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Absorption of ipratropium and L-carnitine into the pulmonary circulation of the *ex-vivo* rat lung is driven by passive processes rather than active uptake by OCT/OCTN transporters

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ABSTRACT

The organic cation transporters OCT and OCTN have been reported to play a significant role in the cellular uptake of substrates within *in vitro* lung cells. However, no studies to date have investigated the effect of these transporters upon transepithelial absorption of substrates into the pulmonary circulation. We investigated the contribution of OCT and OCTN transporters to total pulmonary absorption of L-carnitine and the anti-muscarinic drug, ipratropium, across an intact isolated perfused rat lung (IPRL). The results obtained from the IPRL were contrasted with active transport *in vitro* using three human pulmonary cell lines and primary rat alveolar epithelial cells. *Ex-vivo* studies showed that OCT/OCTN transporters do not play a role in the overall pulmonary absorption of L-carnitine or ipratropium, as evidenced by the effect of chemical inhibition of these transporters upon pulmonary absorption. In contrast, *in vitro* studies showed that OCT/OCTN transporters play a significant role in cellular accumulation of substrates with preferential uptake of ipratropium by OCTs, and of L-carnitine uptake by OCTNs. The results show that *in vitro* uptake studies cannot be predictive of airway to blood absorption *in vivo*. Nevertheless, localised submucosal pulmonary concentrations of inhaled drugs and their pulmonary pharmacodynamic profiles may be influenced by OCT/OCTN transport activity.

1. Introduction

A number of drug candidates for inhaled therapy are cationic and are therefore potential substrates for the *SLC22* superfamily of ATP-independent polyspecific cation transporters at the plasma membrane (Koepsell and Endou, 2004). These transporters include the bidirectional organic cation transporters OCT 1 (*SLC22A1*), OCT2 (*SLC22A2*), *OCT3* (*SLC22A3*) and the sodium-dependent carnitine/cation transporter proteins OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*). The majority of inhaled drugs must cross the rate-limiting pulmonary epithelial barrier to access their pharmacological targets (Grainger et al., 2006), *e.g.* smooth muscle cells. As such the interaction of cationic drugs with transporter pathways expressed in pulmonary epithelium may be important for drug access to underlying pharmacological targets. Indeed this has been the premise of research by a number of groups (reviewed in Bosquillon, 2010; Gumbleton et al., 2011; Salomon and Ehrhardt, 2012) exploring the expression of OCTs/OCTNs within lung epithelium.

The localisation of OCT/OCTN transporters within intact lung has been reported using protein immunohistochemistry in both humans (Bleasby et al., 2006; Horvath et al., 2007; Lips et al., 2007, 2005) and rodents (Bleasby et al., 2006; Lips et al., 2007; Kummer et al., 2006; Tamai et al., 2000). Evidence for expression of OCT and OCTN family members in intact human and rat lung tissue also exists at the mRNA level (Bleasby et al., 2006; Lips et al., 2007; Tamai et al., 2000, 1998; Ishiguro et al., 2005). Nakanishi et al. (2013) showed OCT/OCTN driven accumulation of ipratropium in lung epithelial tissue, namely tracheal epithelium, following drug deposition in the tracheal lumen of the mouse. A number of *in vitro* cell culture studies have reported substrate uptake *via* OCTs and OCTNs in lung epithelial cells including the active uptake of the model OCT/OCTN cationic substrate (4-(4-dimethylaminostyryl)-*N*-methylpyridinium; ASP^+) in normal human bronchial cells (Horvath et al., 2007) and in a range of human bronchial epithelial cell lines (Mukherjee et al., 2012; Salomon et al., 2012, 2015). The

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inhaled anti-muscarinic drug ipratropium has been implicated as a substrate of both OCTN and OCT transporters depending upon the *in vitro* model adopted (Nakamura et al., 2010; Nakanishi et al., 2011). One report exists of the facilitative role of organic cation transporters upon the absorptive and secretory transport of ASP^+ across a cell monolayer (Mukherjee et al., 2012) with the results showing modest effects upon the overall absorptive transport, despite a significant extent of cellular uptake.

Here we hypothesized that in a fully intact lung the OCT/OCTN transporters play little or no role in the transepithelial transport of substrates into the pulmonary vasculature. To test the hypothesis we examined in an intact perfused rat lung (IPRL) the role of OCT/OCTN transport in pulmonary transepithelial permeability of the zwitterionic substrate, L-carnitine, and the inhaled therapeutic cationic drug, ipratropium; archetype substrates for the pathways under study. We found the overall solute transport into the IPRL vasculature was predominantly driven by non-competitive passive processes that eclipse the net effect of any OCT/OCTN-mediated transport.

2. Materials and methods

2.1. Materials

[^{3}H]-ipratropium bromide (70 Ci/mmol) was provided by GlaxoSmithKline (Ware, UK) and [^{3}H]-L-carnitine hydrochloride (70 Ci/mmol) was from American Radiochemicals Inc. (St. Louis, MO). Unlabelled ipratropium, L-carnitine, tetraethylammonium bromide (TEA) and 1-methyl-4-phenylpyridinium iodide (MPP^+) were purchased from Sigma–Aldrich (Poole, UK). Cell culture media and supplements were from Invitrogen (Paisley, UK) with cell culture plastics from Corning Costar (Hemel Hempstead, UK). All other reagents and solvents were from Fisher Scientific (Loughborough, UK) or Sigma–Aldrich. PCR primers were designed in house and supplied by Invitrogen (Paisley, UK).

2.2. Methods

2.2.1. IPRL

All animal experiments adhered to the UK Animal (Scientific Procedures) Act 1986. Rats used for all the experiments in this report weighed 250–350 g. Animals were normally housed with a 12 hour day/night cycle and fed *ad libitum* until the time of surgery.

To examine the transport of ipratropium and L-carnitine across an intact pulmonary barrier an IPRL preparation was employed as previously described (Morris et al., 2011; Sakagami et al., 2006). This model includes an intra-tracheal airway dosing technique that utilises a pressurized metered dose inhaler (pMDI) reproducibly delivering a high extent (>95%) of deposited solute liquid aerosol dose into the lung periphery (Niven and Byron, 1988). Using the pMDI methodology the IPRL was dosed with either vehicle control (100 μL saline) or a competitive inhibitor (in 100 μL saline), *i.e.* either unlabeled solute (125 nmol unlabeled L-carnitine or ipratropium) or the selective OCT inhibitor (MPP^+). Twenty minutes later the lungs were similarly dosed with the radiolabeled substrate (3 μCi of [^{3}H]-L-carnitine or [^{3}H]-ipratropium in 100 μL saline). At discrete timepoints after lung dosing. 200 μL samples were collected from the circulating perfusate and transferred to scintillation vials for radiochemical analysis.

2.2.2. Mathematical modelling

Pulmonary pharmacokinetic absorption parameters were calculated by fitting Eq. (1) to the individual airway to perfusate absorption data using nonlinear regression analysis (Micromath Scientist 3.0, Missouri, USA).

$$\%\text{ofdeposited dose absorbed}(t) = 100 \times f \times (1 - e^{-kt}) \quad (1)$$

where f represents the available fraction to be transported, k is the absorption rate constant (min^{-1}) and t is time in minutes. Modelling of the absorption data was not improved by the use of more complex models and Eq. (1) was deemed the minimum satisfactory model to provide precise parameter estimates for k and f.

2.2.3. In vitro *uptake studies*

The *in vitro* active accumulation of substrates of either OCT and/or OCTN transporters, was studied in a range of continuous lung epithelial cell lines and primary cultures of rat lung epithelium. The human pulmonary adenocarcinoma cell line, A549 (Giard et al., 1973), and the human bronchial epithelial cell line, BEAS-2B, were obtained from ATCC (American Type Culture Collection; Manassas, VA). 16HBE14o$^-$ cells, generated by transformation of normal bronchial epithelial cells, were from Dr D.C. Gruenert (University of California, San Francisco, San Francisco). Culture of these cells was performed as previously described (Endter et al., 2009), and isolation and primary culture of rat alveolar cells to a type I pneumocyte-like phenotype was undertaken as detailed in previous work (Campbell et al., 2003).

Solute uptake studies described hereafter were conducted at incubation temperatures of both 37 °C and 4 °C. Radiolabelled solute was added to each well (24-well format) containing confluent cell monolayers. The dosings were 1 μCi (15 pmol) [^{3}H]-ipratropium or 1 μCi (15 pmol) [^{3}H]-L-carnitine giving a final radiolabel probe concentration in each well of 50 nM (300 μL volume per well). Time- and temperature-dependent solute uptake was quantified at discrete timepoints over a 60 min incubation.

Radiolabelled solute uptake studies were also undertaken in the presence of unlabelled OCT/OCTN competitive inhibitors applied to the cells for a 30 min pre-incubation period prior to addition of the radiolabel probes. The unlabelled inhibitors were used to achieve concentrations of 500 μM ipratropium, 100 μM L-carnitine, 500 μM MPP^+, 5 mM TEA; a no-treatment control comprised radiolabelled solute alone in serum-free DMEM. Following the pre-incubation period, either [^{3}H]-ipratropium or [^{3}H]-L-carnitine was added to each well and the uptake of radiolabel allowed to proceed over a 60 min incubation period. The solute uptake experiments were terminated by two washes of the cell monolayers with ice-cold PBS followed by the addition of ice-cold trypsin-EDTA for 5 min, after which the cells were harvested and suspended in ice-cold DMEM and centrifuged (4 °C, 200 × g) for 10 min after which the supernatant was discarded and the cell pellet collected. This cell washing procedure was performed three times in total. The resulting cell pellets were transferred to scintillation vials and mixed with 3 mL of BioSafe 3 scintillation fluid and the cell-associated radioactivity quantified by liquid scintillation counting. The sodium-dependent nature of solute uptake for both [^{3}H]-ipratropium and [^{3}H]-L-carnitine was similarly conducted but using an incubation buffer where Na^+ was isotonically replaced with *N*-methyl-glucamine as previously described (Horvath et al., 2007).

2.2.4. Real-time quantitative polymerase chain reaction (qPCR)

Human OCT (*SLC22A1, SLC22A3, SLC22A3),* human OCTN (*SLC22A4, SLC22A5)*, rat OCT (*Slc22a1*, *Slc22a2*, *Slc22a3*) and rat OCTN (*Slc22a4*, *Slc22a5*) mRNA sequences were aligned using BLAST2 and the NCBI nucleotide gene search used to identify single exon regions; Oligocalc was used to validate all used transcription variants. Table 1 shows the primer sequences used in qPCR experiments. Total RNA was isolated (RNeasy, Qiagen, UK) from