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Organic cation transporter function in different in vitro models of human lung epithelium

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ABSTRACT

Organic cation transporters (OCT) encoded by members of the solute carrier (SLC) 22 family of genes are involved in the disposition of physiological substrates and xenobiotics, including drugs used in the treatment of chronic obstructive lung diseases and asthma. The aim of this work was to identify continuously growing epithelial cell lines that closely mimic the organic cation transport of freshly isolated human alveolar type I-like epithelial cells (ATI) in primary culture, and which consequently, can be utilised as in vitro models for the study of organic cation transport at the air–blood barrier. OCT activity was investigated by measuring [^{14}C]-tetraethylammonium (TEA) uptake into monolayers of Calu-3, NCI-H441 and A549 lung epithelial cell lines in comparison to ATI-like cell monolayers in primary culture. Levels of time-dependent TEA uptake were highest in A549 and ATI-like cells. In A549 cells, TEA uptake had a saturable and a non-saturable component with $K_m = 528.5 \pm 373.1$ μM, $V_{max} = 0.3 \pm 0.1$ nmol/min/mg protein and $K_d = 0.02$ μl/min/mg protein. TEA uptake into Calu-3 and NCI-H441 cells did not reach saturation within the concentration range studied. RNAi experiments in A549 cells confirmed that TEA uptake was mainly facilitated by OCT1 and OCT2. Co-incubation studies using pharmacological OCT modulators suggested that organic cation uptake pathways share several similarities between ATI-like primary cells and the NCI-H441 cell line, whereas more pronounced differences exist between primary cells and the A549 and Calu-3 cell lines.

1. Introduction

The membranous transport of several physiological regulators of pulmonary cell function and lung fluid homoeostasis has been suggested to be facilitated by members of the organic cation transporter (OCT) family encoded by the *SLC22A1-A3* genes (Salomon and Ehrhardt, 2012). In mammals, OCT1, OCT2 and OCT3 translocate small (i.e. <500 Da) organic cations with broad, overlapping affinities for endogenous substrates, such as choline, acetylcholine and monoamine neurotransmitters, as well as a variety of xenobiotics (Pelis and Wright, 2014). OCTs operate electrogenically and independently of sodium gradients and transport in both directions following the substrates' concentration gradient (Ciarimboli, 2010; Koepsell et al., 2007). OCT physiology in tissues such as the liver, gut and kidneys has by and large been well characterised (Nies et al., 2011), nonetheless, the lungs remain somewhat understudied in this regard. Moreover, OCT expression data concerning the respiratory epithelium is often limited to gene or PCR-based in vitro studies (Bleasby et al., 2006; Courcot et al., 2012; Endter et al., 2009; Mukherjee et al., 2012) and only a small number of reports show OCT immunocytochemistry or indeed transporter function (Lips et al., 2005; Macdonald et al., 2013; Nakanishi et al., 2013). Very recently, it has been shown by our group that β_2 agonists interact with OCT at the lung epithelium (Salomon et al., 2015), however, the clinical relevance of these findings are not fully understood to date.

One confounding factor for this scarcity of data is the limited availability of appropriate in vitro models for the study of ion and solute transport in the human distal lungs (Kim et al., 2001). Whilst freshly isolated human alveolar or bronchiolar epithelial cells in primary culture remain the gold standard for any kind of transport study involving the respiratory zone, limited availability of tissue, ethical restrains in certain countries and often economical and/or logistical shortcomings, hamper the use of human tissue. Accordingly, a number of cell lines

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has been utilised in ion and solute transport experiments, focusing on the respiratory epithelium, including the adenocarcinoma cell line, A549 (Sporty et al., 2008). Although A549 cells have been used as an in vitro model by many investigators, it should be noted that this cell line does not form tight monolayers of polarised cells (Forbes and Ehrhardt, 2005), an essential feature for transmonolayer transport studies. The cell lines 16HBE14o-, VA10, Nuli-1 and Calu-3, all of trachea-bronchial epithelial origin, have functional tight junctions and thus generate reasonable transepithelial electrical resistance (TEER) values indicative of polarised mucosal barriers (Buckley et al., 2011). The cell line NCI-H441 also forms tight monolayers and exhibits features of both alveolar (i.e. type II cell) and bronchiolar (i.e. club cell) epithelial phenotype (Hermanns et al., 2004; Salomon et al., 2014).

It was the aim of this study to determine OCT activity in a number of commonly used cell lines of human respiratory epithelial origin and to compare the obtained data to freshly isolated human alveolar epithelial cells in primary culture, in order to identify a suitable in vitro model for biopharmaceutical and physiological organic cation transport studies at the air–blood barrier.

2. Methods

2.1. Materials

[Ethyl-1-^{14}C] tetraethylammonium chloride (TEA; 55 mCi/mmol) was purchased from American Radiolabeled Chemicals (Herts, UK). Unlabelled TEA, amantadine, corticosterone, 1,1-diethyl-2,2-cyanine (decynium-22), 1-methyl-4-phenylpyridinium (MPP^+), verapamil and all cell culture media and supplements were obtained from Sigma-Aldrich (Dublin, Ireland). Quinidine was bought from Santa Cruz Biotechnology (Heidelberg, Germany). All cell culture plastics were purchased from Greiner BioOne (Frickenhausen, Germany).

2.2. Cell line culture

A549 cells (American Type Culture Collection, ATCC CCL-185) were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). Calu-3 (ATCC HTB-55) and NCI-H441 (ATCC HTB-174) cells were purchased from LGC Promochem (Teddington, UK).

Cell lines were cultured at the following seeding densities: 40,000 cells/cm^2 (A549; passage numbers 65–75), 75,000 cells/cm^2 (Calu-3; passage numbers 48–55) and 100,000 cells/cm^2 (NCI-H441; passage numbers 55–68). A549 cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1 mix) (DMEM/F12) supplemented with 5% foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Calu-3 cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 1% non-essential amino acids solution, 1% sodium pyruvate solution, 0.5% glucose solution and 100 U/ml penicillin and 100 μg/ml streptomycin. NCl-H441 cells were cultured in tissue culture flasks using Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% FBS, 1% sodium pyruvate solution, 100 U/ml penicillin and 100 μg/ml streptomycin. Twenty-four hours post-seeding in well plates, this medium was further supplemented with dexamethasone (100 nM) and insulin-transferrin-sodium selenite (ITS) solution (Roche Diagnostics Limited, West Sussex, UK). All cell lines were maintained at 37 °C in 5% CO_2 atmosphere and the culture media were exchanged every other day.

2.3. Human alveolar epithelial cell isolation and culture

The use of human tissue specimens was approved by Saarland State Medical Board (Saarbrücken, Germany). Human alveolar type II epithelial cells were isolated according to a protocol modified from Demling et al. from non-tumour lung tissue obtained from patients undergoing lung surgery (Demling et al., 2006; Daum et al., 2012). Purified type II cells were seeded at a density of 600,000 cells/cm^2 on collagen/fibronectin-coated plastics using complete small airways growth medium (SAGM; Lonza, Verviers, Belgium) supplemented with 1% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Primary cell monolayers were used after transdifferentiation into an alveolar type I-like (ATI-like) phenotype, following at least one week of culture.

2.4. Uptake studies

Cells were grown to confluent monolayers on 24-well plates for at least 5 (A549), 8 (NCI-H441, ATI-like) and 12 (Calu-3) days, respectively, before being used in uptake studies. Uptake experiments using A549 cells were carried out in extra-cellular fluid buffer (ECF; 122 mM NaCl, 3 mM KCl, 0.4 mM $KHPO_4$, 25 mM $NaHCO_3$, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM HEPES and 10 mM D-glucose, pH 7.4). All other experiments were performed in freshly prepared, bicarbonated Krebs-Ringer buffer (KRB; 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, 15 mM HEPES and 5.55 mM D-glucose, pH 7.4), unless otherwise stated. Both buffer solutions were found not to be significantly different with regards to organic cation uptake (data not shown). Prior to uptake studies, cell monolayers were washed three times with pre-equilibrated ECF or KRB solution. To initiate organic cation uptake, 200 μl of buffer solution containing [^{14}C]-TEA (10 μM) was added to each well. For time-course analyses, organic cation uptake was studied over 30 min. To determine concentration dependency and (self)-inhibitory effects on organic cation uptake, cell monolayers were incubated with TEA in the presence of various concentrations (i.e. 0 to 20 mM) of unlabelled compound for 10 min (Calu-3, NCI-H441, ATI-like) or 30 min (A549). In this case, [^{14}C]-TEA uptake was carried out at 4 °C and 37 °C and values obtained at 4 °C were subtracted from values measured at 37 °C in order to account for adsorption and diffusion processes. In all studies a concentration of 10 μM [^{14}C]-TEA was used. Uptake of TEA was also performed in the presence of several modulators of organic cation transporter function (i.e. amantadine, corticosterone, decynium-22, MPP^+, quinidine and verapamil).

At the relevant time points, the uptake was stopped by washing cell monolayers three times with ice-cold buffer and 400 μl of 1 N NaOH was added to permeabilise the cells for at least 12 h, before 400 μl of 1 N HCl was used for neutralisation of the cell lysate. Five-hundred microlitres of lysate was used to measure the cell-associated radioactivity in a liquid scintillation counter (Tri Carb TR2100 Packard Scintillation Counter, Dublin, Ireland). In parallel, the total cell protein content was quantified using a DC Protein Assay kit (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions.

2.5. RNA interference studies

A549 cells were seeded in 24-well cell culture plates and grown for 24 h. Two microliters of HyperFect reagent (Qiagen, West Sussex, UK), 50 μl of DMEM/F12 and siRNA against OCT1, OCT2 or OCT3 (75 nM; Santa Cruz Biotechnology) or scrambled control siRNA (AllStars, Qiagen) were mixed, incubated at room temperature for 5–10 min and added drop wise to the cells. The concentrations were chosen according to the manufacturer's guidance and did not cause significant toxicity after transfection with any of the siRNA types.

Cells were then incubated at 37 °C for 24 h, before the medium was changed back to the standard cell culture medium. After 24, 48 and 72 h, cell monolayers were lysed in cell extraction buffer containing protease inhibitors (Invitrogen, Karlsruhe, Germany). Protein sample concentration was determined with the DC Protein Assay kit and used for Western blot analysis. Uptake studies were carried out with cells after 4 days of culture, i.e. 72 h post transfection.