Enhanced Expression of Organic Cation Transporters in Bronchial Epithelial Cell Layers following Insults Associated with Asthma – Impact on Salbutamol Transport

M. Mukherjee[#], E.Cingolani, D.I. Pritchard and C. Bosquillon[*]

School of Pharmacy, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom.

[#]Current address: Firestone Institute for Respiratory Health, McMaster University, Hamilton, Ontario, Canada

* Correspondence to: Dr.Cynthia Bosquillon, School of Pharmacy, Boots Science Building, University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom, Telephone: +44 (0) 115 8466078, Fax: +44 (0) 115 9515122, e-mail: cynthia.bosquillon@nottingham.ac.uk

Abstract

Increasing evidence suggests Organic Cation Transporters (OCT) might facilitate the absorption of inhaled bronchodilators, including salbutamol, across the lung epithelium. This is essentially scarred and inflamed in asthma. Accordingly, the impact of epithelial insults relevant to asthma on OCT expression and salbutamol transport was evaluated in air-liquid interfaced layers of the human broncho-epithelial cell line Calu-3. These were physically injured and allowed to recover for 48h or exposed to the pro-inflammatory stimulant lipopolysaccharide (LPS) for 48h and the aeroallergen house dust mite (HDM) for 8 h twice over 48h. Increases in transporter expression were measured following each treatment, with the protein levels of the OCTN2 subtype consistently raised by at least 50%. Interestingly, OCT upregulation upon LPS and HDM challenges were dependent on an inflammatory event occurring in the cell layers. Salbutamol permeability was higher in LPS exposed layers than in their untreated counterparts and in both cases, was sensitive to the OCT inhibitor tetraethylammonium. This study is the first to show epithelial injury, inflammation and allergen abuse upregulate OCT in bronchial epithelial cells, which might have an impact on the absorption of their substrates in diseased lungs.

Keywords

airway epithelium; drug transporters; permeability; inflammation; in vitro model

Abbreviations

ALI: air-liquid interface; AMC: 7-amino-4- methylcoumarin; BSA: bovine serum albumin; CCL17: chemokine (C-C motif) ligand 17; CCR3: C-C chemokine receptor 3; COX-2: cyclo-oxygenase 2; EDTA: ethylenediaminetetraacetic acid; HBSS: Hank's Balanced Salt Solution; HDM: house dust mite; HPLC-MS/MS: high performance liquid chromatography-tandem mass spectrometry; ICW: In-Cell Western^{TM}; LPS: lipopolysaccharide; LY: lucifer yellow VS dilithium salt; OCT: Organic Cation Transporters; P_{app}: Coefficient of apparent permeability; PAR-2: protease activated receptor-2; qPCR: quantitative polymerase chain reaction; TARC: thymus and activation regulated chemokine; TEA: tetraethylammonium; TEER: trans-epithelial electrical resistance.

1. Introduction

Inhaled bronchodilators of the β_2-adrenoceptor agonist and M3 muscarinic receptor antagonist classes are routinely used in the management of asthma and chronic obstructive pulmonary disease. In order to exert their pharmacological activity on the airway smooth muscles, the drugs need to overcome the physical barrier provided by the lung epithelium. However, they bear a net positive charge at physiological pH; hence, are expected to exhibit an intrinsic low permeability across biological membranes, which thus places a question mark over the mechanism by which they cross the epithelium.

In vitro data suggests that carrier proteins, particularly those within the Organic Cation Transporter family (OCT), might facilitate their pulmonary absorption (Bosquillon, 2010; Nickel et al, 2016; Salomon and Ehrhardt, 2012). OCTs are members of the super family of solute-link carriers SLC22A and comprise the electrogenic OCT1 (SLC22A1), OCT2 (SLC22A2), OCT3 (SLC22A3) sub-types as well as the pH-dependent OCTN1 (SLC22A4) and OCTN2 (SLC22A5) transporters (Koepsell et al, 2007). It is now well established that inhaled bronchodilators have the capacity to inhibit OCT proteins (Bosquillon, 2010; Mukherjee et al, 2012; Salomon et al, 2015) and can be transported by them. The M3 antagonists ipratropium and tiotropium are indeed substrates for OCT1, OCT2, (Hendrickx et al, 2013; Nakanishi et al, 2011) as well as OCTN2 (Nakamura et al, 2010); OCT3 transports only the former (Hendrickx et al, 2013) and OCTN1 has a low affinity for both compounds (Nakamura et al, 2010). Data are less comprehensive regarding the β_2 agonists but nevertheless indicate salbutamol is transported by OCT1 (Salomon et al, 2015) and possibly OCTN2 (Gnadt et al, 2012). In addition, formoterol is likely a substrate for at least one member of the transporter family since its uptake by airway smooth muscles was reduced in presence of OCT inhibitors (Horvath et al, 2007).

Evidence for an OCT-mediated absorption across the airway epithelium is currently limited to salbutamol. The drug was transported to a higher extent in the absorptive than the secretory direction in differentiated epithelial layers of the human bronchial cell lines 16HBE14o- and Calu-3 (Ehrhardt et al, 2005) or the bronchiolar cell line NCI-H441 (Salomon et al, 2015). Moreover, OCT inhibitors reduced the drug absorption in the three cell-culture models when added to the transport medium (Ehrhardt et al, 2005; Haghi et al, 2012; Mamlouk et al, 2013; Salomon et al, 2015). Conversely, a study in normal human bronchial epithelial cell layers concluded that passive diffusion was the primary mechanism for salbutamol transport (Unwalla et al, 2012). Permeability values in the cell layers were nevertheless two orders of magnitude greater, both for salbutamol and the paracellular marker mannitol, than those reported in the cell lines (Ehrhardt et al, 2005; Mamlouk et al, 2013; Salomon et al, 2015). The impact of transporters might therefore have been concealed in such permeable layers.

Transepithelial permeability data for salbutamol have so far been collected in cell layers representing a healthy epithelium. However, in a clinical setting, the drug is most commonly administered to asthmatic lungs. Those are characterised by chronic inflammation, often consecutively to a disproportionate reaction of the pulmonary tissue to inhaled environmental agents. In addition, they exhibit areas of scarring due to defective repair mechanisms following repetitive injury of a hypersensitive epithelium (Holgate, 2011). Epithelial inflammation and wounding might alter OCT expression profile in the lung epithelium, as reported in other tissues. For instance, OCT2 was shown to be downregulated in rat kidneys following injury (Ji et al, 2002) while the renal expression of both OCT1 and OCT2 was decreased upon tissue inflammation induced by administration of the pro-inflammatory agent lipopolysaccharide (LPS) (Heemskerk et al, 2008). In contrast, OCTN1 and OCTN2 levels were raised in rat liver during tissue regeneration post partial resection (Dransfeld et al, 2005). A similar increase in the expression of the latter transporter was also observed in inflamed sections of the intestine

in humans (Fujiya et al, 2011). More significantly, as of direct relevance to asthma, variations in OCT1-3 pulmonary expression were measured in rat and murine lungs following their exposure to the model allergen ovalbumin (Lips et al, 2007).

Since inhaled bronchodilators essentially face an inflamed and wounded epithelium when administered to patients, the aim of this study was to evaluate the effect of epithelial insults relevant to asthma on OCT expression in Calu-3 bronchoepithelial cell layers and consequences on salbutamol transepithelial transport. Calu-3 layers maintained at an air-liquid interface (ALI) provide an *in vitro* model anatomically close to the native bronchial epithelium (Grainger et al, 2006). Importantly, they have been shown to express the same OCT subtypes as normal human bronchial epithelial cell layers (OCT1, OCT3, OCTN1 and OCTN2) on their apical side (Mukherjee et al, 2012) and were deemed a reliable cell culture system for asthma research (Stewart et al, 2012).

ALI Calu-3 layers were physically injured or exposed to environmental agents relevant to asthma, i.e., LPS, a component of Gram negative bacteria wall present in organic dusts and well known to cause lung inflammation (Thorn, 2001) and the aeroallergen house dust mite (HDM), a major trigger for allergic asthma (Gandhi et al, 2013). Changes in OCT expression resulting from the insults were quantified at the gene and protein level by quantitative polymerase chain reaction (qPCR) or In-Cell Western™ (ICW), respectively. Salbutamol absorptive transport was then measured in LPS challenged and control layers in presence of an OCT inhibitor in order to investigate whether the drug is differentially handled by a healthy or an inflamed epithelium.

2. Materials and methods

2.2. Materials

Calu-3 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). LPS from *E. coli* 0:111B4 was purchased from Sigma-Aldrich (Dorset, UK) and HDM extracts from Indoor Biotechnologies (Warminster, UK). Primers for qPCR analysis were designed using a Beacon Designer Version 7.0 and obtained from Invitrogen, UK (Table 1). The rabbit primary antibodies used for OCT detection by ICW were from Alpha Diagnostics and the anti-GAPDH primary antibody raised in mouse from Sigma-Aldrich. The corresponding secondary antibodies goat anti-rabbit IgG (IRDye® 800CW Conjugate) and goat anti-mouse IgG (IRDye® 680CW Conjugate) were from LI-COR Biosciences UK Ltd (Cambridge, UK). Salbutamol sulfate was purchased from Alfa Aesar (Heysham, UK). Cell culture media and reagents as well as all other chemicals were from Sigma-Aldrich.

2.3. Characterisation of HDM extracts

The dry HDM extracts were reconstituted in Phosphate Buffer Saline (PBS) and stirred overnight at 4ºC. The preparation was centrifuged at 13,000 rpm for 20 min and the collected supernatant stored at -20°C. The total protein content was estimated using the method developed by Lowry. One mL of Lowry AB solution consisting of 20 ml of 2% sodium carbonate, 100 µL NaK tartrate and 100 µL 1% copper sulphate was added to 0.9 mL of the reconstituted extracts or bovine serum albumin (BSA) standards. After 10 min incubation at room temperature, 100 µL of 1:1 Folin reagent in dH_2O was added and each solution vortexed. 200 µL of each sample was transferred in triplicate into a clear 96-well plate. Samples were read at 750 nm using a Dynex Technologies MRX II plate reader running the Dynex Revelation version 4.02 software. A protein content equivalent to 13.5 µg/µL was obtained after dissolution of 2.2 grams of dry extracts in 14 mL of PBS.

The fluorogenic substrate Boc-Gln-Ala-Arg-AMC was used to measure the activity of the cysteine protease Der p1. 20 µL of HDM preparation was reacted with 180 µL of PBS pre-warmed at 37°C containing 5 mM cysteine and 0.9 µL/mL of Boc-Gln-Ala-Arg-AMC (10 mM) in a black 96-well plate in triplicate. The fluorescence of 7-amino-4- methylcoumarin (AMC) released from the substrate was measured using a DYNEX Microtiter Plate MFX Revelation Fluorometer equipped with the Dynex Revelation v4.21 software at t = 0, 10 and 20 min. Relative fluorescence units were converted into picomoles using a standard curve obtained with increasing concentrations of free AMC. The specific Der p1 activity of the HDM extracts was calculated as 345 picomoles degraded/mg/min.

2.4. Cell culture and maintenance

Calu-3 cells were cultured in the same conditions as reported by Grainger et al [21]. Briefly, they were maintained in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham supplemented with 10% foetal bovine serum (non USA origin, sterile filtered) , 100 UI/mL penicillin and 100 µg/mL streptomycin, 20 mM L-glutamine and 1% non-essential amino acids at 37°C in a humidified 5% CO_2 atmosphere. When approximately 90% confluence was reached, they were passaged using 0.25% trypsin/0.1% ethylenediaminetetraacetic acid (EDTA) and seeded at a density of 10^5 cells/cm^2 onto 0.4 µm pore size, 1.13 cm^2 surface area polyester Transwell® cell culture inserts (Co-Star Corning, Corning, UK) with 0.5 and 1.5 mL of cell culture medium in the apical or basolateral chamber, respectively. After 24 hours, cells were raised at an ALI by removing medium from the apical chamber and lowering the volume of basolateral medium to 0.5 mL. For quantification of OCT protein expression by ICW, cells were grown on the reverse side of the Transwell® membrane as described previously (Mukherjee et al, 2013) and ALI conditions were created by feeding the cells from the apical chamber. The medium was replaced every 2-3 days for up to 21 days when cell layers were used for experimentation. The integrity of the layers was assessed by measuring the trans-

epithelial electrical resistance (TEER) using an epithelial voltohmmeter with chopstick electrodes (World Precision Instruments, Stevenage, UK) and only cell layers with TEER values above 400 $\Omega.cm^2$ were included in the study. Cells were used at passage 27 to 39 and cells from the same passage were treated in parallel for quantification of OCT expression at the gene or protein level.

2.5. Physical injury

Nineteen day old Calu-3 layers were mechanically scraped using a sterile 10 µL pipette tip to form three homogenous and parallel scratches. After scraping, the wells were washed with media and the TEER was measured. The layers were then maintained in supplemented cell culture medium under submerged conditions and their healing was monitored *via* TEER measurements and time-lapse microscopy. Immediately after wounding of the layers, the Transwell® plate was transferred to the built-in chamber of a Leica DR IRBE microscope which provided a humidified CO_2 atmosphere at 37°C. Images of the scratches were captured by an ORCA-285 1394 high resolution digital camera (Hamamatsu, Japan) every 30 min over 48 hours. OCT expression was measured in the healed layers 48 h after injury by qPCR and ICW.

2.6. LPS and HDM challenges

Nineteen day old Calu-3 layers were exposed to 10 µg/mL LPS on their apical side in presence or absence of 1 µM of the anti-inflammatory steroid hydrocortisone in 500 µL of cell culture medium while 1.5 mL of medium was added to the basolateral compartment. TEER measurements were taken at regular time intervals over 48h before cells were harvested for quantifying mRNA levels of the OCT and inflammatory markers, or the cell layers were processed for ICW analysis.

For the allergen challenge, solutions of reconstituted HDM extracts were prepared at various protein concentrations in serum-free cell culture medium supplemented with 1 mM cysteine in order to maintain Der p1 activity. The presence of serum in the medium, even when heat

inactivated, indeed resulted in a loss of the allergen enzymatic activity (data not shown). Nineteen day old Calu-3 layers were exposed to 500 µL of the HDM preparations on their apical side for 8 h, an exposure time that allowed the detection of the chemokine CCL17 released in the cell culture medium (data not shown). During HDM exposure, cells were maintained in serum-free conditions but then were allowed to recover at an ALI in full culture medium. The TEER was regularly measured over a 48 h period spanning the exposure and recovery phases. Subsequently, the layers were subjected to two similar 8 h long exposures to HDM at a total protein concentration of either 2.0 or 3.0 µg/µL, each followed by a 16 h recovery phase. Cells were collected at t = 0, 8, 24, 32 and 48 h for assessing the mRNA levels of the OCT and inflammatory markers. OCT protein expression was quantified at the end of the second recovery period.

2.7. Quantification of gene expression

Changes in gene expression upon cell treatment were measured by qPCR following the MIQE guidelines (Bustin et al, 2009). Calu-3 layers were rinsed with ice cold PBS and harvested from the Transwell® using non-enzymatic cell dissociation buffer. cDNA was synthesised using a µMacs RNA extraction and cDNA synthesis kit (Miltenyi Biotech, UK) according to the manufacturer's instructions. Nucleic acid quantity and purity were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA). The Brilliant Sybr Green qPCR Master Mix (Stratagene, La Jolla, CA, USA) was used for the amplification reactions. The reaction mixture was made of 12.5 µl of Sybr Green Mix, 1 µl of primer mix (forward and reverse, 5 µM), 0.5-1.0 µl of cDNA template and DEPC-treated water up to 25 µl. The cycling conditions included 15 min polymerase activation at 95°C, and 40 cycles at 95°C for 15 s, 55/60°C for 30 s, and at 72°C for 30 s. Reactions were run on an Mx3005P (Stratagene) Real-

Time PCR Detection System according to the manufacturer's protocol (Software: MxPro-Mx3005P v4.01 (c) 2007, Stratagene). The primers were designed using Beacon Designer Version 7.0 and validated on a gradient using untreated Calu-3 cDNA in order to determine the annealing temperature. The melt curves were generated and the amplification efficiency of each primer was determined for each gene by serial dilutions of the basal Calu-3 cDNA. Two reference genes, i.e., 18S and GAPDH were assessed for normalising expression of the genes of interest between cell layers. Minimum variation of the Ct value was observed between samples for GAPDH. Therefore, relative gene expression levels were calculated by the ΔΔCt method as followed:

$$\text{Gene expression} = \frac{\text{Efficiency}_{(\text{gene of interest})} \wedge (Ct_{\text{basal control}} - Ct_{\text{treatment}})}{\text{Efficiency}_{(\text{GAPDH})} \wedge (Ct_{\text{basal control}} - Ct_{\text{treatment}})}$$

2.8. Quantification of protein expression

Variations in OCT protein levels induced by the different challenges were quantified in intact cell layers by In-Cell Western™ (ICW) according to a protocol previously validated (Mukherjee et al, 2013). Briefly, Calu-3 layers were washed with ice cold PBS, fixed with 4% paraformaldehyde, permeabilised with PBS containing 1% BSA and finally blocked with 1:25 human serum in PBS. A solution containing the primary antibody against the OCT subtype of interest (raised in rabbits) as well as the primary antibody against GAPDH (raised in mice) was prepared in PBS containing 0.1% BSA to achieve 1:1000 dilutions of the antibodies. This, together with the blocking serum, was added as a 100 µL droplet on top of the cell layers. After an overnight incubation at 4°C, the layers were washed with 0.1% BSA and 0.05% Tween in PBS. They were then exposed to 100 µL of 1:1000 dilutions of the secondary antibodies in

PBS containing 0.1% BSA before a rigorous wash with PBS supplemented with 0.1%BSA and 0.05% Tween followed by a quick wash with Millipore grade water on a Stuart® SSM1orbital shaker at 50 rpm (Bibby Scientific Limited, Staffordshire, UK).

Each individual Transwell® insert was scanned using the LI-COR Odyssey® NIR Imaging system (LI-COR Biosciences, Cambridge, UK) in order to detect the two simultaneous IRDye® signals (green for OCT and red for GAPDH). These were then quantified by the LI-COR Odyssey® Software V3.0 (LI-COR Biosciences).

The red intensity corresponding to GAPDH expression was used to normalise variations in cell number between the different wells. The background signal was obtained by treating Calu-3 layers with the anti-GAPDH primary antibody followed by both secondary antibodies. OCT protein levels were quantified based on the ratio of the green to the red intensity within the same well. Protein expression in treated layers was expressed as a percentage increase from basal expression in control layers.

2.9. Transepithelial transport of salbutamol after LPS challenge

The permeability of salbutamol sulfate was measured in the presence or not of the OCT inhibitor tetraethylammonium (TEA) in Calu-3 layers after LPS challenge and in control layers for comparison.

At day 19 after seeding on the Transwells®, cell layers were exposed to 10 µg/mL LPS as described above or covered with the same volume of medium.

After 48 h, the layers were washed once with 0.5 mL of pre-warmed PBS, and moved to clean 12-well plates. They were then equilibrated in Hank's Balanced Salt Solution (HBSS) at 37°C for 30 min before the TEER was recorded. 200 µL of 5.0 µg/mL solution of salbutamol in pre-warmed HBSS ± 5 mM TEA was placed in the apical chamber of the Transwells® and 900 µL of HBSS ± 5 mM TEA at 37°C was added to the basolateral chamber. Cell layers were placed

on an orbital shaker (60 rpm) inside the incubator. 200 µL samples were withdrawn from the basolateral compartments at different time points over 4 h and replaced with the same volume of corresponding buffer. Directly after collection of the last sample, the final TEER was measured and the permeability of the paracellular marker lucifer yellow VS dilithium salt (LY) across the layers was quantified. Briefly, 300 µL of 100 µM LY solution in pre-warmed HBSS was placed in the apical chamber while 900 µL of the buffer was added to the basolateral chamber. 100 µL were sampled from the basolateral chamber after 30 and 60 minutes and transferred to a black 96 well-plate (Nunc F96, Scientific Laboratory Supplies, Nottingham, UK). The fluorescence was assayed using a Tecan (SPARK 10M) plate-reader at λ_{em}= 437 nm and λ_{ex}= 535 nm.

During both salbutamol and LY permeability measurements, samples (100 µL) were taken from the apical side at the beginning and at the end of the experiment for determination of the mass balance.

Salbutamol transport data were accepted if the TEER remained above 400 $\Omega.cm^2$, the LY flux was less than 2.0% over 60 minutes and the mass balance was in the 75-110% range.

The coefficient of apparent permeability (P_{app}) was calculated according to the following equation:

$$P_{app} = \frac{dQ/dt}{AC_0}$$

where dQ/dt (mol. s^{-1}) is the transport rate, A (cm^2) is the surface area of the filter supporting the cell layer, and C_0 (mol. cm^{-3}) is the initial substrate concentration in the donor chamber.

2.10. Salbutamol analysis

Salbutamol sulfate was quantified by high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). The HPLC system consisted in an Agilent Hewlett Packard

series 1100 coupled with a Micromass Quattro Ultima Pt mass spectrometer (Waters, Milford, USA) equipped with an electrospray ion source operated in positive mode. An ACE3 C18 (3 μm, 150 mm x i.d. 2.1 mm) column fitted with a C18 guard cartridge was used for all analysis. Salbutamol sulfate samples were diluted 1:1 with methanol (HPLC grade), vortexed for 1 min and centrifuged at 5,000 rpm for 5 min at 4°C. The supernatant was diluted 1:1 with phase A consisting of an aqueous solution containing 0.1% v/v formic acid, ammonium formate 20 mM (pH 3.8), and 50 µL of the resulting solution was injected in the HPLC-MS/MS system for quantification. Samples were run isocratically at 0.2 mL min^{-1} using a mixture of phase A and MeOH (50:50) as the mobile phase. Salbutamol was detected in multi reaction monitoring (MRM) mode at m/z 240.1 → 148.1. MS parameters were as follows: capillary voltage, 3.5 kV; cone voltage, 35 V; source temperature, 125°C; desolvation temperature, 350°C; collision energy, 20 kV.

2.11. Statistical analysis

All experiments were conducted on 3-4 layers over at least two different passages. Data are presented as the mean ± standard error of the mean (SEM). A Student t-test was performed for comparing two groups while ANOVA followed by post-hoc tests (Bonferroni's or Tukey's for expression or transport data, respectively) was used for comparing more than two groups. A p value < 0.05 was considered to be statistically significant. Graphpad prism software version 6.02 was used for statistical analysis of the data.

3. Results

3.1. Increased OCT expression following recovery from a scrape wound

Calu-3 layers were mechanically scraped in order to mimic epithelial injury and scarring observed in asthma. Immediately after inflicting the scratches, the TEER dropped to ~ 150 $\Omega.cm^2$ for all experimental wells but returned to values above 600 $\Omega.cm^2$ after 48 h (data not shown). The formation of a physical wound and its repair within 48 h was evident from time-lapse microscopy images (Figure 1).

A significant increase in the mRNA levels of OCT1, OCT3 and OCTN2 was observed in healed layers when compared to basal levels in their unwounded counterparts (Figure 2A). OCT1 and OCTN2 protein expression in broncho-epithelial cells was concomitantly raised by more than 50% after recovery from the injury while only a marginal increase in expression was observed for OCT3 and OCTN1 (Figure 2B)

3.2. Increase OCT expression upon LPS challenge

Cell layers were treated with LPS primarily to induce an inflammatory reaction and thus, mimic the condition of an asthmatic epithelium. Exposure of Calu-3 layers to LPS from *E. coli* 0:111B4 over 48 h did not affect their physical integrity as assessed via regular TEER measurements (data not shown). Nevertheless, a significant up-regulation of the inflammatory markers cyclo-oxygenase 2 (COX-2), chemokine (C-C motif) ligand 17 (CCL17) - also called thymus and activation regulated chemokine (TARC) - and C-C chemokine receptor 3 (CCR3) was observed in treated cells (Figure 3A), which indicated that LPS was able to trigger an inflammatory event in Calu-3 cell layers. This was associated with fold increases in OCT1, OCT3 and OCTN2 gene expression (Figure 3A) as well as an enhancement of protein levels by approximately 20% for OCT1 or 50% for OCTN1 and OCTN2 (Figure 3B).

As expected, the presence of hydrocortisone in the test medium curbed the LPS-induced inflammatory cascade, as evidenced by COX-2, CCR3, and CCL17/TARC remaining at basal levels in cell layers exposed to both compounds simultaneously (Figure 3A). Interestingly, in these conditions, LPS impact on OCT expression was also abolished (Figure 3A), suggesting upregulation of the transporters was driven by epithelial inflammation.

3.3. Increased OCT expression following HDM challenge

As asthma is known to very commonly have an allergic component, cell layers were also challenged with the widespread airborne allergen HDM. Cell layers were initially treated with various concentrations of reconstituted HDM extracts for 8 h in order to monitor their effects on epithelial tight junctions. It had indeed previously been shown that HDM proteases have the capacity to cleave tight junction proteins (Gandhi et al, 2013). Protein concentrations above 3 µg/µL irreversibly damaged the cell layers while a concentration of 3 µg/µL disrupted these to an extent where the TEER dropped below 400 $\Omega.cm^2$ but reformation of functional tight junctions occurred within 48 h (Figure 4). Finally, even though 2 µg/µL HDM proteins caused a statistically significant reduction in TEER after 8 h exposure (Figure 4), the absolute TEER values remained above 400 $\Omega.cm^2$, indicating that the barrier properties of the cell layers were mainly preserved.

Based on those preliminary results, layers were then challenged with the allergen at a disruptive but non-lethal dose of 3 µg/µL as well as the highest safe dose of 2 µg/µL twice, over the total time span of 48h with a 16 h recovery period following each challenge in order to mimic a daily inhalation of the allergen. Although cell layers exposed to the highest concentration were unable to fully restore their barrier properties between the two challenges, this experimental set up provided an *in vitro* model exhibiting defective tight junctions, as observed in the bronchial epithelium of asthmatic patients (Xiao et al, 2011).

In an event where the Calu-3 tight junctions were reversibly disrupted, mRNA levels of all OCT as well as the inflammatory markers COX-2, CCR3, CCL17/TARC and protease activated receptor-2 (PAR-2) were raised at the end of the first 8h HDM exposure. They nevertheless returned to basal levels within 24h (Figure 5A). Interestingly, the second HDM exposure led to lower fold increases in the expression of the OCTs, COX-2, CCR3 and CCL17/TARC genes than the initial treatment (Figure 5A). Furthermore, expression remained elevated during the subsequent recovery period (Figure 5A) despite TEER values returning to their initial values (data not shown). At the end of this second recovery period, the OCT1 and OCT3 protein levels were slightly increased while those of OCTN2 were raised by around 60% (Figure 6).

In contrast, when the HDM challenge did not affect tight junction functions, it failed to induce an inflammatory reaction in the epithelial layers. In that case, no change in the expression of inflammatory markers was indeed noticed (Figure 5B). Quite strikingly, only the OCTN2 gene was actually overexpressed and showed sustained high mRNA levels over the course of the experiment (Figure 5B). Expression of the other OCTs was unmodified, indicating that their upregulation by HDM was conditional to the development of an inflammatory reaction in the layers likely triggered by the loss of their barrier function. It is however noteworthy that the fold increase in OCTN2 mRNA levels was significantly higher after exposure to the tight junction disruptive concentration of the allergen than to the lower milder concentration (20.3 ± 2.2 *vs.* 10.1 ± 3.8, $p < 0.001$). This suggests that inflammation also played a role in the upregulation of the gene but this had nevertheless the capacity to respond to an allergen independently of epithelial damage or the inflammation cascade.

3.4. Increased salbutamol transport after LPS challenge

How enhanced OCT expression in inflamed Calu-3 layers impacts on salbutamol permeability was assessed following cell exposure to LPS for 48 h since this treatment caused upregulation

of the OCT1, OCTN1 and OCTN2 proteins, without affecting the TEER or epithelial morphology.

Salbutamol P_{app} in control cell layers (Table 2) was slightly lower than in other studies (Ehrhardt et al, 2005; Haghi et al, 2012) in which the drug permeability was measured at a much higher concentration across 10-14 day old Calu-3 layers. However, as previously reported (Ehrhardt et al, 2005; Haghi et al, 2012, Mamlouk et al, 2013), the drug transepithelial transport was reduced when the layers were exposed to TEA (Figure 7), thus confirming an OCT involvement.

Despite similar TEER and LY flux between all cell layers, salbutamol permeability was more than doubled in LPS challenged layers as compared to non-treated layers and importantly, was still sensitive to TEA (Table 2, Figure 7). This indicated that the increase in salbutamol transport following LPS exposure was not promoted by enhanced passive diffusion but more likely by the inflammation driven overexpression of the OCT.

4. Discussion

In vitro transport data in airway epithelial cell layers suggest that the pulmonary absorption of salbutamol, and possibly other cationic inhaled bronchodilators, might be facilitated by the OCT family of transmembrane carriers. Those data have nevertheless been obtained in models of a 'healthy' epithelium while, in a clinical setting, the drugs have to cross an inflamed and remodelled epithelium that might exhibit a different OCT expression pattern. The impact of epithelial injury, inflammation and allergen exposure on OCT broncho-epithelial levels was therefore investigated in Calu-3 ALI layers. Salbutamol permeability was subsequently compared in inflamed and healthy layers. All three epithelial insults were shown to upregulate OCT, with variable effects on the different subtypes. Furthermore, inflammation enhanced salbutamol transepithelial transport, most likely *via* increased OCT expression.

Upregulation of the different OCT sub-types upon cell treatment did not follow similar trends when assessed by qPCR or ICW. This is not an unusual observation since poor correlations are commonly found between mRNA and protein levels because of post-transcriptional regulation and/or degradation of proteins (Vogel and Marcotte, 2012). Nevertheless, epithelial inflammation was found to be a major factor in the enhanced expression of the transporters post LPS and HDM challenges. Epithelial injury caused similar fold increases in OCT levels although it is unclear whether this was promoted by cell proliferation or linked to a possible post-wounding inflammatory reaction. Expression data in Calu-3 layers were in agreement with previous studies which had reported changes in OCT levels following an inhaled allergen challenge (Lips et al, 2007), kidney (Ji et al, 2002) or liver (Dransfeld et al, 2005) resection and in inflamed renal (Heemskerk et al, 2008) and intestinal (Fujiya et al, 2011) tissues. However, this study is the first to show both allergic and non-allergic inflammations together with physical injury regulate OCT in epithelial cells through direct stimulation, i.e., without the involvement of other cell types, particularly of the immune system.

The development of an inflammatory reaction in the Calu-3 cell layers was confirmed by the increased expression of COX-2, CCL17/TARC and CCR3, as well as PAR-2 during the HDM challenge. COX-2 is an ubiquitously expressed enzyme responsible for the synthesis of prostaglandins and is induced at inflammation sites (Dubois et al, 1998). It was therefore used as a general inflammatory marker in this study. CCL17/TARC and CCR3 are both overexpressed in airway epithelial cells *in vitro* following exposure to pro-inflammatory stimulants (Heijink et al, 2007; Stellato et al, 2001) but also, in the airways of asthmatic patients (Beck et al, 2006; Sekiya et al, 2002). Their upregulation in Calu-3 cell layers upon stimulation by LPS and HDM therefore indicated that the inflammatory event orchestrated in the cell line was relevant to asthma.

Both LPS and HDM are known to activate the transcription factor NFκB following their recognition by Toll-like receptors (principally TLR-4) or protease-activated receptors (principally PAR-2) respectively, located at the surface of airway epithelial cells, which then stimulates the synthesis and release of various cytokines and chemokines (Gandhi et al, 2013; Chen et al, 2011). OCT genes appear therefore under the control of the NFκB pathway in the bronchial epithelium.

Although both environmental agents increased OCT expression as part of the inflammation cascade, the inflammatory reaction triggered by LPS was independent of tight junction disruption whereas it was associated with a loss of barrier function in case of HDM exposure. It has previously been shown that the proteolytic components of the HDM extracts could cleave tight junction proteins in vitro but also that PAR-2 activation by the allergen led to a decrease in the adhesion properties of the tight junction protein E-cadherin (Gandhi et al, 2013). Tight junction disruption by the calcium chelating compound EDTA did not modify OCT levels in Calu-3 layers (data not shown), suggesting that inflammation was the only process behind OCT upregulation by HDM.

The OCTN2 subtype was nevertheless an exception since its expression was enhanced in a HDM challenge even in absence of an inflammatory reaction. Expression of the transporter was, in addition, the most largely increased amongst OCT subtypes during epithelial non-allergic inflammation or post recovery from a wound. Interestingly, single nucleotide polymorphisms (SNPs) in the OCTN2 gene have been associated with an increased susceptibility to develop asthma (Moffatt et al, 2010). The transporter carries the endogenous antioxydant L-carnitine (Koepsell et al, 2007) and was upregulated in Calu-3 cells after treatment with rosiglitazone, an agonist of the nuclear receptor Peroxisome Proliferator-Activated Receptor γ (PPARγ) (Mukherjee et al, 2013). PPARγ is a ligand-activated transcription factor which is known to control immunity and inflammation and is being

explored as a potential pharmacological target in asthma (Park and Lee, 2008). This suggests that OCTN2 might participate in anti-inflammatory and/or anti-allergic events in bronchial epithelial cells until those are overridden by pro-inflammatory processes.

Overexpression of the OCT1, OCTN1 and OCTN2 proteins in LPS challenged Calu-3 layers resulted in a two-fold increase in salbutamol transport as compared to that in untreated cells. The drug transport in both inflamed and control layers was reduced in presence of the OCT inhibitor TEA, indicating it was partly mediated by at least one member of the transporter family. While the drug has been shown to be a substrate for OCT1 (Salomon et al, 2015), only indirect evidence suggests it might also be transported by OCTN2 Gnadt et al, 2012). On the other hand, its interactions with OCTN1 have not been investigated to date. Considering LPS raised OCT1 levels by only ~ 20%, upregulation of this transporter alone cannot be accounted for the increase in salbutamol permeability across the inflamed layers. The OCTN1 and/or OCTN2 protein(s) whose expression was enhanced by around 50% upon LPS exposure are therefore likely to contribute to the drug active transport in the Calu-3 model.

Although the inflammation reaction induced in the cell line following LPS or HDM exposure was representative of epithelial inflammation in asthma, it is unknown whether broncho-epithelial cells from asthmatic patients actually respond to pro-inflammatory stimulants by upregulating the OCTs and thus, differentially express the transporters as compared to healthy cells. Until such data are available, the clinical significance of the enhancement in OCT expression and salbutamol absorption observed in Calu-3 layers during an inflammatory event will remain speculative. Our work nevertheless suggests that common features of asthma such as epithelial injury and inflammation, both of allergic and non-allergic origin, have the potential to alter transporter expression in the pulmonary epithelium with possible consequences on the fate of their substrates in diseased lungs.

5. Conclusions

This study showed that epithelial insults relevant to asthma have the capacity to upregulate OCTs in bronchial epithelial cell layers *in vitro* without the participation of other cell types, such as immune cells. It also demonstrated that an inflammatory event in the layers enhanced the transepithelial permeability of the bronchodilator salbutamol *via* OCT overexpression. The clinical significance of these findings remains nevertheless to be determined. Quantifying OCT expression in asthmatic *vs.* healthy bronchial epithelial cells would, for instance, be particularly informative.

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Note: all raw data created during this research are openly available from the corresponding author.

Figure legends

Fig 1: Time lapse microscopy images showing the healing within 48 h of a scrape wound inflicted to air-liquid interfaced Calu-3 layers. The scale bar represents 250 µm.

Fig 2: Variation in Organic Cation Transporter (OCT) expression profile in Calu-3 air-liquid interfaced layers following recovery from a scrape wound. (A) fold increases in OCT mRNA levels. (B) percentage increases in OCT protein levels. *, **, *** indicate statistically significant increases from basal expression at $p < 0.05$, $p < 0.001$, $p < 0.0001$, respectively.

Fig 3: Variation in Organic Cation Transporter (OCT) expression profile in Calu-3 air-liquid interfaced layers following exposure to lipopolysaccharide (LPS) for 48h. (A) fold increases in the mRNA levels of OCT and inflammatory markers. Cell layers were exposed to LPS alone, LPS and the anti-inflammatory drug hydrocortisone or the drug alone (B) percentage increases in OCT protein levels in layers exposed to LPS alone. * indicates statistically significant increases from basal expression at $p < 0.05$.

Fig 4: Effect of house dust mite (HDM) exposure on the integrity of Calu-3 air-liquid interfaced layers. Layers were exposed to different HDM protein concentrations for 8 h and the transepithelial electrical resistance (TEER) was monitored over 48h from t = 0h. * indicates a statistically significant difference in the TEER value as compared to the initial TEER at t = 0 h ($p < 0.05$).

Fig 5: Variations in the mRNA levels of Organic Cation Transporters (OCT) and inflammatory markers upon exposure of air-liquid interfaced Calu-3 layers to house dust mite (HDM) at a protein concentration of (A) 3.0 µg/µl, i.e., causing a reversible drop of the TEER below 400 $\Omega.cm^2$ or (B) 2.0 µg/µl, i.e., maintaining the TEER above 400 $\Omega.cm^2$. Layers were exposed to HDM for 8 h twice over 48h with a 16h recovery period between each exposure. * indicates statistically significant increases from basal expression ($p < 0.05$); # indicates statistically significant higher levels than at other time points ($p < 0.05$).

Fig 6: Variations in Organic Cation Transporter (OCT) protein expression in Calu-3 air-liquid interfaced layers after two 8h exposure to house dust mite (HDM) at a protein concentration of 3.0 µg/µl followed each by a 16h recovery period. * or ** indicate statistically significant increases from basal expression at $p < 0.05$ or $p < 0.001$, respectively.

Fig 7: Salbutamol apical to basolateral cumulative transport in untreated Calu-3 layers or layers exposed to lipopolysaccharide (LPS) for 48h in presence or absence of the Organic Cation Transporter (OCT) inhibitor tetraethylammonium (TEA). * indicates a statistically significant increase from transport in untreated cells at $p < 0.05$; # indicates a statistically significant decrease from transport in absence of TEA at $p < 0.05$.

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Table 1: details of primers used for qPCR

Protein (Gene)	Forward and Reverse sequences	T_m	Annealing temp. (°C)	Efficiency (E value)	Size (bp)
GAPDH	F: 5'-ACAGTCAGCCGCATCTTC-3' R: 3'-GCCCAATACGACCAAATCC-5'	53.8/53.1	55	1.95	101
18s	F: 5'-CATCTGCCGTTGGAACCTGAAG-3' R: 3'-CTTGCGGTGTTCTTCCTGGCAT-5'	55.9/57.8	55	1.95	130
OCT1 (SLC22A1)	F: 5'- TGAAGGACGCCGAGAACC-3' R: 3'-AGGAAGAATACAGAGAAGTGAAGG-5'	55.7/55.6	50	1.84	188
OCT3 (SLC22A3)	F: 5'-CCACTCCACCATCGTCAG-3' R: 3'-ACACCAAGGCAGGATAGC-5'	53.5/53.0	60	1.98	168
OCTN1 (SLC22A4)	F: 5'-TGTCATCACCCGTAGTTG-3' R: 3'-ACATACCATTGAAGCCATTG-5'	50.3/50.9	50	2.1	156
OCTN2 (SLC22A5)	F: 5'-GCTACATGGTGCTGCCACTGTT-3' R: 3'-CTGCCTCTTCAAATCGTCCCTG-5'	57.7/55.4	50	2.4	156
TARC (CCL17)	F: 5'-ACTTCTCCCGGGACTACCT-3' R: 3'-TCCCTCACTGTGGCTCTTCT-5'	52.9/54.1	58	1.95	111
COX-2	F: 5'-CGGTGAAACTCTGGCTAGACAG-3' R: 3'-GCAAACCGTAGATGCTCAGGGA-5'	54.9/56.7	60	2.3	156
CCR3	F: 5'-TACTCCCTGGTGTTCACTGTGG-3' R: 3'-ACGAGGAAGAGCAGGTCCGAA-5'	52.9/54.1	55	1.86	134
iNOS	F: 5'-GCTCTACACCTCCAATGTGACC-3' R: 3'-CTGCCGAGATTTGAGCCTCATG-5'	54.8/55.8	60	1.94	136
PAR-2 (F2RL1)	F: 5'-CTCCTCTCTGTCATCTGGTTCC-3' R: 3'-TGCACACTGAGGCAGGTCATGA-5'	53.7/58.1	58	1.90	152

T_m : Melting temperature

Table 2: Coefficient of apparent permeability (P_{app}) of salbutamol in untreated Calu-3 layers or layers exposed to lipopolysaccharide (LPS) for 48h.

	P_{app} (10^{-7} cm/s)
Control layers	4.3 ± 0.6
Control layers + TEA	2.5 ± 0.2
Layers exposed to LPS	10.2 ± 3.0
Layers exposed to LPS + TEA	3.7 ± 1.5

Salbutamol transport was measured in the presence or absence of the Organic Cation Transporter (OCT) inhibitor tetraethylammonium (TEA)

Figure 1, Mukherjee et al

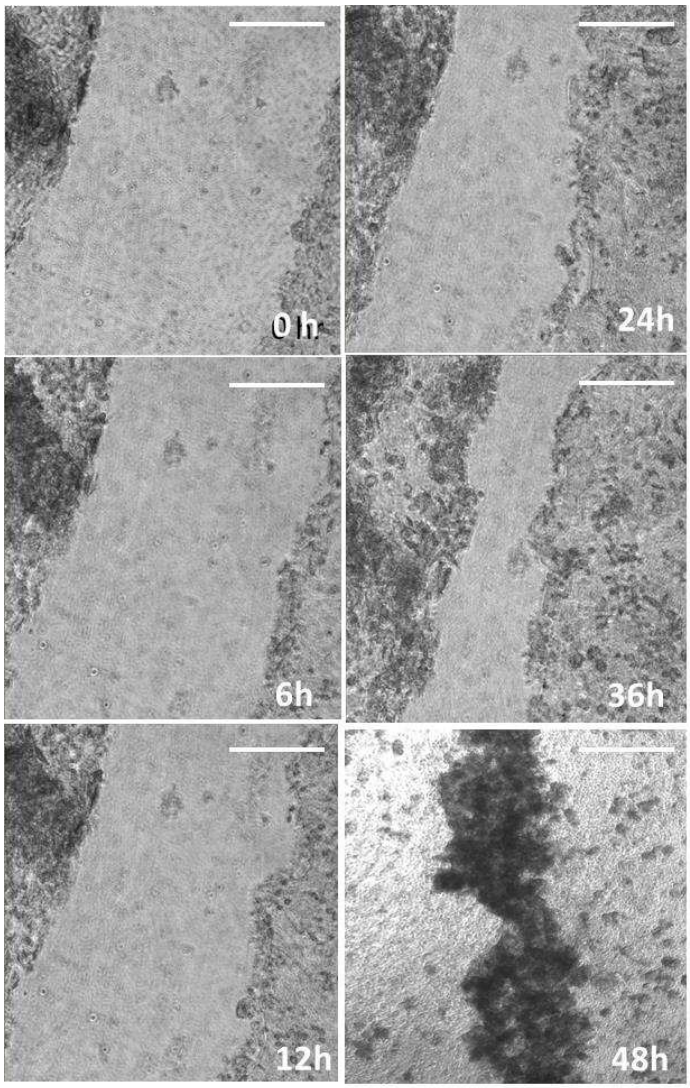

Figure 2, Mukherjee et al

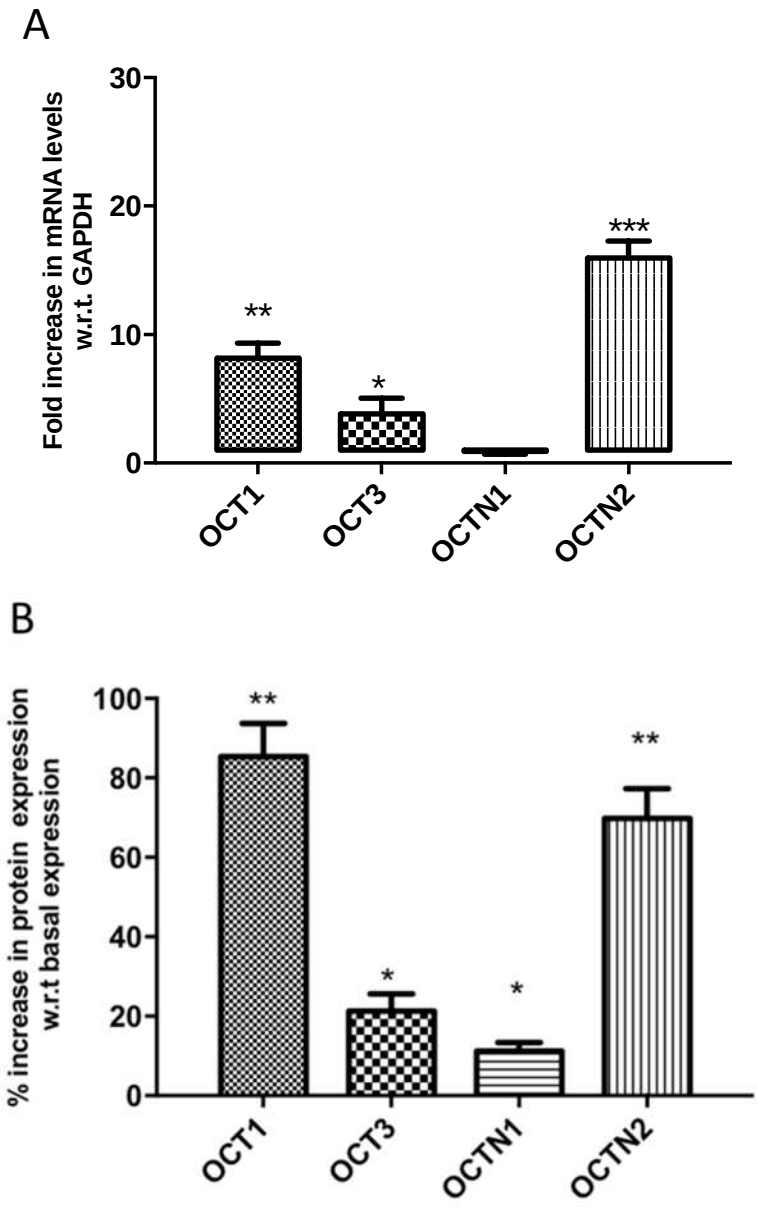

Figure 3, Mukherjee et al

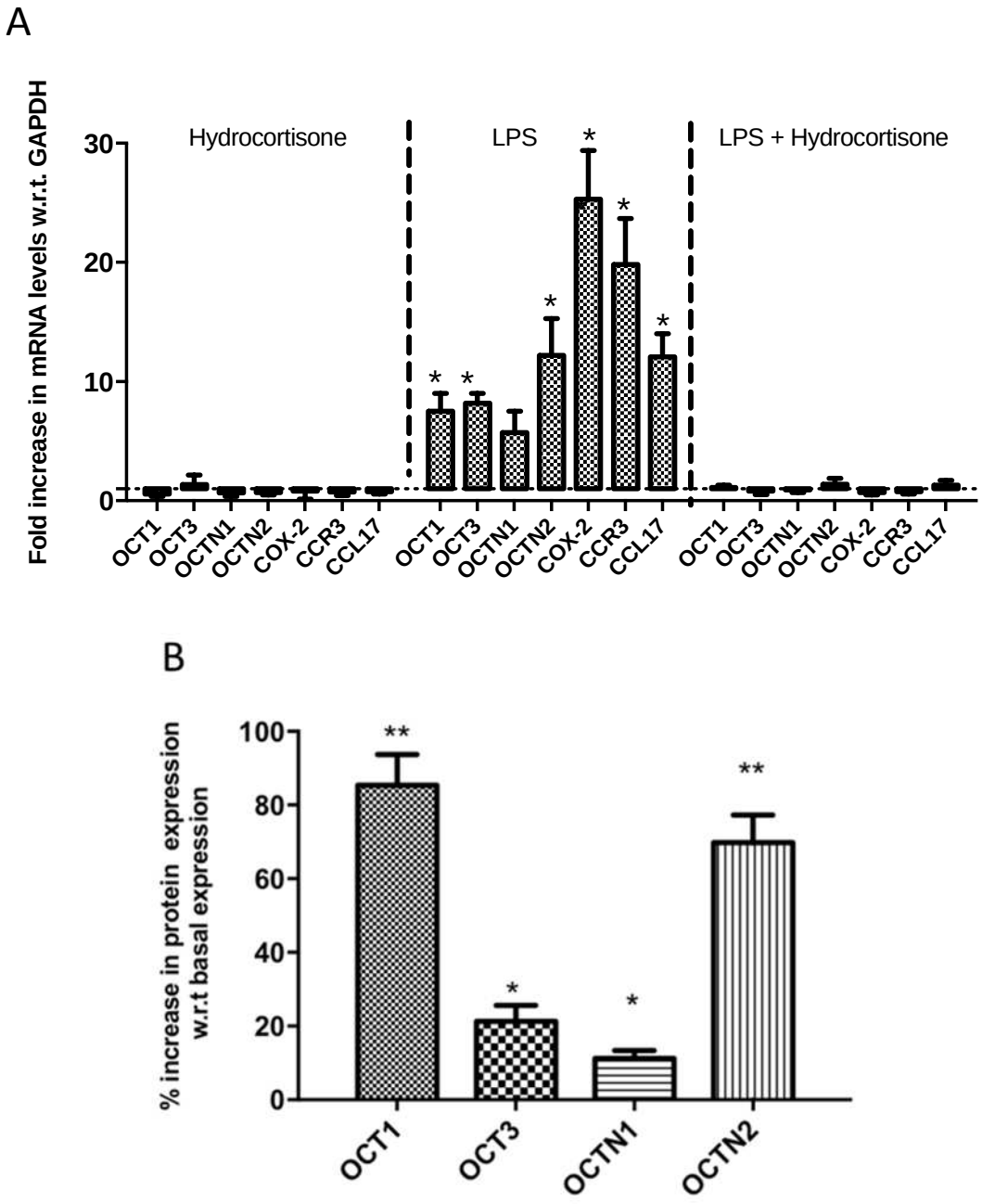

Figure 4, Mukherjee et al

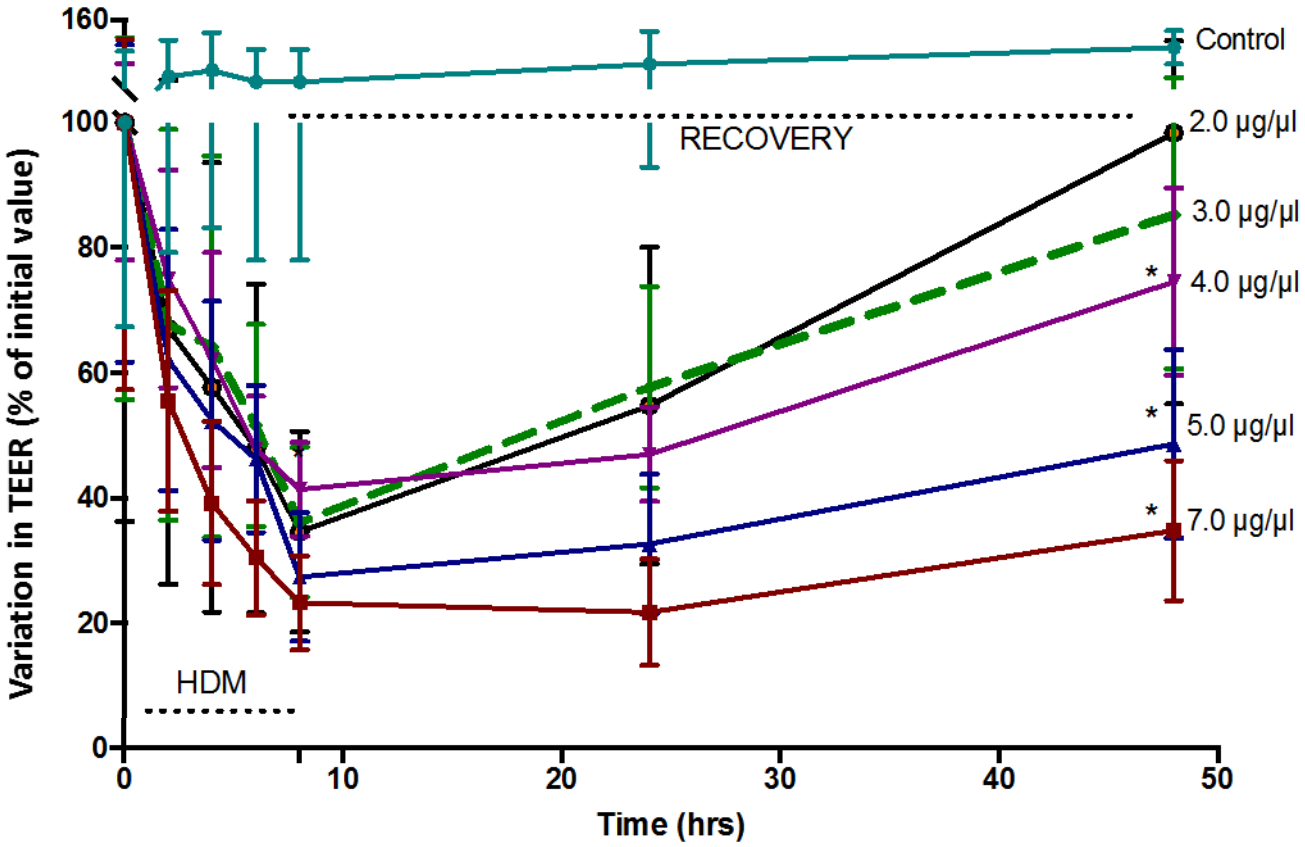

Figure 5, Mukherjee et al

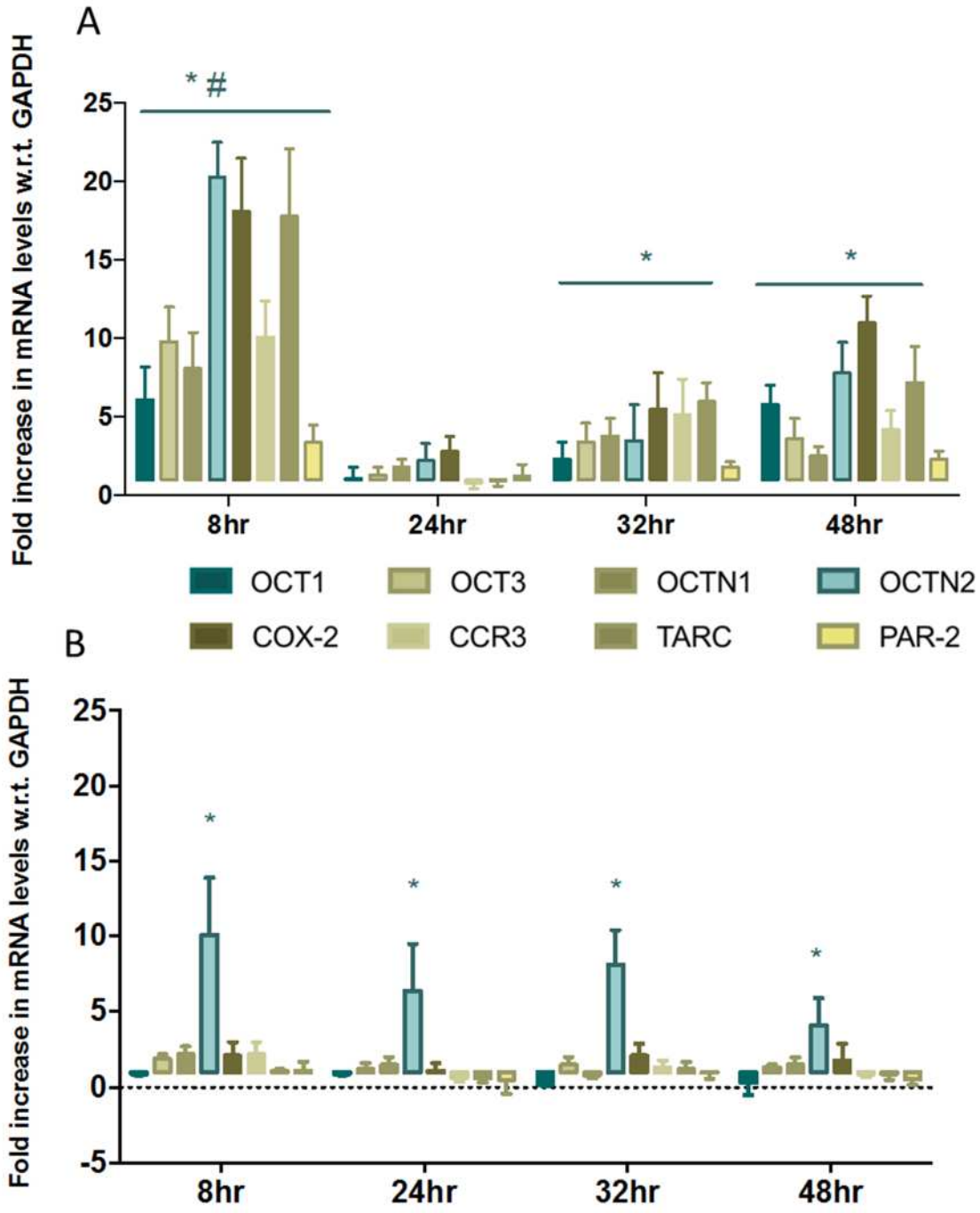

Figure 6, Mukherjee et al

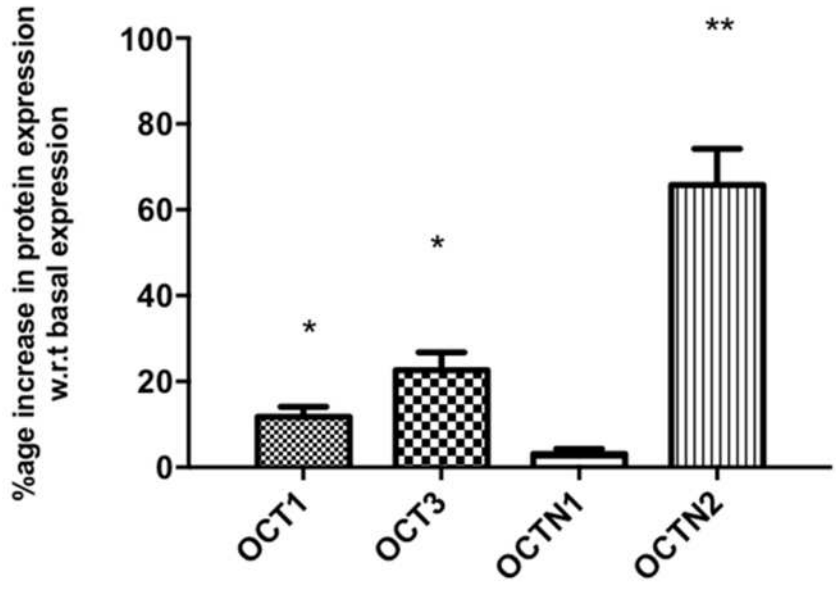

Figure 7, Mukherjee et al