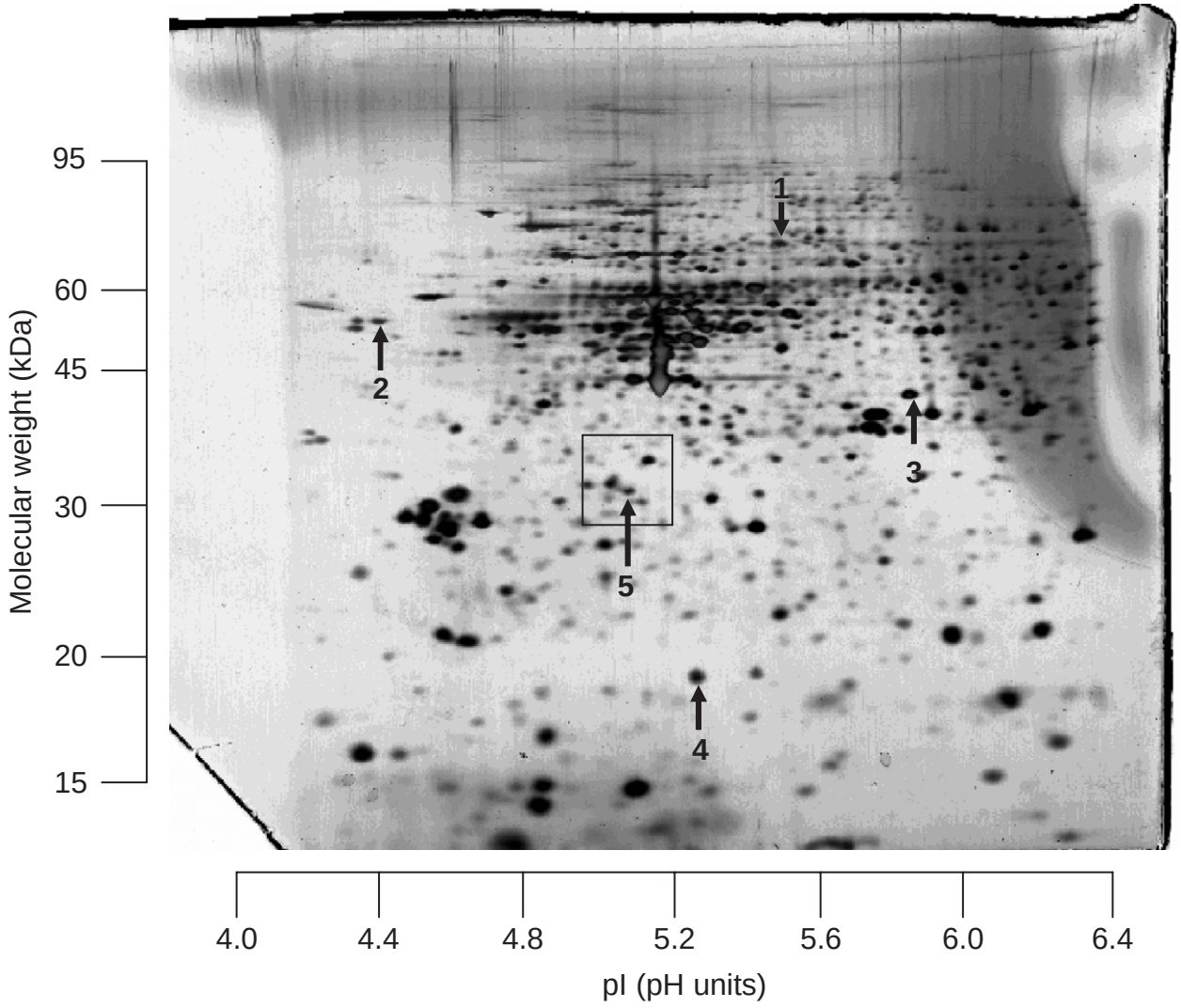

Fig. 1. Representative 2D electrophoresis protein gel from rainbow trout gill cells cultured as intact epithelia (DSI culture) under asymmetrical conditions; i.e. apical water and basolateral cell culture media. Candidate proteins, selected for identification analysis (refer to Results and Table 1), are indicated by arrows. The rainbow trout pre-apoAI (spot number 5) area of interest is expanded in Fig. 2.

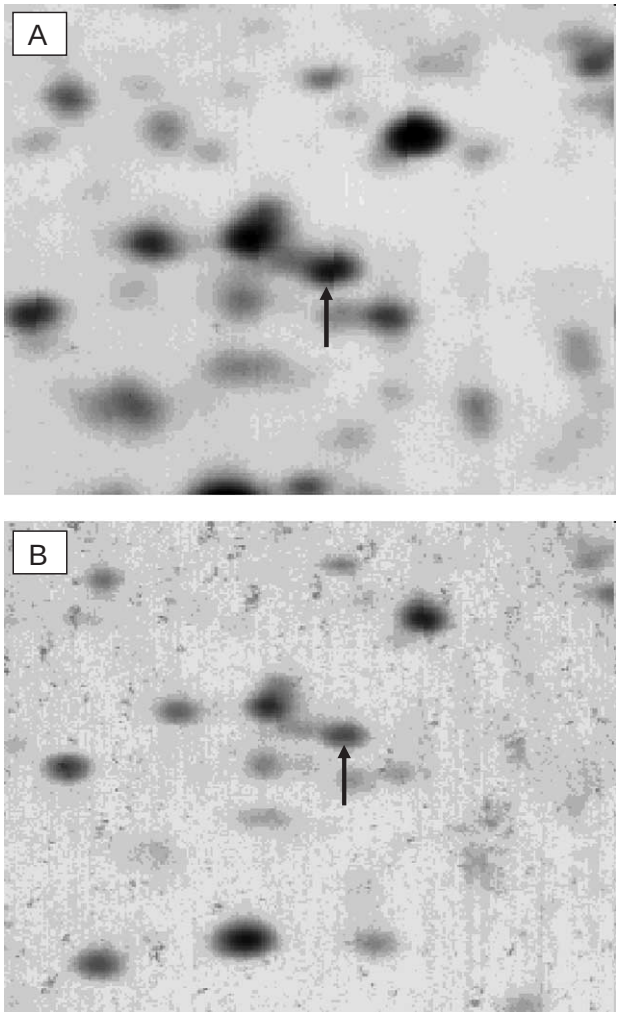

Fig. 2. Regions of homologous (A) asymmetrical and (B) symmetrical gill cell cultures (refer to Fig. 1) illustrating the minimum extent (a factor of ×2.9) of pre-apoAI (arrow) enhanced expression (as defined by normalised spot volume) in asymmetrical epithelia measured in this investigation.

a significant increase in normalised spot volume (a parameter offered by the Phoretix analytical software, which combines spot area and peak height to give an overall expression index), relative to the same spot on 2D gels from symmetrical cultures. Five candidate proteins (refer to Fig. 1) demonstrated an increase in normalised spot volume following the imposition of asymmetrical culture conditions (Fig. 3). The result of the identification analysis of these proteins is summarised in Table 1. Of these, the only protein spot which was positively identified as a rainbow trout protein and which achieved a statistically significant MASCOT search score was spot 5; i.e. rainbow trout pre-apoAI (Table 1). NCBI database rainbow trout pre-apoAI information: p*I*=5.3, molecular weight=29,686 Da; MASCOT database searches of peptide maps from spots 1 and 3 (refer to Fig. 1) also listed fish proteins as possible identities: spot 1=ventricular natriuretic peptide, from rainbow trout or bone morphogenetic protein 7, from zebrafish (*Brachydanio rerio*), and spot 3=troponin I, from Atlantic herring (*Clupea harengus*) (Table 1). However the MASCOT search scores were below that required for a statistically positive identification. The nearest identification to the other protein spots apparently associated with increased TER were mammalian proteins and the MASCOT identification scores for all of these were also below a statistically significant value (Table 1).

A total of seven separate DSI preparations was used in this investigation; three used FBS as the media supplement and four used rainbow trout plasma. However the pattern of pre-apoAI expression was consistent irrespective of whether FBS or pooled rainbow trout plasma was used to supplement the basolateral media. Using FBS, pre-apoAI expression was up-regulated by a factor of 9.9 (i.e. the increase in normalised spot volume relative to the corresponding symmetrical culture) in one DSI preparation and completely induced (i.e. pre-apoAI was not detected in the corresponding symmetrical cultures) in two preparations. Using rainbow trout plasma, pre-apoAI expression was up-regulated under asymmetrical culture conditions by factors of 2.9 and 3.7 in two DSI preparations and completely induced in the two remaining preparations. Fig. 2 illustrates the minimum extent of pre-apoAI up-regulation detected in this investigation (see above); other gel images are not shown.

The rainbow trout genome is less well characterised than mammalian genomes are. Therefore, to maximise our chances of naming any of the other proteins found to be associated with increased TER (refer to Fig. 1), we also attempted a second method of protein identification. Using the "backtranslate" facility of the ExPASy proteomics server (Swiss Institute of Bioinformatics) the nucleotide sequences corresponding to the peptide sequences of the proteins listed in Table 1 were generated. These were used to search The Institute for Genomic Research (TIGR) BLAST genome databases for all 3 of the fish species available: rainbow trout (the species in question here), and also Atlantic salmon (i.e. a related species) and zebrafish. However, although this second search confirmed protein spot 5 (refer to Fig. 1) as rainbow

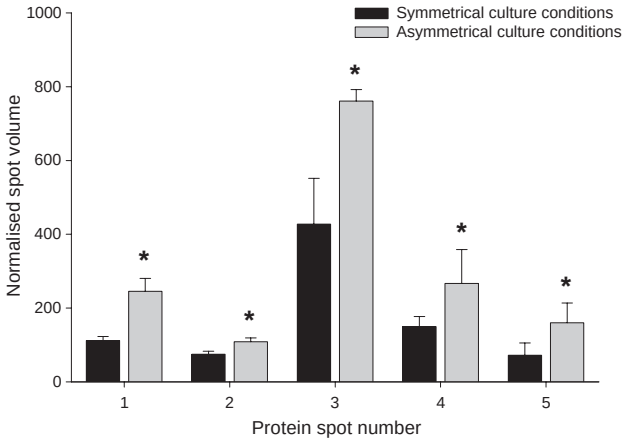

Fig. 3. Changes in normalised spot volumes of the 5 candidate proteins indicated in Fig. 1 following the imposition of asymmetrical culture conditions. Asterisks (*) indicate a significant difference between symmetrical and asymmetrical cultures. Data derived from 7 separate DSI preparations.

Table 1
Result of identification analysis for proteins indicated in Fig. 1

Protein spot number[a]	Accession number	Score	MASCOT protein description[b]
1	gi 176728	61	MHC class II (species not given)
	gi 176662	61	MHC class II (*Pan troglodytes*)
	gi 913160	59	Ventricular natriuretic peptide (*Oncorhynchus mykiss*)
	gi 18858369	49	Bone morphogenetic protein 7 (*Brachydanio rerio*)
2	gi 16758828	54	Voltage-gated K channel (*Rattus norvegicus*)
	gi 2505579950	50	RIKEN cDNA (*Mus musculus*)
	gi 13623433	50	Similar to KIAA1191 protein (*Homo sapiens*)
3	gi 4505889	55	Lysine hydroxylase (*Homo sapiens*)
	gi 22713625	54	Similar to lysine hydroxylase (*Homo sapiens*)
	gi 2505248	41	Hypothetical protein XP 195821 (*Mus musculus*)
	gi 2133832	38	Troponin I (*Clupea harengus*)
4	gi 353526	57	Gastricin N terminus (species not given)
	gi 26347021	52	Unnamed protein product (*Mus musculus*)
	gi 999901	50	Progastricsin (species not given)
5	gi 6686384	88	Apolipoprotein AI precursor (*Oncorhynchus mykiss*)
	gi 27665720	77	Similar to kinetochore-associated protein 1 (*Rattus norvegicus*)
	gi 27729953	58	Similar to RNA polymerase II transcription factor subunit (*Rattus norvegicus*)

Only protein identification scores of > 68 are significant ($P<0.05$).
[a] Refers to protein spot numbers given in Fig. 1.
[b] The 3 highest scoring MASCOT identities are listed, together with any other proteins identified as being of piscine origin (spots 1 and 3).

trout pre-apoAI, no further statistically significant search result scores, for rainbow trout protein identities, were obtained. Since this second method did not contribute to our MASCOT findings these non-significant data are not shown.

Therefore, in the present investigation, only the action of apoAI on the cultured epithelia was studied further. This is not to say that further analysis will not confirm the importance of other proteins or that spots 1–4 (refer to Fig. 1) will not be identified as our knowledge of the rainbow trout proteome increases. However these results suggest that maintaining the barrier functions of the rainbow trout gill may not involve widespread changes to the branchial proteome, a finding which is similar to the proteomic changes associated with thermal acclimation in gill cells from the log-jawed mudsucker [20].

3.2. Apolipoprotein AI treatment, TER and [^{3}H]polyethylene glycol-4000 permeability

Fig. 4 illustrates the TER profiles of 9 separate DSI preparations following the induction of asymmetrical culture conditions and simultaneous treatment with basolateral L15 only (Fig. 4A), basolateral L15+NH_4HCO_3 (Fig. 4B) and basolateral L15+apoAI (and NH_4HCO_3) (Fig. 4C). The homologous epithelia (i.e. composed of cells from the same preparation) in each of these treatments are indicated by the same symbol. The general TER development trends, with time, were similar to previous measurements [6,11]. Note that we have used log TER to emphasise the effect of apoAI (as described below). Fig. 4 also clearly illustrates the variation in TER between individual DSI epithelia preparations; in 7 out of 9 DSI preparations the TER did not rise above 5000 kΩ cm^2 whereas 2 out of 9 TER preparations attained maximum TERs of > 8000 kΩ cm^2. Presumably as a result of the extent of this variation, no significant change in mean TER was detected during the time course, irrespective of basolateral media composition, or significant effect of the L15 composition on TER at any given time point. However the addition of an additional 50 μg ml^{-1} apoAI to the basolateral L15 at the onset of asymmetrical culture resulted in a rapid effect on median TER; i.e. within 10 min the median TER epithelia had increased (Fig. 4C): 2.7 (min/max=2.2/11.5) kΩ cm^2, compared to 1.7 (min/max=1.3/9.2), with basolateral L15 only (Fig. 4A) and 2.2 (min/max=1.6/9.8) kΩ cm^2 with basolateral L15+NH_4HCO_3 (Fig. 4B). This continued for 30 min after apoAI addition (Fig. 4C): 2.6 (min/max=1.5/12.4) kΩ cm^2, compared to 1.6 (min/max=1.2/8.9), with basolateral L15 only (Fig. 4A) and 1.7 (min/max=1.1/10.1) kΩ cm^2 with basolateral L15+NH_4HCO_3 (Fig. 4B), respectively. By 1 h the median TER of apoAI treated epithelia (Fig. 4C) had declined to untreated or NH_4HCO_3 treatment only values (Fig. 4A and B, respectively).

Further analysis of the data presented in Fig. 4 revealed that this increase in median TER was due to apoAI acting preferentially to elevate TER in those epithelia which displayed the lower TER values prior to apoAI treatment, rather than causing a further increase in those epithelia with an already higher TER. Therefore each epithelium was defined as "high resistance" (Fig. 5A) or "low resistance" (Fig. 5B), according to whether the TER was greater, or less, than the experimental median, respectively. The addition of a further 50 μg ml^{-1} ApoAI had no effect on TER in the high resistance epithelia (Fig. 5A) but, within 10 min of addition to the basolateral compartment, caused an increase in TER in low resistance epithelia which then lasted for 2 h (Fig. 5B). NH_4HCO_3 had no effect on TER in either the low (Fig. 5A) or high (Fig. 5B) resistance epithelia.

Irrespective of basolateral media composition the relationship between TER and PEG conformed to a common model (Fig. 6). This indicates that the addition of an additional 50 μg ml^{-1} apoAI to the basolateral compartment of the epithelial cultures did not affect paracellular flux. It is therefore concluded that any increase in TER was the result of apoAI action on the trans-cellular

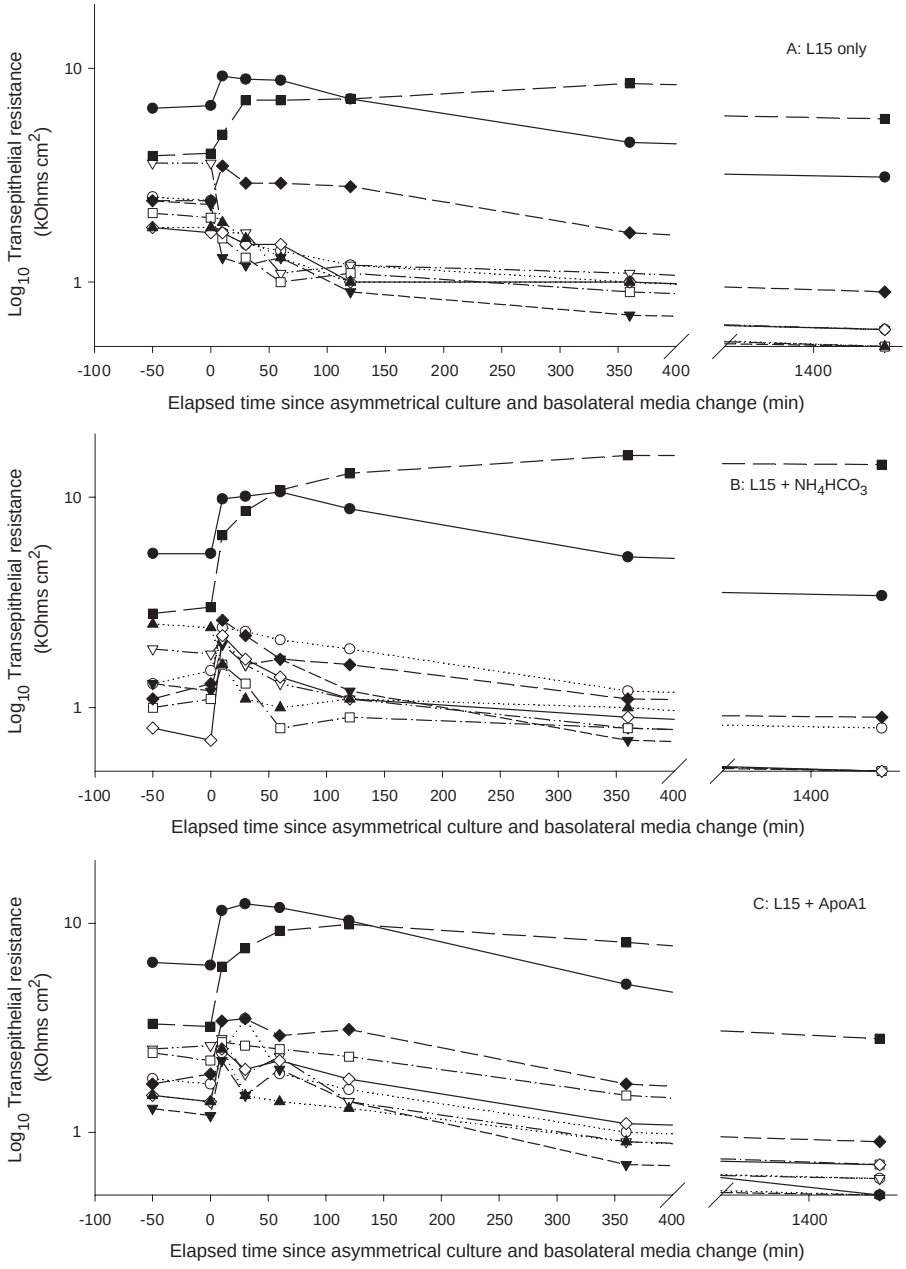

Fig. 4. TER profiles of 9 separate DSI rainbow trout gill epithelia cultures, following the induction of asymmetrical culture conditions, with (A) L15 only, (B) L15+NH_4HCO_3 or (C) L15+50 μg ml^{-1} apoAI (+NH_4HCO_3) in the basolateral compartment. Homologous gill cell cultures are indicated by the same symbol and line trace.

flux pathway; i.e. changes in cell membrane properties rather than changes in the junctions between adjacent cells.

4. Discussion

4.1. Methodology

The advantages of homologous cell cultures in mechanistic studies are evident from the present investigation and the benefits of standardising experimental conditions [21] are well illustrated. In particular the difficulties of making comparisons between completely separate fish in solutions of differing osmolarity (i.e. comparable with asymmetrical and symmetrical cell culture conditions) are avoided. Direct cellular responses are also isolated from more complex secondary (e.g. hormonal and metabolic) influences [22], a point of considerable importance given the nature of this investigation.

It is unlikely that the effects of apoAI on TER and paracellular permeability were a general response to the addition of protein to the basolateral compartment, which would have arisen irrespective of the protein added, since a protein

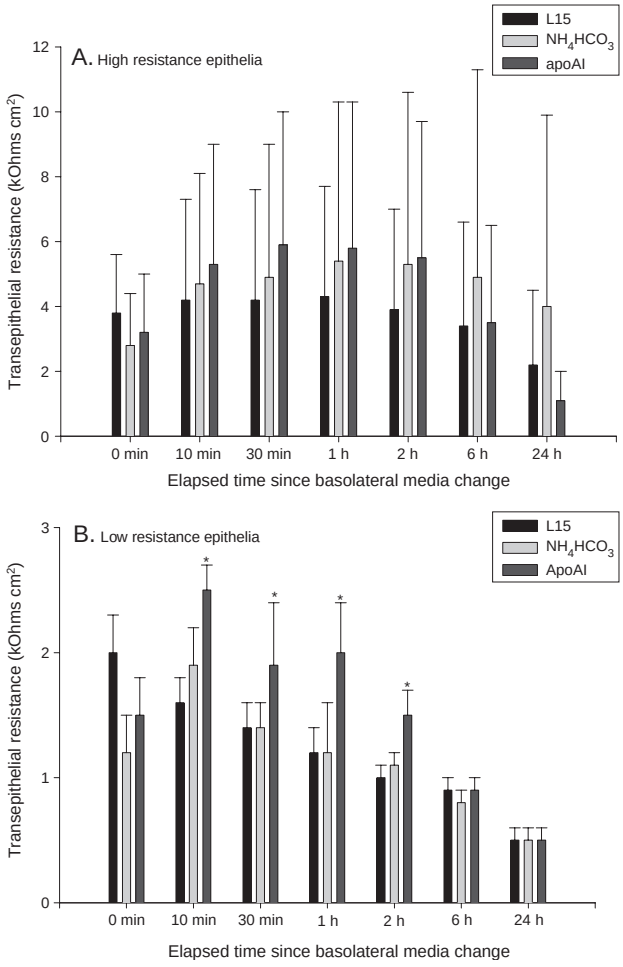

Fig. 5. ApoAI treatment and TER in (A) high and (B) low resistance epithelia. High and low resistance epithelia are defined, from Fig. 4, as those with TER values which are either equal or greater or equal or less than the experimental median, respectively. Asterisks (*) indicate the elevation of mean TER by 50 μg ml^{-1} apoAI to both L15 only (untreated) and NH_4HCO_3 (apoAI solvent) controls. Data are derived from 5 separate DSI preparations.

analysis of FBS and trout plasma [23] showed total protein concentrations of approximately 82–116 mg ml^{-1}. Thus, since the 2.0 ml L15+5% FBS or plasma in the basolateral compartments contained approximately 8–12 mg protein before the addition of apoAI, the addition of an extra 100 μg of apoAI (i.e. to give 50 μg ml^{-1}) only increased the total protein concentration in the cell culture media by approximately 0.8–1.3%.

If in vitro models are to be used effectively it is advantageous to establish a degree of parity with the equivalent tissue in vivo. Previously DSI cultures have been found to have similar unidirectional Na^+ and Cl^- flux rates to those recorded in SSI cultures [4], which are similar to the whole gill [3]. SSI cultures also replicate the protein synthesis rate and intracellular Na concentration found in whole gills [7]. The present investigation continues to demonstrate the sensitivity of cultured gill epithelia to biologically active macromolecules, evident from the investigations on cortisol and prolactin [6,8].

Prior to the present investigation there has only been one investigation into the fish gill proteome; i.e. from freshly isolated gill cells from the long-jawed mudsucker [20]. However, although similar p*I* (4.4–7.2) and molecular weight ranges (100–20 kDa) were employed, a cursory visual comparison between the representative gel illustrated by Ref. [20] and that shown in the present study suggests significant inter-species differences in piscine branchial proteomics. More comprehensive data are therefore required for fish gill proteomes, utilising the improved protein resolution from larger format gels and more sensitive staining techniques, to compare between species as well as between in vitro models and the whole gill.

4.2. Apolipoprotein A1 and gill epithelial integrity

The primary role reported for apoAI is in lipid metabolism (e.g. [24,25]). ApoAI confers water solubility on the lipoprotein complex thus facilitating lipid transport and metabolism (for review, refer to [26]). For example, in humans apoAI transports cholesterol to the liver for excretion (for review, refer to [24,27]), thus protecting against atherosclerosis (e.g. [28]) and other heart diseases (for review, refer to [29]).

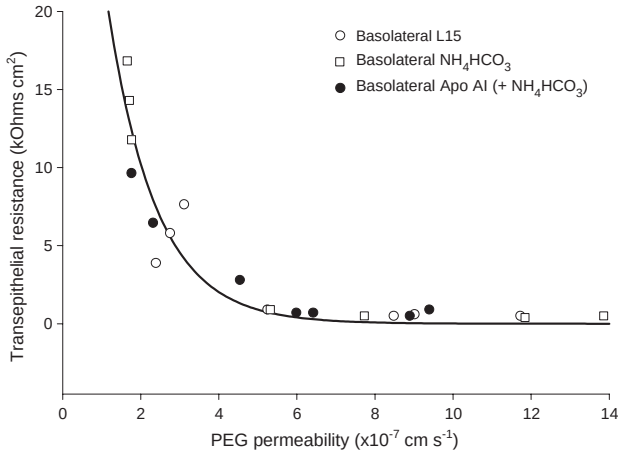

Fig. 6. Para-cellular PEG permeability and TER in intact rainbow trout gill cell epithelia cultured under asymmetrical conditions. This relationship may be described as follows: $\mathrm{Log}_{10}\ Y = 1.47 - 1.79\ \mathrm{Log}_{10}\ X$ ($r = -0.957$), where Y=TER (kΩ cm^2) and X=PEG permeability ($\times 10^{-7}$ cm s^{-1}), respectively. Each data point refers to a single gill cell culture. Data are derived from 2 separate DSI preparations.

In mammals apoAI synthesis occurs predominantly in the liver and intestine (for reviews, refer to [30,31]) although recently apoAI synthesis has also been found to occur in the human heart [32]. In contrast, in lower vertebrate species apoAI is also synthesised in a number of peripheral tissues, e.g. chicken muscle and kidney, and pigeon brain [33], and carp optic nerve [34,35] and skin [36]. ApoAI has also been detected, by analysis of 2D protein gels, in rainbow trout liver [37], and in fish hepatocytes, apoAI accounts for approximately 10% of all protein synthesised [38,39]. Relatively little is known about apoAI in the peripheral tissues [40] but, in carp, apoAI promotes nerve regeneration [34,35] and demonstrates antimicrobial properties in the skin [36] whilst in the cod (*Gadus morhua*) apoAI is closely associated with the C3 component of the compliment system [41]. Additionally apolipoprotein E has also been shown to promote fin repair in zebrafish [42]. Therefore the emerging picture of piscine apolipoproteins (particularly apoAI) as having a restorative or protective role is continued by the present investigation.

To our knowledge this is the first study to provide evidence that apoAI may exert a physiological action on the gill epithelium of a freshwater fish. Therefore we propose that apoAI can be added to the three factors already shown to influence branchial epithelial integrity: cortisol [6], prolactin (mixed with cortisol) [8], and T_3, both alone and when combined with cortisol [11]. Cortisol (the most potent TER modulator), prolactin and T_3 are all exogenous factors, originating in inter-renal, adenohypophyseal and thyroid tissue, respectively, which are transported to the site of action (i.e. the gill).

In the present investigation, spot 5 (refer to Fig. 1) was identified specifically as pre-apoAI (i.e. the precursor, not the mature apoAI molecule). However this identification was made without peptide information from the N-terminus (refer to Table 2). It is this section of the molecule which contains the peptide sequences characteristic of pre-apoAI and these are cleaved off during the synthesis of mature apoAI [43–45]. Without detecting the N-terminal peptides specific to pre-apoAI, it is not possible to provide a definitive answer, based solely on trypsin digest, as to whether this protein was the apoAI precursor mature apoAI. Unfortunately we also found there was insufficient material in spot 5 (refer to Fig. 1) or its equivalent on other gels for a specific N-terminal amino acid analysis to complement our peptide digests. Nevertheless we propose this study provides strong evidence that this protein was indeed pre-apoAI and that the cultured gill cells were the site of synthesis.

The only alternative explanation would be an uptake of any pre-apoAI present in the culture media but this is negated by the fact that only rainbow trout pre-apoAI sequence information were obtained for spot 5 (refer to Fig. 1) irrespective of whether the media supplement was FBS or trout plasma. To confirm this FBS was run on an identical 2D gel to those described in the Materials and methods (gel not shown). The protein spot, on the FBS 2D gel, which corresponded to rainbow trout gill cell pre-apoAI, on the gill cell 2D gels, was excised and identified (using the methods previously described) as bovine apoAI. The amino acid sequence data for rainbow trout and bovine pre-apoAI and the peptide sequences used for identification are indicated on Table 2. The obvious lack of homology between rainbow trout and bovine pre-apoAI (refer to Table 2) suggests that it is impossible to falsely identify one species for the other. Therefore the contamination of the rainbow trout gill cell 2D gels with bovine apoAI would be readily apparent and this did not occur. Thus, since rainbow trout pre-apoAI was identified in gill cells that had access to only bovine apoAI in the culture medium, this indicates that the gill cell proteome does not contain (pre-)apoAI carried over from the cell culture medium; i.e. all protein detected are intracellular in origin.

In addition the well documented details of the apoAI synthesis and metabolism pathway suggest that mature apoAI is unlikely to be present in the intracellular proteome. ApoAI is initially synthesised as pre-apoAI which then remains in the trans-golgi network for some considerable time [46]. After cleavage in the cell [43] the resulting pro-apoAI is exported to the plasma, where it is cleaved again to form functional apoAI [44,45]. In other words the initial apoAI precursor, pre-apoAI, does not cross the cell membrane. Instead it is pro-apoAI (i.e. the next stage in apoAI synthesis) which undergoes membrane translocation and is secreted intact before the final conversion to apoAI; the proteolytic processing of pro-apoAI to apoAI is an exclusively extra-cellular event (for review, refer to Ref. [47]). Thus mature apoAI does not exist within the cell during synthesis. Furthermore mature apoAI uptake only occurs as part of a lipoprotein complex. However the uptake of low and high density lipoprotein complexes is directly linked to apoAI proteolysis [48] or elastase digestion [49],

Table 2
Comparison of rainbow trout and bovine pre-apoAI amino acid sequences (as listed by the SWISS-PROT protein database)

```
                          10        20        30        40        50        60
                          |         |         |         |         |         |
TROUT    MKFLALALTILLAAGTQAFPMQADAPSQLEHVKAALSMYIAQVKLTAQRSIDLLDDTEYK
BOVINE   MKAVVLTLAVLFLTGSQARHFWQQDDPQSSWDRVKDFATVYVEAIKDSGRDYVAQFEASA
                          a         b

                          70        80        90       100       110       120
                          |         |         |         |         |         |
TROUT    EYKMQLTQSLDNLQQYADATSQSLAPYSEAFGTQLTDATAAVRAEVMKDVEELRSQLEPK
BOVINE   LGKQLNLKLLDNWDTLASTLSKVREQLGPVTQEFWDNLEKETASLRQEMHKDLEEVKQKV

                 130       140       150       160       170       180
                 |         |         |         |         |         |
TROUT    RAELKEVLDKHIDEYRKKLEPLIKEHIELRRTEMEAFRAKMEPIVEELRAKVAINVEETK
BOVINE   QPYLDEFQKKWHEEVEIYRQKVAPLGEEFREGARQKVQELQDKLSPLAQELRDRARAHVE

                 190       200       210       220       230       240
                 |         |         |         |         |         |
TROUT    TKLMPIVEIVRAKLTERLEELRTLAAPYAEEYKEQMIKAVGEVREKVSPLSEDFKGQVGP
BOVINE   TLRQQLAPYSDDLRQRLTARLEALKEGGGSLAEYHAKASEQLKALGEKAKPVLEDLRQGL

                          250       260
                          |         |
TROUT    AAEQAKQKLLAFYETISQAMKA
BOVINE   LPVLESLKVSILAAIDEASKKLNAQ
```

The peptides used to identify protein spot 5 (refer to Fig. 1) as rainbow trout pre-apoAI are highlighted in black and the peptides used to identify bovine pre-apoAI are identified by a black outline. Bovine pre-apoAI amino acids which show homology with the rainbow trout pre-apoAI sequence are highlighted in grey. Arrows refer to amino acids cleaved from bovine apoAI during synthesis: a=pre-peptide cleavage (amino acids 1–18); b=pro-peptide cleavage (amino acids 19–24).

respectively. Thus mature apoAI, as part of a lipoprotein complex, does not remain intact once it is taken up by the cell. Clearly all this evidence indicates that pre-apoAI synthesis occurs in the rainbow trout gill. However we emphasise this is a working hypothesis which now needs to be definitively proven or refuted.

We concede there may also be a degree of species specificity in branchial apoAI expression. For example, in the sea bream (*Sparus aurata*), a marine fish which is able to tolerate brackish water, apoAI mRNA could not be detected in the gill [50], although the non-detection of message does not conclusively prove or disprove protein expression. An inter-species difference in tissue specific apoAI expression has been observed in the muscle with apoAI mRNA being apparently absent from sea bream muscle [50] but present in salmon muscle [51] (a fact acknowledged by Ref. [50]). It is possible that the sea bream could be entirely reliant on liver apoAI synthesis to supply the gill but it has also been demonstrated that prolactin minimises plasma ion loss after sea bream transfer from sea water to brackish water and increases plasma ion loss after transfer from brackish water to sea water [52]. Therefore the question of whether freshwater (or diadramous) fish may be more reliant on apoAI to maintain gill barrier function, than a marine euryhaline species, is intriguing and warrants further investigation.

Although apoAI derived from liver synthesis presumably does exert an effect on rainbow trout gill epithelia, branchial pre-apoAI synthesis (with subsequent localised extracellular conversion to apoAI) would offer an additional, autonomous aspect to gill barrier property regulation, particularly in providing an immediate response in situations where the gill displays an otherwise lower degree of branchial integrity. Since it has been recently demonstrated that feeding rainbow

trout a diet which reduces dietary protein conversion efficiency causes a decline in liver apoAI [37] the potential advantage of synthesis for the freshwater gill is evident.

The use of an extra 50 μg ml^{-1} apoAI, to treat the gill epithelia, was primarily selected to reflect the lower end of the concentrations measured in human and turtle (*Chrysemys picta*) blood plasma: 7–2150 μg ml^{-1} [53–55] and 100–650 μg ml^{-1} [56], respectively, and rat serum (male = 336 ± 41, female = 514 ± 89 μg ml^{-1}) [57]. Fifty μg ml^{-1} apoAI also ensured that the NH_4HCO_3 solvent concentration was kept to a minimum, since any increase in apoAI concentration automatically incurs an increase in NH_4HCO_3 in the basolateral media. Finally there was an economic consideration as the protein is extremely expensive.

Despite the similarity in overall amino acid percentage composition (for review, refer to [34]) the considerable variation in actual apoAI amino acid sequence between species (as illustrated in Table 2) is well documented (e.g. for reviews, refer to [24,58]). Yet, even with these differences to the primary structure, the critical lipid binding aspects of the secondary structure remain conserved. Indeed deletions of certain amino acids sequences throughout the molecule have no effect on apoAI lipid binding properties as long as the secondary structure is not modified (for review, refer to [24]). Also the similarity between human and rainbow trout lipoproteins [59] suggests that human and rainbow trout apoAI share common binding properties. Therefore the effectiveness of 50 μg ml^{-1} of human apoAI, on gill epithelial TER, may demonstrate the extent of functional conservation between human and trout apoAI. In fact it has been suggested that rainbow trout and human apoAI genes arose from a common ancestor [47] and apoAI function was determined before the teleost/mammalian evolutionary split [58]. However it remains possible that rainbow trout apoAI may exert an even more potent effect on rainbow trout gill cells than demonstrated by human apoAI in the present study.

A major benefit of using the gill cell model, as opposed to whole fish, is the ability to isolate the effects of apoAI at the cellular level alone. Given the effectiveness of the addition of a further 50 μg ml^{-1} apoAI to the basolateral compartment, we can hypothesise that apoAI could be as significant as cortisol as a determinant of branchial barrier properties. However a crucial difference between cortisol and apoAI is that cortisol acts on intercellular tight junctions and thus determines para-cellular permeability [6], whereas apoAI appears to act specifically on the cell membrane and therefore determines trans-cellular permeability. In fact the present study provides a possible explanation for the recently discovered increase in gill epithelia membrane lipid metabolism, which follows the induction of asymmetrical culture conditions [60], particularly since this is not hormonally controlled [60].

Thus there appear to be at least two major mechanisms by which branchial barrier properties are maintained, depending on whether the barrier regulation involves the para-cellular or trans-cellular flux route. ApoAI appears to be particularly important in rapidly reducing trans-cellular conductance in those epithelia which otherwise display lower TER. Consequently an investigation of the combined effects of apoAI and cortisol on this gill cell model, such as those carried out using prolactin plus cortisol [8] and T_3 plus cortisol [11] combinations, would make a valuable addition to gill physiological research, as would a comparison of the relative importance of hepatic and branchial apoAI synthesis to fish gill barrier properties. Other related issues to be resolved are whether pre-apoAI exists in a single gill cell type or in all rainbow trout gill cells and if apoAI exerts more influence on a specific cell type (e.g. human heart apoAI is synthesised in cardiac myocyte cells only [32]). This is not clear from the present investigation, since DSI cultures contain both pavement epithelial and mitochondrial rich cells, but these questions could be answered by further comparative proteomic investigations between this and the SSI (pavement epithelial cells only) version of the reconstructed gill model. Also, whilst this study suggests that apoAI is part of a physical response (possibly even a stress response) by the freshwater fish gill to an osmotic challenge, it would be useful to know if apoAI is also instrumental in more functional aspects of gill physiology, such as the regulation of branchial ion flux.

In conclusion, by extending a proteomic finding to the manipulation and functional measurements of the action of a specific protein, this study has identified and provided evidence for the possible importance of apoAI as a determinant of the barrier properties of the freshwater fish gill, particularly in terms of modulating trans-cellular permeability. This study also provides evidence that pre-apoAI may be synthesised in rainbow trout gill cells, which would support a recent suggestion that the physiological relevance of apoAI synthesis in the peripheral tissues of lower vertebrates has been underestimated [36]. We therefore propose that the addition of apoAI to the existing suite of regulatory factors suggests a higher degree of sophistication to maintaining gill barrier function than simply the reliance on hormonal or steroidal regulation.

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