

Metformin prevents the effect of *Pseudomonas aeruginosa* on airway epithelia epithelial tight junctions and restricts hyperglycaemia-induced bacterial growth

Wishwanath R. A. Patke¹, Georgina Carr², Emma H. Baker¹, Deborah L. Baines¹⁺, James P. Garnett^{2*+}.

1- Institute for Infection and Immunity, St George's, University of London, Tooting, London, UK.

2- Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, UK.

*Corresponding Author - Dr James P. Garnett, Institute of Cellular Medicine, Newcastle University, Newcastle-upon-Tyne, NE2 4HH, UK. Email: james.garnett@ncl.ac.uk; Telephone: +44 (0) 191 208 7009.

⁺Joint senior authors.

Running Title: Metformin reduces airway *P. aeruginosa* growth

Abstract

Lung disease and elevation of blood glucose are associated with increased glucose concentration in the airway surface liquid (ASL). Raised ASL glucose is associated with increased susceptibility to infection by respiratory pathogens including *Staphylococcus aureus* and *Pseudomonas aeruginosa*. We have previously shown that the anti-diabetes drug, metformin reduces glucose-induced *S. aureus* growth across *in vitro* airway epithelial cultures. The aim of this study was to investigate whether metformin has the potential to reduce glucose-induced *P. aeruginosa* infections across airway epithelial (Calu-3) cultures by limiting glucose permeability. We also explored the effect of *P. aeruginosa* and metformin on airway epithelial barrier function by investigating changes in tight junction protein abundance. Apical *P. aeruginosa* growth increased with basolateral glucose concentration, reduced transepithelial electrical resistance (TEER) and increased paracellular glucose flux. Metformin pre-treatment of the epithelium inhibited the glucose-induced growth of *P. aeruginosa*, increased TEER and decreased glucose flux. Similar effects on bacterial growth and TEER were observed with the AMPK agonist, AICAR. Interestingly, metformin was able to prevent the *P. aeruginosa*-induced reduction in the abundance of tight junction proteins, claudin-1 and occludin. Our study highlights the potential of metformin to reduce hyperglycaemia-induced *P. aeruginosa* growth through airway epithelial tight junction modulation, and that claudin-1 and occludin could be important targets to regulate glucose permeability across airway epithelia and suppress bacterial growth. Further investigation into the mechanisms regulating metformin and *P. aeruginosa* action on airway epithelial tight junctions could yield new therapeutic targets to prevent/suppress hyperglycaemia-induced respiratory infections, avoiding the use of antibiotics.

Keywords: Glucose, *P. aeruginosa*, Airway Epithelium, Tight Junctions, Metformin, AICAR, AMPK, Claudin-1, Occludin, Respiratory Infection, Diabetes.

Introduction

The airway epithelium is covered by a thin layer of surface liquid (ASL), which is vital for maintaining a healthy respiratory tract. The ASL contains many potential growth substrates for bacterial growth including glucose. Glucose concentration in human ASL is normally ~0.4 mM, 12.5 times lower than that of blood [1]. ASL glucose concentrations are elevated in people with respiratory disease including acute viral rhinitis [2], chronic rhinosinitis [3], chronic obstructive pulmonary disease [4] and cystic fibrosis [1]. ASL glucose concentrations are also increased in experimental and diabetic hyperglycaemia [2,5] and are further increased in people with both respiratory disease and diabetes mellitus [1,6]. Such elevations in ASL glucose are associated with increased susceptibility to infection, particularly with *Staphylococcus aureus* and *Pseudomonas aeruginosa* [4,6,7].

We developed an *in vitro* model of airway glucose homeostasis and showed that ASL glucose concentration is the net effect of the paracellular diffusion of glucose from the blood/interstitial fluid across the airway epithelium into ASL and its removal from ASL by epithelial uptake through facilitative glucose transporters [8-11]. We showed that paracellular glucose diffusion was increased not only by elevating basolateral glucose concentrations (mimicking hyperglycaemia of blood/interstitial fluid), but also by inflammation. In human epithelial cell monolayers, we found that application of either apical pro-inflammatory cytokines [9] or apical bacterial pathogens [12,13] increased paracellular glucose flux, increasing glucose concentrations in ASL. Under pro-inflammatory conditions, airway epithelial glucose uptake through apical GLUTs is elevated, but this is insufficient to compensate for the increased paracellular glucose leak and does not prevent elevation of ASL glucose concentrations [9]. Control of paracellular glucose movement thus appears to be the main rate-limiting step for maintaining low ASL glucose concentrations.

We have previously shown that pre-treatment of airway epithelial monolayers with the anti-diabetes drug, metformin, attenuated the effects of *S. aureus* on paracellular glucose flux and reduced bacterial growth in ASL by restricting glucose availability [12]. Reducing glucose permeability could therefore be a therapeutic target in the prevention and treatment of respiratory *S. aureus* infections.

Epithelial barrier dysfunction during bacterial infections is characterised by alterations in tight junction protein abundance. Tight junctions, the most apically located of the intercellular junctional complexes, regulate the passage of fluid, ions, macromolecules, and

inflammatory cells through the paracellular space between epithelial cells. Tight junctions are formed by three classes of integral membrane proteins: claudins, occludins, and junctional adhesion molecules. Directly underneath tight junctions, are another specialized cell–cell contact domain, adherens junctions, which are mainly composed of cadherin family members. The composition of these protein complexes regulates epithelial permeability to solutes and ions. Factors that alter the abundance and localisation of specific tight junction proteins within this complex could therefore alter glucose permeability.

The aim of the present study was to determine whether metformin also has the potential to reduce glucose-induced *P. aeruginosa* infections across airway epithelial cultures by limiting glucose permeability. We used the adenocarcinoma cell line, Calu-3, derived from human tracheobronchial submucosal glands, which form a heterogeneous population of mucus (mucin-secreting) and serous (fluid-secreting) cell types [14,15]. Calu-3 cells secrete a fluid rich in antimicrobial secretions to suppress bacterial growth [16] and form polarised airway epithelial monolayers with functional tight junctions expressing many of the integral membrane proteins found *in vivo* [17,18]. We explored the effect of *P. aeruginosa* and metformin on airway epithelial barrier function by investigating changes in tight junction protein abundance.

Materials and Methods

Airway epithelial cell culture

The human adenocarcinoma-derived cell line, Calu-3 (from ATCC), was grown in Eagle's minimal essential medium (EMEM) plus 10% FCS, 2mM L-Glutamine, 100 units/ml penicillin, 100µg/ml streptomycin, and 1% non-essential amino acids (Sigma) and incubated in humidified air containing 5% CO₂ at 37°C. Calu-3 cells were seeded onto clear Costar Transwell inserts (0.45-µm pore size) at 250,000 cells/cm² to form confluent polarised monolayers, as previously described [19]. Experiments were carried out 10–14 days post-seeding. 24 hours before the experiment, monolayers were washed to remove the mucus layer and placed in Krebs salt solution consisting of 115 mM NaCl, 5 mM KCl, 25 mM NaHCO₃, 1 mM MgCl₂, 1 mM CaCl₂ and 5 mM D-glucose (equilibrated with 5% CO₂ to pH 7.4) to remove antibiotics and components of the media that could influence bacterial growth.

Calu-3 monolayers were pre-treated with (or without) metformin (0.03 or 1 mM) or 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR; 0.5 mM), added to the basolateral medium 18 hours prior to apical inoculation with *P. aeruginosa*. Metformin was applied to the basolateral membrane to mimic the situation *in vivo*, in which metformin would reach the airway epithelium via the blood/interstitial fluid. Calu-3 cells and other bronchial cell types express organic cation transporter 3 on the basolateral membrane which is known to facilitate metformin uptake [20-22].

Using a chopstick style Epithelial Voltohmmeter (EVOM, WPI, UK), Transepithelial Electrical Resistance (TEER) measurements were made, a widely accepted quantitative technique to measure the presence of tight junctions, the major component of epithelial cell barrier integrity in cell culture models of epithelial monolayers. TEER measurements quantify the transport of ions across the cells with paracellular ion transport representing the most substantial contribution to such readings. An intact cellular barrier displays high TEER values. Loss of barrier integrity leads to decreased TEER values.

Airway epithelia - *Pseudomonas aeruginosa* co-culture

A single colony of *P. aeruginosa* strain PA01 was incubated overnight at 37°C in RPMI media (Life Technologies, Paisley, UK) and diluted with glucose-free RPMI to produce a culture of approximately 10^7 colony forming units (CFU) per ml. 100µl of PA01 culture (1×10^6 CFU total) was applied to the apical surface of Calu-3 monolayers. Co-cultures were bathed in Krebs salt solution. Normal blood glucose or hyperglycaemia was modelled by applying basolateral glucose concentrations of 5 and 15 mM, respectively. Co-cultures were placed in a CO₂ incubator at 37°C for 7 hours, after which either cell lysates were collected for western blotting or cells were homogenised and CFU calculated by plating out serial dilutions.

Paracellular L-glucose flux experiments

Paracellular movement of glucose across Calu-3 monolayers was measured by analysis of radiolabelled [¹⁴C]-L-glucose transepithelial flux. Experiments were initiated by adding 1 ml of Krebs salt solution containing 1.0µCi of [¹⁴C]-L-glucose plus 10 mM of non-radiolabelled equivalent glucose to the basolateral side of the transwells and 0.1 ml of glucose-free Krebs

salt solution to the apical side. Apical and basolateral samples were taken after 1h and the concentration of radiolabelled glucose was analysed using a scintillation counter.

Western blotting for tight junction proteins

After cells were co-cultured they were lysed in ERK Phosphorylated buffer containing 20mM Tris-HCL pH 7.5, 150mM NaCl, 1mM EDTA, 50mM NaF, 1mM NaVO₄, 1% w/v Triton-X, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 1 µg/ml protease inhibitor cocktail (P8340, Sigma, UK). Cells were sonicated and cell debris was removed by centrifugation at 10,000 RPM at 4°C for 10 mins. Total protein concentration was determined by Bradford Assay and between 30µg - 60 µg was electrophoresed on NuPage Novex 10% Bis-Tris Protein Gels (Invitrogen). Protein was then transferred for 70 mins onto polyvinylidene difluoride membranes (Merck Millipore). Nonspecific reactivity was blocked in Odyssey Blocking Buffer diluted 1:1 in PBS for 1h at room temperature. Blots were incubated overnight with the following antibodies obtained from Life Technologies (Paisley, UK) and used at 1:500 dilution; mouse anti E-Cadherin (33-4000), rabbit anti-Claudin-1 (51-9000), and rabbit anti-Occludin (H-279; Santa Cruz Biotechnology). The blots were then washed in Tris-buffered saline/0.2% Tween 20 and incubated with either Donkey anti-mouse IgG (926-3221) (Licor) or goat anti-rabbit IgG (Licor) for 1hr at room temperature and kept in the dark. Blots were subsequently probed with a mouse monoclonal antibody to β-actin. Detection of antigen-antibody complexes was assessed using a Licor Odyssey western blot imaging system. Integrated density of each fluorescent band was determined using Image Studio Lite Ver 3.1 (Licor). Results are expressed as a ratio to β-actin control within the same sample.

Chemicals and reagents

All chemical and reagents were obtained from Sigma, Poole, UK unless otherwise stated.

Statistical Analysis

Values are reported as mean ± SEM. Statistical analysis was performed using analysis of variance (ANOVA) and post hoc Tukey's or Student's t test. P values <0.05 were considered significant.

Results

Metformin and AMPK agonist AICAR restrict glucose-induced *P. aeruginosa* growth in airway epithelia-bacteria co-cultures

Elevating basolateral glucose from 5 to 15 mM in Calu-3 airway epithelial co-cultures increased apical *P. aeruginosa* growth by $66 \pm 12\%$ ($p < 0.01$; $n = 5$; Figure 1A). Pre-treatment of the epithelium with 1 mM metformin (18 hours) significantly reduced apical *P. aeruginosa* growth in the presence of normoglycemic (5 mM) and hyperglycaemic (15 mM) basolateral glucose concentrations ($p < 0.05$; $n = 5$). A significant reduction in hyperglycaemic-induced *P. aeruginosa* growth was also observed using clinically-relevant concentrations of metformin (Figure 1B; 20 μM metformin; usual metformin dose is approximately 15–30 mg/kg/day, producing plasma concentrations $\sim 5\text{--}20 \mu\text{M}$) [23,24].

It is well documented that metformin activates AMP activated protein kinase (AMPK) [25], which is known to play an important role in tight junction assembly [26]. In addition the AMPK agonist, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) has previously been shown to partially prevent tight junction disassembly (induced by calcium depletion) [27]. We therefore tested the effect of epithelium pre-treatment with AICAR (0.5mM, 18 hours) and found that this also reduced *P. aeruginosa* growth by $52 \pm 9\%$ under hyperglycaemic conditions ($p < 0.05$; $n = 6$; Figure 1B).

Metformin attenuates the effects of *P. aeruginosa* on Calu-3 transepithelial electrical resistance (TEER) and transepithelial glucose flux

Pre-treatment with 1 mM metformin increased Calu-3 monolayer TEER from 676 ± 33 to $756 \pm 54 \Omega \cdot \text{cm}^2$ ($p < 0.05$; $n = 5$; Figure 2A). The addition of *P. aeruginosa* to the apical surface produced a significant decrease in TEER to $477 \pm 21 \Omega \cdot \text{cm}^2$ after 6 hours of co-culture ($p < 0.01$; $n = 5$; Figure 2A). Metformin treatment (both 1 mM and 20 μM) attenuated the fall in TEER produced by apical *P. aeruginosa* ($p < 0.05$; $n = 5\text{--}6$), which was prevented by pre-treatment with the AMPK inhibitor compound C (Figure 2B). AICAR pre-treatment also attenuated the *P. aeruginosa*-induced reduction in Calu-3 TEER ($p < 0.05$; $n = 6$; Figure 2B).

The decrease in TEER evoked by apical *P. aeruginosa* induced an increase in the rate of basolateral-to-apical paracellular glucose flux by $47.6 \pm 5.8\%$ ($p < 0.01$, $n = 4$; Figure 2C), which could be partially overcome following metformin treatment ($p < 0.05$; $n = 4$). Metformin had no significant effect on paracellular glucose flux in the absence of *P. aeruginosa* ($p > 0.05$; $n = 4$; Figure 2C), despite increasing Calu-3 TEER, consistent with the non-linear relationship between TEER and paracellular glucose permeability (large changes in TEER are required at high TEER to induce small changes in paracellular flux; conversely small changes in TEER at low TEER produce relatively large changes in paracellular flux) [8].

Metformin increases claudin-1 and occludin protein abundance to prevent *P. aeruginosa*-induced tight junction disruption

We investigated the effects of *P. aeruginosa* and metformin on the abundance of tight junction (claudin-1 and occludin) and adherens junction (E-cadherin) proteins.

Claudin-1 protein abundance was elevated by $16 \pm 8\%$ after 18h in the presence of 1 mM metformin ($p < 0.05$; $n = 4$; Figure 3). Co-culture with *P. aeruginosa* produced a small decline in claudin-1 abundance ($p < 0.05$; $n = 4$), although this could be overcome by metformin pre-treatment ($p < 0.01$; $n = 4$).

Metformin pre-treated Calu-3 cells exhibited significantly increased occludin abundance by $38 \pm 9\%$ compared to the untreated controls ($p < 0.01$; $n = 7$; Figure 4). *P. aeruginosa* addition resulted in reduced abundance of occludin (65kDa band), as well as the appearance of occludin cleavage fragments consistent with previous studies of *P. aeruginosa*-induced changes in airway epithelia tight junctions [28]. Interestingly, the abundance of the *P. aeruginosa*-induced occludin cleavage fragments (~45kDa and ~35kDa) was significantly reduced by metformin pre-treatment (by $34 \pm 7\%$ and $18 \pm 5\%$, respectively; $p < 0.05$; $n = 4$).

E-cadherin abundance was not significantly altered by either metformin or *P. aeruginosa* addition ($p > 0.05$; $n = 3$; Figure 5).

Discussion

Our study highlights the potential of metformin to reduce hyperglycaemia-induced *P. aeruginosa* growth through modulation of airway epithelial tight junctions. Metformin pre-

treatment of Calu-3 airway epithelial cells reduced hyperglycaemia-induced apical *P. aeruginosa* growth consistent with our previous observations where metformin decreased hyperglycaemia-induced apical *S. aureus* growth on H441 and primary human bronchial epithelial cells (HBEC) [12]. A similar attenuation of *P. aeruginosa*-induced changes in TEER and restricted bacterial growth were observed with the AMPK agonist, AICAR, and metformin failed to significantly alter the bacteria-induced reduction in TEER in the presence of the AMPK inhibitor, compound C. These data therefore indicate a potential AMPK-dependence in metformin's ability to prevent *P. aeruginosa*-induced impairment of airway epithelial barrier function.

Metformin increased occludin and claudin-1 abundance which correlated with increased TEER and reduced paracellular glucose flux across Calu-3 cells. The route by which glucose crosses airway epithelial tight junctions has not been defined. We have shown that, although glucose permeability is related to TEER which is a measurement of ionic permeability it is not a direct linear relationship [8]. Moreover, permeability of ions can be restricted by the presence of specific tight junction proteins which influence charge selectivity of the tight junction [29]. Thus, the alterations in tight junction composition that determine glucose permeability as a larger non-ionic solute are likely to be different to that of ions. Exploring the tight junction proteins modified by metformin to reduce glucose permeability in the presence of bacteria may highlight key proteins involved in regulating paracellular glucose movement.

P. aeruginosa addition reduced the abundance of full length occludin (~65kDa) and produced occludin cleavage products consistent with previous reports [28]. Furthermore, we showed that this was associated with increased glucose permeability confirming previous findings that reduced abundance of occludin increased the permeability of epithelial cells to ethanolamine and mannitol, non-charged solutes of similar size to glucose [30].

Cleavage of occludin was shown to occur via TLR-2-dependent activation of calpains in airway epithelial cell monolayers co-cultured with *P. aeruginosa* strain PA01 [28]. The abundance of cleaved occludin proteins was significantly reduced by metformin, suggesting that metformin may inhibit this pathway. Metformin is known to have anti-inflammatory effects in airway epithelial cells [31] and in a study of 30 diabetic patients, those on metformin therapy exhibited lower blood TLR-2 expression [32]. Calpains are Ca^{2+} -dependent proteases that are ubiquitously expressed in cells and tissues. Knockdown of

calpains 1 and 2 reduced cleavage of occludin in epithelial cells [28]. Currently, there is no evidence for any effect of metformin on calpain activity in airway cells although metformin treatment efficacy in patients has been linked with polymorphisms in the gene for calpain-10 [33].

Claudin-1 abundance was also reduced in the presence of *P. aeruginosa*, which has similarly been shown to be regulated by TLR2-dependent signalling pathways in Calu-3 cells [34]. Claudin-1 is expressed ubiquitously in most epithelial tissues of the body [35], including the airway epithelium and to a lesser extent the alveolar epithelium [36, 37]. *In vitro* studies have shown that claudin-1 acts predominantly as a barrier-forming tight junction protein, with overexpression leading to an increase in TEER [38, 39]. Knockout of claudin-1 in mice leads to loss of the tight junctional barrier to water and macromolecules across the epidermis [40]. Consistent with our findings, downregulation of claudin-1 increased paracellular permeability to non-charged molecules in airway epithelial cells [41].

Interestingly, TLR2 agonists were able to modify claudin-1 expression through atypical PKC ζ [34], which is a known target of AMPK in alveolar epithelial cells [42]. Given that metformin is an AMPK agonist, and similar effects on TEER and bacterial growth were produced by the AMPK agonist AICAR, it is possible that claudin-1 expression is regulated by metformin through AMPK-dependent phosphorylation of PKC ζ .

The changes in abundance shown in this study highlight potential roles for claudin-1 and occludin in the action of metformin to prevent the *P. aeruginosa* induced increase in glucose permeability and hyperglycaemia-induced growth. Although metformin treatment is likely to affect other tight junction proteins, both claudin-1 and occludin could be important targets to reduce glucose permeability across airway epithelia and suppress bacterial growth. Further investigation into the mechanisms regulating metformin and *P. aeruginosa* action on airway epithelial tight junctions are now required. Such studies could yield potential alternatives to antibiotics to prevent/suppress hyperglycaemia-induced respiratory infections.

Acknowledgements

This work was supported by a grant from the Medical Research Council (Grant No: MR/K012770/1) to DLB and a grant from the Newcastle University Wellcome Trust Institutional Strategic Support Fund to JPG.

Conflicts of Interest

The authors confirm that there are no conflicts of interest.

Author contributions

DLB, EHB and JPG designed the research study; WP, GC and JPG performed the research; WP, GC, DLB and JPG analysed the data; DLB and JPG wrote the paper. GC contributed to revision of the manuscript.

References

1. Baker EH, Clark N, Brennan AL, *et al.* Hyperglycemia and cystic fibrosis alter respiratory fluid glucose concentrations estimated by breath condensate analysis. *J Appl Physiol.* 2007; 102: 1969-75.
2. Philips BJ, Meguer JX, Redman J, Baker EH. Factors determining the appearance of glucose in upper and lower respiratory tract secretions. *Intensive Care Med.* 2003; 29: 2204-10.
3. Lee RJ, Kofonow JM, Rosen PL, *et al.* Bitter and sweet taste receptors regulate human upper respiratory innate immunity. *J Clin Invest.* 2014; 124: 1393-405.
4. Baker EH, Janaway CH, Philips BJ, *et al.* Hyperglycaemia is associated with poor outcomes in patients admitted to hospital with acute exacerbations of chronic obstructive pulmonary disease. *Thorax.* 2006; 61: 284-9.
5. Wood DM, Brennan AL, Philips BJ, Baker EH. Effect of hyperglycaemia on glucose concentration of human nasal secretions. *Clin Sci.* 2004; 106: 527-33.
6. Brennan AL, Gyi KM, Wood DM, *et al.* Airway glucose concentrations and effect on growth of respiratory pathogens in cystic fibrosis. *J Cyst Fibros.* 2007; 6: 101-109.
7. Philips BJ, Redman J, Brennan A, *et al.* Glucose in bronchial aspirates increases the risk of respiratory MRSA in intubated patients. *Thorax.* 2005; 60: 761-4.

8. Kalsi KK, Baker EH, Fraser O, *et al.* Glucose homeostasis across human airway epithelial cell monolayers: role of diffusion, transport and metabolism. *Pflugers Arch.* 2009; 457: 1061-70.
9. Garnett JP, Nguyen TT, Moffatt JD, *et al.* Proinflammatory mediators disrupt glucose homeostasis in airway surface liquid. *J Immunol.* 2012; 189: 373-80.
10. Garnett JP, Baker EH, Baines DL. Sweet talk - insights into the nature & importance of glucose transport in lung epithelium. *Eur Respir J.* 2012; 40: 1269-76.
11. Garnett JP, Braun D, McCarthy AJ, *et al.* Fructose transport-deficient *Staphylococcus aureus* reveals important role of epithelial glucose transporters in limiting sugar-driven bacterial growth in airway surface liquid. *Cell Mol Life Sci.* 2014; 71: 4665-73.
12. Garnett JP, Baker EH, Naik S, *et al.* Metformin reduces airway glucose permeability and hyperglycaemia-induced *Staphylococcus aureus* load independently of effects on blood glucose. *Thorax.* 2013; 68: 835-45.
13. Garnett JP, Gray MA, Tarran R, *et al.* Elevated paracellular glucose flux across cystic fibrosis airway epithelial monolayers is an important factor for *Pseudomonas aeruginosa* growth. *PLoS One.* 2013; 8(10): e76283.
14. Shen BQ, Finkbeiner WE, Wine JJ, *et al.* Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl⁻ secretion. *Am J Physiol.* 1994; 266(5 Pt 1): L493-501.
15. Kreda SM, Okada SF, van Heusden CA, *et al.* Coordinated release of nucleotides and mucin from human airway epithelial Calu-3 cells. *J Physiol.* 2007; 584(Pt 1): 245-59.
16. Joo NS, Lee DJ, Wings KM, *et al.* Regulation of antiprotease and antimicrobial protein secretion by airway submucosal gland serous cells. *J Biol Chem.* 2004; 279(37): 38854-60.
17. Grainger CI, Greenwell LL, Lockley DJ, *et al.* Culture of Calu-3 cells at the air interface provides a representative model of the airway epithelial barrier. *Pharm Res.* 2006; 23(7): 1482-90.
18. Wan H, Winton HL, Soeller C, *et al.* Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o-. *Eur Respir J.* 2000; 15(6): 1058-68.

19. Garnett JP, Hickman E, Burrows R, *et al.* Novel role for pendrin in orchestrating bicarbonate secretion in cystic fibrosis transmembrane conductance regulator (CFTR)-expressing airway serous cells. *J Biol Chem.* 2011; 286: 41069-82.
20. Salomon JJ, Endter S, Tachon G, *et al.* Transport of the fluorescent organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium iodide (ASP+) in human respiratory epithelial cells. *Eur J Pharm Biopharm.* 2012; 81(2): 351-9.
21. Ingoglia F, Visigalli R, Rotoli BM, *et al.* Functional characterization of the organic cation transporters (OCTs) in human airway pulmonary epithelial cells. *Biochim Biophys Acta.* 2015; 1848(7): 1563-72.
22. Chen L, Pawlikowski B, Schlessinger A, *et al.* Role of organic cation transporter 3 (SLC22A3) and its missense variants in the pharmacologic action of metformin. *Pharmacogenet Genomics.* 2010; 20(11): 687-99.
23. Sum CF, Webster JM, Johnson AB, *et al.* The effect of intravenous metformin on glucose metabolism during hyperglycaemia in type 2 diabetes. *Diabet Med.* 1992; 9: 61-5.
24. Radwan MA, Al Taweel ES, Al-Moghairi AM, *et al.* Monitoring metformin in cardiac patients exposed to contrast media using ultra-high-performance liquid chromatography tandem mass-spectrometry. *Ther Drug Monit.* 2011; 33: 742-9.
25. Zhou G, Myers R, Li Y, *et al.* Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest.* 2001; 108(8): 1167-74.
26. Zhang L, Li J, Young LH, Caplan MJ. AMP-activated protein kinase regulates the assembly of epithelial tight junctions. *Proc Natl Acad Sci U S A.* 2006; 103(46): 17272-7.
27. Zheng B, Cantley LC. Regulation of epithelial tight junction assembly and disassembly by AMP-activated protein kinase. *Proc Natl Acad Sci U S A.* 2007; 104(3): 819-22.
28. Chun J, Prince A. TLR2-induced calpain cleavage of epithelial junctional proteins facilitates leukocyte transmigration. *Cell Host Microbe.* 2009; 5: 47-58.
29. Anderson JM, Van Itallie CM. Physiology and function of the tight junction. *Cold Spring Harb Perspect Biol.* 2009; 1: a002584.

30. Yu AS, McCarthy KM, Francis SA, *et al.* Knockdown of occludin expression leads to diverse phenotypic alterations in epithelial cells. *Am J Physiol Cell Physiol.* 2005; 288: C1231-41.
31. Myerburg MM, King JD Jr, Oyster NM, *et al.* AMPK agonists ameliorate sodium and fluid transport and inflammation in cystic fibrosis airway epithelial cells. *Am J Respir Cell Mol Biol.* 2010; 42: 676-84.
32. Andrews M, Soto N, Arredondo M. Effect of metformin on the expression of tumor necrosis factor- α , Toll like receptors 2/4 and C reactive protein in obese type-2 diabetic patients. *Rev Med Chil.* 2012; 140: 1377-82.
33. Tkáč I, Javorský M, Klimčáková L, *et al.* A pharmacogenetic association between a variation in calpain 10 (CAPN10) gene and the response to metformin treatment in patients with type 2 diabetes. *Eur J Clin Pharmacol.* 2015; 71: 59-63.
34. Ragupathy S, Esmaili F, Paschoud S, *et al.* Toll-like receptor 2 regulates the barrier function of human bronchial epithelial monolayers through atypical protein kinase C zeta, and an increase in expression of claudin-1. *Tissue Barriers.* 2014; 2: e29166.
35. Furuse M, Fujita K, Hiiragi T, *et al.* Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol.* 1998; 141: 1539–50.
36. Chen SP, Zhou B, Willis BC, *et al.* Effects of transdifferentiation and EGF on claudin isoform expression in alveolar epithelial cells. *J Appl Physiol.* 2005; 98: 322–328.
37. Wang F, Daugherty B, Keise LL, *et al.* Heterogeneity of claudin expression by alveolar epithelial cells. *Am J Respir Cell Mol Biol.* 2003; 29: 62–70.
38. Inai T, Kobayashi J, Shibata Y. Claudin-1 contributes to the epithelial barrier function in MDCK cells. *Eur J Cell Biol.* 1999; 78: 849–855.
39. McCarthy KM, Francis SA, McCormack JM, *et al.* Inducible expression of claudin-1-myc but not occludin-VSV-G results in aberrant tight junction strand formation in MDCK cells. *J Cell Sci.* 2000; 113: 3387–98.
40. Furuse M, Hata M, Furuse K, *et al.* Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol.* 2002; 156: 1099–1111.

41. Gan H, Wang G, Hao Q, *et al.* Protein kinase D promotes airway epithelial barrier dysfunction and permeability through down-regulation of claudin-1. *J Biol Chem.* 2013; 288: 37343-54.

42. Gusarova GA, Dada LA, Kelly AM, *et al.* Alpha1-AMP-activated protein kinase regulates hypoxia-induced Na,K-ATPase endocytosis via direct phosphorylation of protein kinase C zeta. *Mol Cell Biol.* 2009; 29: 3455-64.

Figure Legends

Figure 1. Metformin and AMPK agonist AICAR inhibits hyperglycaemia-induced *P. aeruginosa* growth across Calu-3 airway epithelial monolayers. **A:** Airway epithelia–bacteria co-cultures grown in the presence of 5 or 15mM basolateral glucose, without (grey bars) or with pre-treatment with 1 mM metformin (MF; 18 hour pre-treatment; black bars). Bacterial CFU were measured 7 h after infection (percentage compared with mean growth in the presence of 5 mM basolateral glucose control). n=4. *p<0.05. **B:** Airway epithelia–bacteria co-cultures grown in the presence of 15mM basolateral glucose, without (grey bars) or with pre-treatment with metformin (MF; 20 uM or 1 mM, 18 hour pre-treatment; black bars) or AICAR (0.5 mM, 18 hour pre-treatment; hatched bars). Bacterial CFU were measured 7 h after infection (percentage compared with mean growth in the presence of 15 mM basolateral glucose control). n=6. *p<0.05, **p<0.01 compared to 15 mM basolateral glucose control.

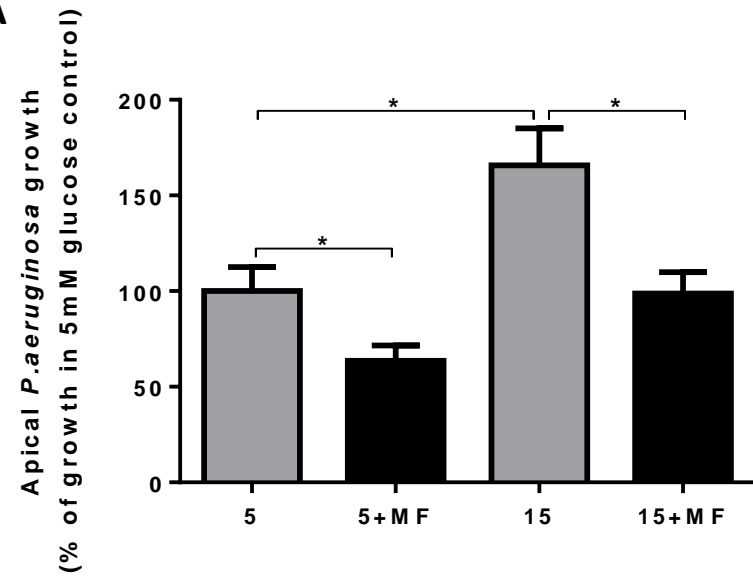
Figure 2. Metformin prevents the effect of *P. aeruginosa* infection on transepithelial resistance and paracellular glucose flux across the airway epithelium. **A:** The effect of metformin pre-treatment (MF; 1 mM, 18 hours prior to addition of *P. aeruginosa*) on Calu-3 monolayer transepithelial electrical resistance (TEER; $\Omega \cdot \text{cm}^2$), in the presence and absence of *P. aeruginosa* (7 hour co-culture), under normoglycaemic conditions (5 mM basolateral glucose). n=5. *p<0.05, **p<0.01. **B:** The effect of metformin (MF; 20 μM or 1 mM, 18 hour pre-treatment), AICAR (0.5 mM, 18 hour pre-treatment) and AMPK inhibitor compound C (CC; 80 μM pre-treatment for 1 hour prior to addition of metformin) on Calu-3 monolayer TEER (percentage compared with mean TEER in uninfected control Calu-3 cultures) in co-culture with *P. aeruginosa* (7 hour co-culture), under normoglycaemic conditions (5 mM

basolateral glucose). n=6. *p<0.05, **p<0.01. **C:** Paracellular glucose flux across uninfected and *P. aeruginosa* infected Calu-3 monolayers, with and without pre-treatment with 1 mM metformin. Paracellular flux measured by adding radiolabelled L-glucose to the basolateral surface and monitoring its appearance at the apical surface. n=4. *p<0.05.

Figure 3. Metformin attenuates *Pseudomonas aeruginosa*-induced cleavage of epithelial occludin and increases claudin-1 abundance. Calu-3 airway epithelial monolayers with and without 1 mM metformin pre-treatment (18 hours), were infected with PA01 for 7 hours, in the presence of 5mM basolateral glucose. **A:** Claudin-1 western blot band densities normalised against β -actin control and expressed as comparison to untreated Calu-3 control lysates. n=4. *p<0.05, **p<0.01. **B:** Occludin western blot band (65kDa) densities normalised against β -actin control and expressed as comparison to untreated Calu-3 control lysates. n=7. *p<0.05, **p<0.01. Arrows indicate ~35kDa and 45kDa occludin cleavage fragments. **C:** E-cadherin western blot band densities normalised against β -actin control and expressed as comparison to untreated Calu-3 control lysates. n=3.

Figure 1

A



B

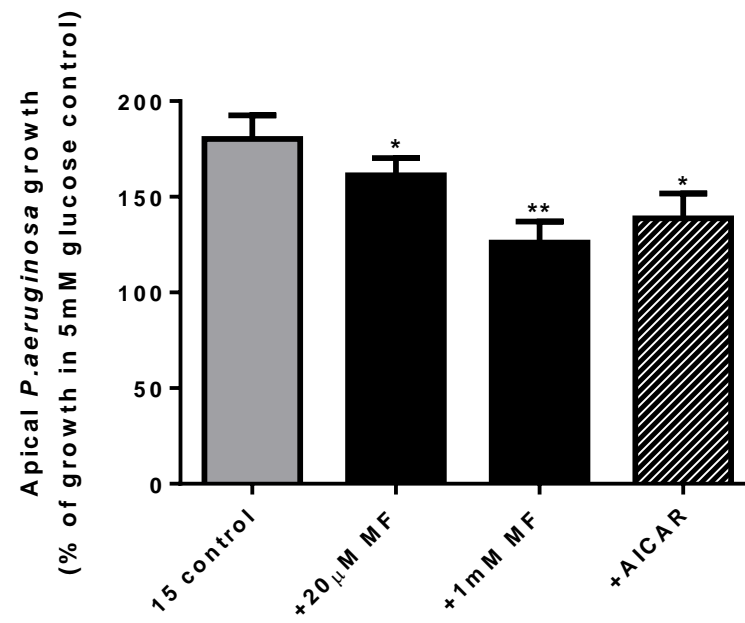


Figure 2

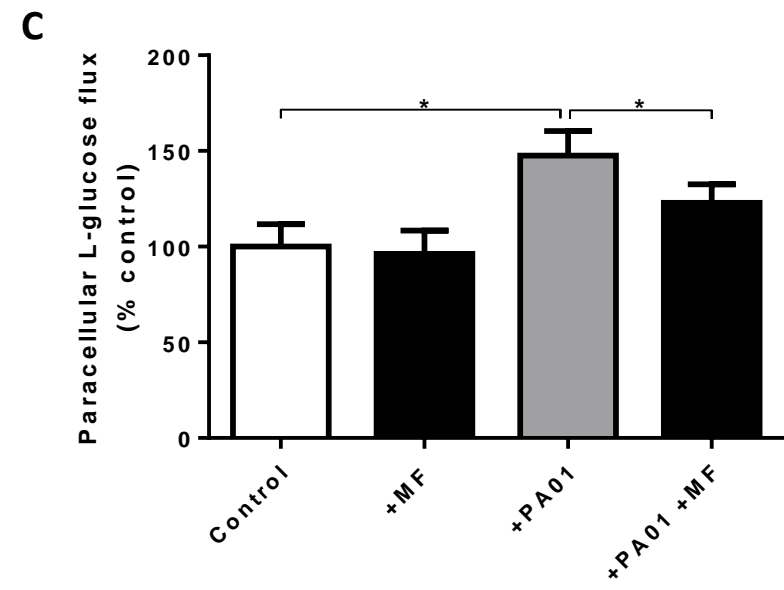
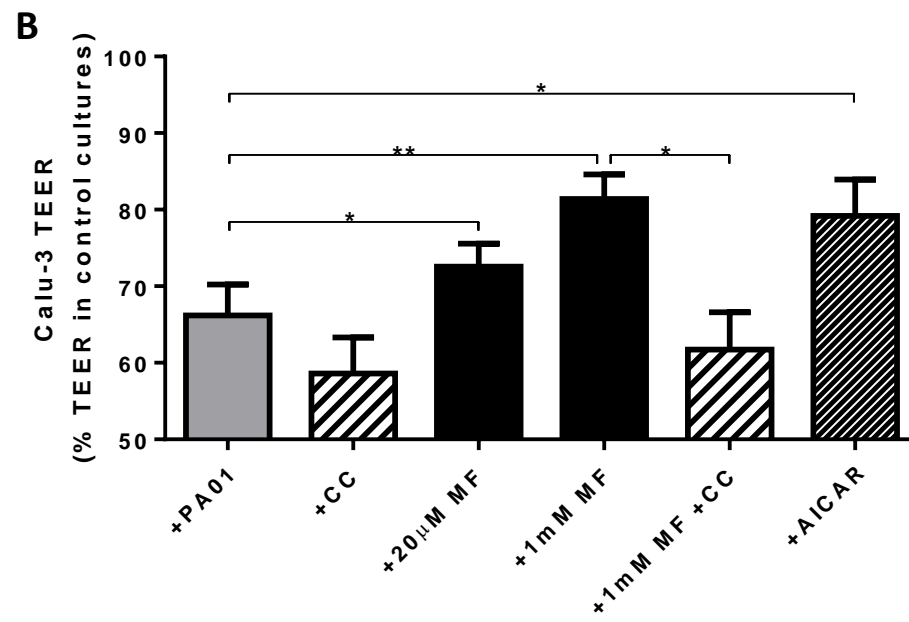
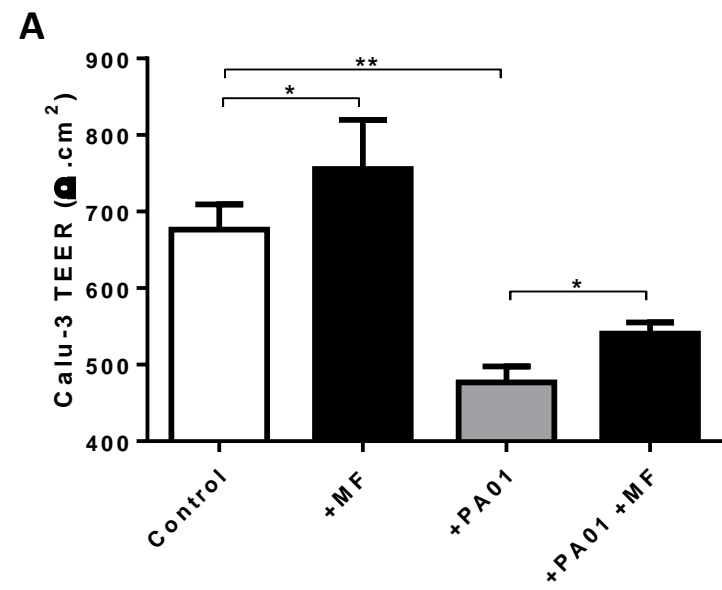
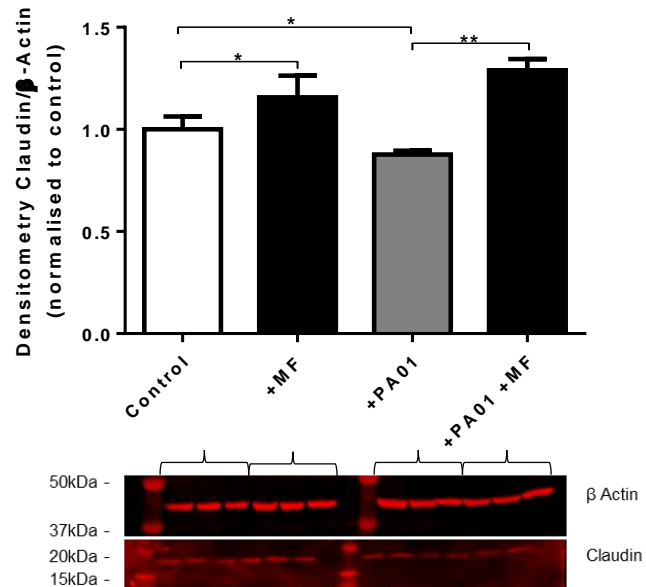
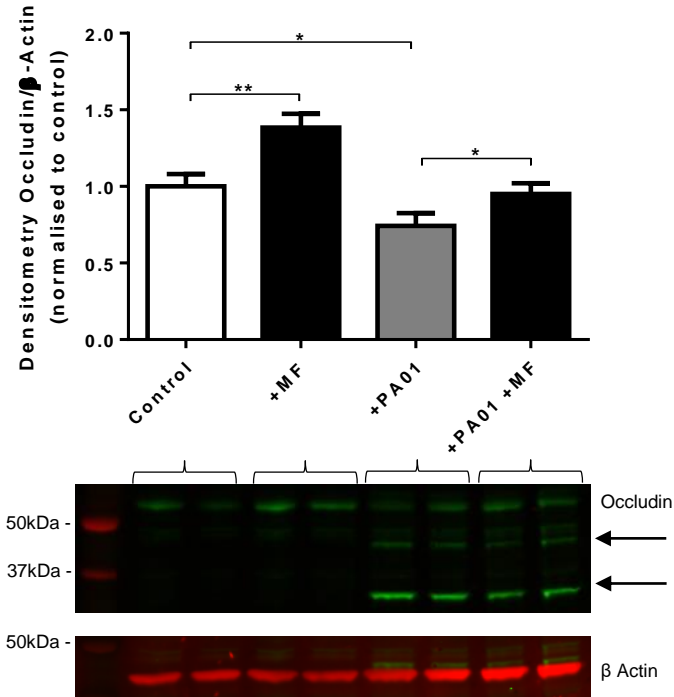


Figure 3

A



B



C

