Pulmonary Pharmacology & Therapeutics 40 (2016) 30–38

Contents lists available at ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

Effect of polyunsaturated fatty acids (PUFAs) on airway epithelial cells' tight junction

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ARTICLE INFO

Article history:
Received 19 May 2016
Received in revised form
21 June 2016
Accepted 20 July 2016
Available online 22 July 2016

Keywords:
Airway tight junctions
PUFA
Polyunsaturated fatty acids

1. Introduction

Airway epithelial cells form a barrier against the environment, but are also required for the regulated exchange of molecules between the body and its surroundings. Epithelial cells are characterised by a polarization of their plasma membrane, evidenced by the appearance of structurally, compositionally, and functionally distinct surface domains [1]. One of these domains is tight junction (TJ) that restricts paracellular movement of solutes and macromolecules across epithelia. Tight junctions are located in the apical region of the lateral plasma membrane in epithelial tissue layers. The essential transmembrane proteins of the TJ are claudin, junction adhesion proteins, as well as cytoplasmic proteins like, ZO-1, and cingulin [2]. Three main functions have been related to TJs. These functions are 1) regulation of the passage of ions, water, and molecules through the paracellular pathway [3]; 2) limiting the diffusion of proteins and lipids between the apical and basolateral domains of the plasma membrane [4]; and 3) recruiting cytoskeletal and signalling molecules involved in cell polarity, growth, differentiation and apoptosis [2,5].

Tight junction transmembrane proteins are located in detergent-insoluble glycolipid (DIG) rafts in the cell membrane [6]. It has also been shown that TJ proteins are in cholesterol-enriched micro-domains and a rapid reduction of membrane cholesterol by methyl-b-cyclodextrin decreases transepithelial electrical resistance (TEER) and enhances mannitol paracellular transport, modifying tight junctions integrity [7,8]. Since TJ proteins are embedded within the phospholipid bilayer of the membrane, a change in the structure of cell membrane can affect the structure and function of these transmembrane proteins. It has been shown that PUFAs may integrate into phospholipids and modify the physical properties of cell membranes, thus increasing membrane fluidity [9,10]. Immunofluorescence staining of embedded proteins in the cell membrane has shown there are structural changes in the TJ-related proteins after treating cells with the fatty acids [11]. Among unsaturated fatty acids, PUFAs have the potential to modulate TJ and enhance the permeation of drugs through the intestinal epithelial cell barrier via the paracellular route [12–14]. This effect can be attributed to their interaction with lipid rafts and lipid raft-mediated signalling [10,15]. Specifically PUFAs can change the membrane's biophysical property [16] and membrane's morphology and fluidity [17]. Membrane fluidity plays an important role in cellular functions since the surrounding lipid environment modulates the activity of membrane proteins, such as TJ proteins. For instance, it was shown that arachidonic acid affects the cell membrane of Calu-3 lung epithelia cells, inducing changes in the morphology of the membrane and its fluidity, leading to changes in the paracellular transport of fluorescein sodium (Na-flu) [18]. Therefore, it can be assumed that PUFAs can alter TJs by perturbing the lipid environment in which TJ proteins are located. PUFAs are unsaturated fatty acids with more than one double bond in their structure and have been classified in different groups by their chemical structure. Among PUFAs, omega-3 and 6 are the most commonly considered essential fatty acids [19]. PUFAs not only have shown TJ modulatory effect but have also been shown to

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http://dx.doi.org/10.1016/j.pupt.2016.07.004
1094-5539/

modulate mucus production and immunomodulatory processes. For example, *in vivo* [20] and *in vitro* [21] studies have shown an inverse association between PUFA and mucus hypersecretion. Mucus is a viscoelastic and adhesive gel that protects the lung airways. Most foreign particulates, including conventional inhaled drug delivery systems, are trapped in mucus. Trapped inhaled particles are eliminated from the lung within seconds to a few hours depending on the anatomical location, thereby strongly hindering the duration of drug delivery [22]. Immunomodulatory effects of PUFAs have also been demonstrated in many experimental and clinical studies where polyunsaturated fatty acids, especially n-3 PUFAs, exert immunosuppressive effects making them potential therapeutic agents for inflammatory and autoimmune diseases [23]. The n-3 fatty acids, particularly EPA (Eicosapentaenoic acid) and to a lesser extent DHA (docosahexaenoic acid), are capable of competing with arachidonic acid to produce eicosanoids with beneficial effects on inflammation [24].

While the effect of PUFAs on intestinal tight junctions has been investigated previously [12,13], the effect on pulmonary tight junctions has, to-date, not been explored. As such, in this study the *in vitro* effect of PUFAs, specifically docosahexaenoic acid (DHA)-22:6 (n-3) (an omega 3 fatty acid), linoleic acid (LA)-18:2 (n-6), arachidonic acid (AA) −20:4 (n-6), and gamma-linoleic acids (GLA)-18:3 (n-6) (all omega 6 fatty acids) and palmitoleic acid (PA)-16:1 (n-9) (a monounsaturated fatty acid with one double bond) on airway epithelial cells (Calu-3 cells) with regards to their tight junction modulatory effect, mucus production and inflammatory response was investigated. In addition, the impact of PUFAs on the bulk rheological properties of artificial mucus was investigated since these long chain molecules have surfactant-like properties that could potentially alter surface tension and thus viscoelastic response.

2. Materials and methods

Palmitoleic acid (PA) ≥ 98.5%, linoleic acid (LA) ≥ 99%, gamma-linoleic acid (GLA) ≥ 99%, arachidonic acid (AA) ≥ 99%, and docosahexaenoic acid (DHA) ≥ 98% were all purchased from (Sigma-Aldrich- Australia) and used as supplied. A stock solution of 10 mM of each unsaturated fatty acid was prepared in 100% ethanol and maintained in the dark at −20 °C. Samples were freshly dissolved in complete medium to the required concentration prior to use. Controls were run in parallel and contained the same percentage of ethanol used for the sample experiments.

An artificial mucus (AM) model was modified from a previously published mucus model [25]. Artificial mucus was prepared by dissolving 500 mg of deoxyribonucleic acid sodium salt from salmon testes (DNA) (Sigma Aldrich-Australia) in 10 ml water and addition of 11.3 ml of an autoclaved solution containing 300 mg of mucin from procine stomach type III (Sigma Aldrich-Australia). Then 250 mg NaCl, 110 mg KCl, 0.295 mg diethylenetriaminepentaacetic acid (DTPA), 12.5 mg amino acids mixture (amino acids kit) (all purchased from Sigma Aldrich-Australia) were mixed with the former solution and finally complemented with 250 μl of sterile egg yolk emulsion (Thermofisher Scientific-Australia).

2.1. Cell culture

Calu-3 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's medium/F-12 (Gibco-Australia), 1% non-essential amino acids (Sigma Aldrich-Australia), and 1% L-glutamine solution (Sigma Aldrich-Australia). Cells were maintained in humidified atmosphere of 5% CO_2/95% air at 37 °C. The medium was replaced three times a week and cells were passaged at a ratio of 1:3. For TEER measurement, paracellular permeability and mucus secretion experiments, Calu-3 cells were seeded at an initial density of 1.65×10^5 cells/insert on Transwell® polyester inserts (pore size 0.4 μm, surface area 0.33 cm^2, Corning-Australia). After 24 h, an air-liquid interface (ALI) model was created by aspirating the apical medium and the cells were maintained with 0.5 ml of culture medium in the basolateral chamber. The ALI conditions stimulated differentiation of the cell layer to form a polarized and bio-electrically tight epithelial layer [26].

2.1.1. Cytotoxicity assay

To evaluate the cytotoxicity of the PUFAs, a range of concentration (between 100 nM and 1 M) for each PUFA was prepared aseptically in complete cell culture medium and tested on Calu-3 cells. Cytotoxicity was determined by MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega). Calu-3 cells were seeded into 96-well plate at a density of 5×10^4 cells/well and incubated overnight. On the second day, the medium was replaced with 200 μl of pre-warmed PUFAs solution. After 24 h of incubation with PUFAs, the cells were incubated with 20 μl of MTS solution for another 2 h. After incubation, the plate was read at 490 nm using a SpectraMax plate reader for optical density that is directly correlated with cytotoxicity of treatments and expressed as the IC_{50} (50% inhibitory concentration) of each treatment. Considering the viability concentration range of each PUFA from the MTS assay, the experimental concentration of 100 μM was selected.

The LDH (lactate dehydrogenase) assay measures the cell membrane damage via released LDH into the cell culture media. Extracellular LDH in the media can be quantified by a coupled enzymatic reaction in which LDH catalyses the conversion of lactate to pyruvate [27]. LDH release measurements were performed using a commercial LDH assay kit (RayBio LDH-Cytotoxicity) to monitor cell membrane damage of each PUFA with the selected concentration (100 μM). Calu-3 cells were seeded into a 96-well plate at a density of 5×10^4 cells per well overnight. Then cells were incubated to each PUFA (100 μM) for 24 h, then 100 μl of the cell culture supernatants were withdrawn and transferred to a new well plate followed by addition of 100 μl of reaction mixture from LDH kit. The absorbance was measured with SpectraMax plate reader at 490–500 nm. HBSS and 1% (v/v) Triton®X-100 were used as negative and positive controls, respectively. Cytotoxicity was calculated according the following equation and presented as the percentage of total LDH leakage of each treatment compared to the positive control:

$$\text{Total LDH leakage (\%)} = [(\text{Test sample} - \text{Negative control}) / (\text{Positive control} - \text{Negative control})] \times 100$$

2.1.2. Cell viability assay

Cell viability assays of Calu-3 after incubation with each PUFA was performed using trypan blue exclusion test. Calu-3 cells were grown to confluence on 60 mm culture dishes. Then Cells were treated with 100 μM of each PUFA for 24 h. Subsequently, the cells were rinsed in Hanks Balance Salt Solution (HBSS, Gibco Invitrogen, USA), trypsinised, centrifuged and resuspended in 1 ml of medium. Then, 100 μl trypan blue (0.4%) and 500 μl cell solution was added to a centrifuge tube and mixed thoroughly. Solution was allowed to incubate at room temperature for 5 min. Viable and non-viable cells were counted using a hemocytometer. Complete medium and 1% (v/v) Triton®X-100 were used as negative and positive controls, respectively.

2.2. Measurement of transepithelial resistance (TEER)

2.2.1. Ohmmeter

The TEER of Calu-3 cells cultured in Transwells was monitored using an ohmmeter (EVOM; World Precision Instruments, Sarasota, FL) with chop-stick electrodes. HBSS was added to the apical surface and basolateral compartments and resistance measured between electrodes before and 1 h after cells treatment with each PUFA. Resistance was normalised by subtracting the blank inserts and multiplied by the area of the Transwell inserts.

2.2.2. Electric cell-substrate impedance sensing (ECIS)

In addition, epithelial barrier function was also measured using the electric cell-substrate impedance sensing system (ECIS; Applied BioPhysics, Troy, NY). This technique records transepithelial resistance changes in real time. Both TEER and ECIS are complimentary techniques since the former is capable of studying air interface cultures grown in Transwells while the later studies liquid covered cultures in real-time. Transepithelial impedance was measured every 5 min over the course of the experiment. Calu-3 cells were seeded at 5×10^5 cells per well in collagen coated 8EW10 + arrays (8W10E + Applied Biophysics, Troy, NY) and allowed to grow for 24 h, until confluent. Confluent Calu-3 cells were incubated with each PUFA (100 μM) until the resistance was stable. Baseline values were established with culture media alone and compared to electrodes covered with a monolayer of cells. Three separate experiments were run for each PUFA, data analysed using ECIS software and resistance normalized by dividing the resistance values from electrodes confluent with cells by the corresponding quantities for the cell-free electrodes using ECIS software [28,29].

2.3. Transepithelial cell permeability

Permeation of fluorescein sodium (Na-flu), a marker for paracellular transport, across Calu-3 cells was measured after treatment with PUFAs to evaluate barrier integrity and paracellular transport of Na-flu. Briefly, Calu-3 cell layer was treated with 100 μM of each PUFA for 1 h in the incubator. Then inserts washed three times with HBSS, followed by adding 250 μl of 2.5 mg/ml Na-flu solution on the apical chamber, while 600 μl of HBSS were added into the basolateral chamber. At pre-determined time points up to 4 h, 100 μl samples were taken from the basolateral chamber and subsequently replaced with fresh buffer to maintain sink conditions. Samples were placed in a black, 96 well plate and fluorescence reading was subsequently measured using Spectromax plate reader with excitation and emission wavelengths settings of 485 and 520 nm, respectively. The apparent permeability coefficient (Papp) of Na-flu across the epithelial cells was calculated using this equation: $Papp = (V/AC_0)(dC/dt)$, where V is the volume of solution in the basolateral chamber, A is the surface area of the Transwell membrane (cm^2), C_0 is the initial concentration in the apical chamber (μg/mL) and dC/dt is the flux (rate of change in cumulative mass transport) of Na-flu.

2.4. Pro-inflammatory marker expressions

The expression of cytokines IL-6 (Interleukin-6), IL-8 (Interleukin 8) and TNF (Tumour necrosis factor) in Calu-3 cells treated by PUFAs were evaluated. Briefly, 5×10^5 cells were seeded in 6 well plates and incubated with 100 μM of each PUFA. After 24 h of adding PUFAs 1 ml of medium (cell-free) were collected and kept at −80 °C until cytokine assays were conducted. This supernatant was used to measure all the markers. Cells induced with 5 μg/ml LPS (lipopolysaccharide) as positive control and cells without any treatment were expressed as negative control. Finally, IL-8, IL-6 and TNF markers were measured using enzyme immunoassay kits (BD Bioscience, Australia) according to the manufacturer's protocol.

2.5. Immunofluorescence staining

To investigate the effect of PUFAs on the structure of TJ related proteins in Calu-3 cells, Zona occluden-1 (ZO-1) which is one of the main TJ related proteins from the family of occludins, was labelled and visualised with confocal microscopy. In brief, Calu-3 cells were plated in sterile 8 chamber glass slide (Nunc™ Lab-Tek® Chamber Slides) with the density of 50×10^4 cells per well and incubated overnight with one of the PUFAs (100 μM). After treatment for 24 h cells were rinsed with PBS, fixed in 4% paraformaldehyde for 20 min, permeabilized and blocked for 15 min (0.1% Triton X-100, 1% BSA). Then primary antibody (rabbit anti-ZO-1- Abcam) was added and incubated for 1 h at room temperature. Cells were rinsed in 2% BSA/PBS solution three times and then incubated with secondary antibody (Alexa flour 488, goat and rabbit IgG; Invitrogen by Life Technologies) for 30 min at room temperature in the dark. Then cells were counterstained with DAPI for 30 min. Following incubation, coverslips were rinsed in PBS and mounted using ProLong gold (Invitrogen - Life Technologies). Imaging was performed using an Olympus confocal microscope (Olympus IX71).

2.6. Effect of PUFAs on airway mucus production

Previous *in vivo* [20] and *in vitro* [21] studies showed an inverse association between PUFA and mucus hypersecretion. Therefore, an *in vitro* experiment was conducted to investigate the effect of PUFAs on mucus secretion by Calu-3 cells. In brief, Calu-3 cells were cultured using the ALI model allowing cells differentiation until day 11. Then PUFAs were added to the basolateral chamber to a concentration of 100 μM. Cells were incubated for 4 days and then stained for mucus secretion using alcian blue as previously described [30]. This was performed to allow the visualisation of mucus on the surface of Calu-3 cells and results compared with untreated cells (control). Cells were subsequently stained for mucus using alcian blue (1% w/v alcian blue in 3% v/v acetic acid/water at pH 2.5) (Fronine Laboratory, Sydney, Australia) followed by washing until the rinsate ran clear. Afterwards, inserts were air dried and the filter membrane cut and mounted onto glass slides using mounting agent (Glycerol). The slides were imaged using an Olympus BX60 microscope (Olympus, Hamburg, Germany) attached to a DP71 camera (Olympus). The images were post processed using Apple Automater (v 2.0.4 Apple Inc, California, USA) to centre crop 400 × 300 pixel JPEG images. Each image was analysed using Image J (v1.42q, NIH) with colour profile (Dimiter Prodanov; Leiden University Medical Centre, Netherlands) and colour inspector 3D v2.0 (Kai Uwe Barthel; Internationale, Medien informatik, Berlin, Germany) plugins. A three-dimensional colour space was produced representing the 8-bit red-green-blue (RGB) value of each image. The ratio of blue (RGB_b) which is a semi-quantitative scale of mucus secretion was calculated by dividing the average RGB_b by the sum of the RGB values ($RGB_r + RGB_g + RGB_b$) for each image.

2.7. Rheological analysis

Effect of PUFAs on the rheological property of artificial pulmonary mucus was assessed using rheometry (Advanced Rheometer 2000, TA Instruments; New Castle, DE). Rheological properties of AM were measured as previously described [31]. In brief, 100 μl of AM was incubated for 10 min with each PUFA at room temperature according to previous publication [32]. Each experiment was conducted on 23 μl aliquot of incubated AM with PUFA using the

rheometer equipped with 20 mm, 0.5° aluminium cone and plate with a gap width of 19 μm at room temperature. Stress sweep test was performed to determine the linear viscoelastic region (LVR) across a range of oscillatory stresses (0.1–1000 Pa). A frequency sweep (0.1–10 Hz) was performed at 0.2 Pa within the LVR where dynamic elasticity (G′) and viscosity (G″) were determined.

2.8. Statistics

Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA) version 6.07 for windows by one-way analysis of variance (ANOVA), followed by Tukey post hoc analysis for multiple comparisons. Values are presented as mean ± SD of three independent experiments (n = 3) ($^{**}P < 0.01$ and $^{***}P < 0.001$).

3. Results and discussion

3.1. Cytotoxicity assay

The cytotoxicity of each PUFA on Calu-3 cells was assessed via changes in cell metabolic activity (MTS assay) and cell membrane damage (LDH assay). Results obtained from MTS assay are expressed as IC 50 for each PUFA and were 490, 440, 400, 390, 240 μM for AA, GLA, DHA, LA and PA, respectively. Consequently, 100 μM of each PUFA was selected for the study since this concentration was lower than the measured IC50 of each PUFA on Calu-3 cells. A study by Roig-Pérez et al., also reported that 100 μM DHA was the lowest concentration that showed significant changes in paracellular permeability assessed via TEER and d-mannitol flux in Caco-2 cells [33].

Subsequently, the viability of Calu-3 cells was tested in the presence of 100 μM PUFAs for 24 h (Fig. 1A). In general, the Calu-3 cells were viable up to 70% of the non-treated control cells at the selected concentration studied. The LDH leakage of each PUFA was measured and data presented in Fig. 1B. The effect of selected concentration (100 μM) on cell membrane damage was also investigated. The LDH leakage showed that Calu-3 cells generated an amount of LDH significantly higher ($P < 0.001$) in the presence of AA, compared to the other PUFAs. In general, the effect of AA on high LDH leakage can be related to the fact that PGE2 (prostaglandin E2), a cyclooxygenase, is a metabolite of AA which is a potent mediator of inflammation [34]. Therefore, PGE2 can cause membrane damage and increase LDH leakage. All other PUFAs did not show high LDH release.

3.2. TEER and Na-flu permeability assessment on Calu-3 after incubation with PUFAs

PUFAs exerted effects on tight junctions by changing the paracellular permeability as measured by Na-flu transport and TEER. Permeability increased in all PUFA treated cells (Fig. 2A). The increase was prominently higher with GLA and PA treatments (8.5×10^{-6} cm/s) compared to the control with the Papp increasing 1.5 times over control. This increase in Papp mirrored the concurrent drop in TEER to 198 ± 12.7 Ωcm^2 in Calu-3 cells treated with GLA (Fig. 2B). The permeability of Na-flu in untreated samples (control) was 8.5×10^{-7} cm/s, and the normalized TEER value was 310 ± 15 Ωcm^2. Results for control samples were consistent with findings by Grainger et al. [35]. Resistance dropped significantly ($P < 0.001$) after incubating Calu-3 cells with PUFAs compared to non-treated control cells and permeability increased following the rank order: GLA ≥ PA > DHA > AA > LA.

While there are no specific studies focussing on lung epithelia permeability with respect to PUFAs, our findings can be compared to previous studies focussing on intestinal epithelia (since this is a natural barrier to the adsorption of orally ingested therapeutics). For example, there are a handful of *in vivo* [36,37] and *in vitro* [37–39] studies on the effect of omega 3 and omega 6 PUFAs on intestinal tight junctions. These studies demonstrated similar findings. Usami et al. reported that a 24-hr treatment of Caco-2 cells with eicosapentaenoic acid (EPA - 20:5 (n-3)) or alpha linoleic acid (ALA- 18:3 (n-3)) enhanced tight junction permeability [40]. The same research group also investigated the effect of PUFA on paracellular permeability of fluorescein sulfonic acid across Caco-2 cell monolayer and it was demonstrated that GLA and DHA enhanced fluorescein sulfonic acid permeability, and lowered TEER, compared to non-treated samples in a concentration-dependent manner without cell injury [41]. Another study also reported that high concentrations of DHA, i.e. 5–30 mM for 90 min, decreased TEER and increased paracellular transport of ^{14}C-mannitol [42]. The investigations on the effect of PUFAs on tight junctions are not only limited to intestinal tight junctions; other tight junction forming cells have also been studied. For instance, in a study on hepatic tight junctions, the effect of n-3 PUFAs resulted in structural change in TJ proteins expression and localisation [43]. The levels of tight junction occludin decreased significantly, whereas claudin-3 and ZO-1 levels increased 2- or 3-fold over control levels [43]. In a study on

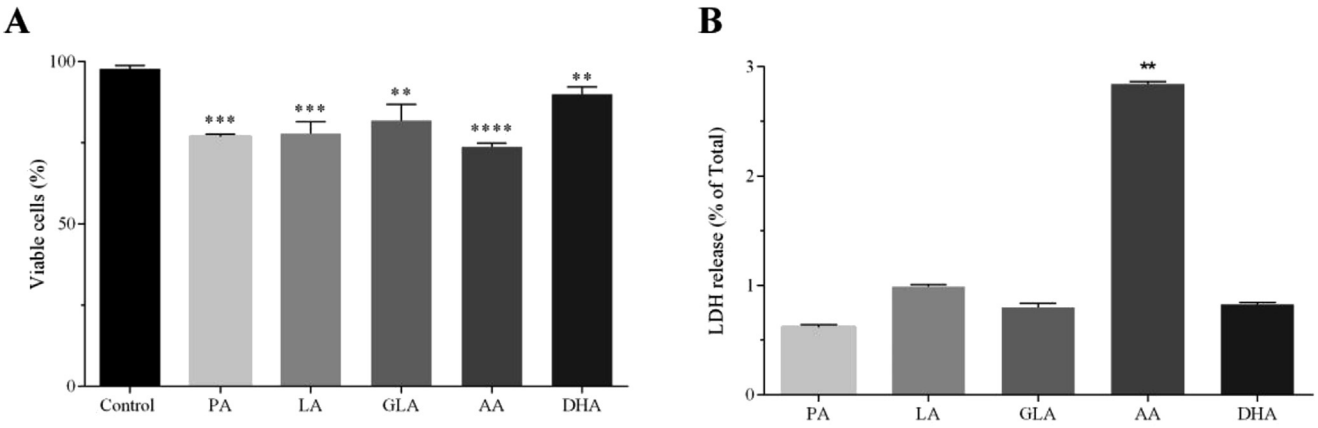

Fig. 1. A- Viability of Calu-3 cells after incubation with 100 μM of PUFAs treatment with Trypan blue exclusion test. B- Cytotoxicity of PUFAs assessed by LDH leakage. Data are means ± SD of three independent replicates. * ($p < 0.05$) and *** ($p < 0.001$) shows statistically significant between groups.

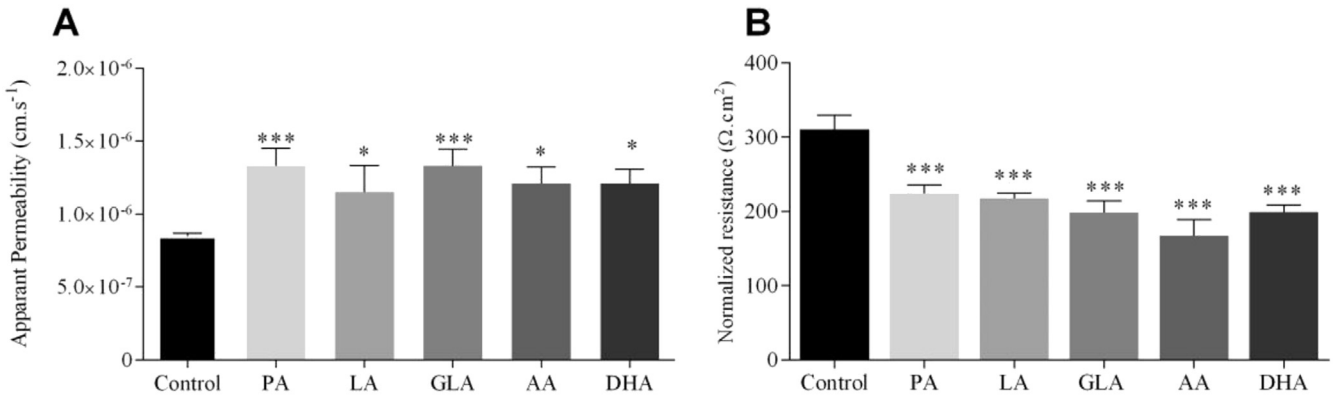

Fig. 2. A- TEER measurements and B- apparent permeability of Calu-3 cells after treatment with PUFAs. Data are means ± SD of three independent replicates. * ($P < 0.05$) and *** ($p < 0.001$) show statistically significant difference between groups.

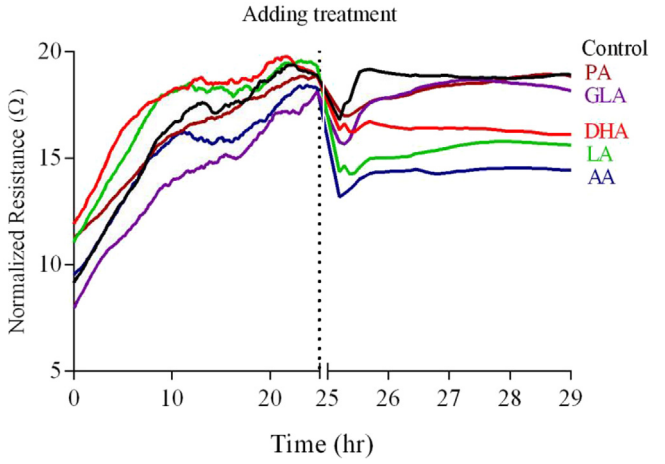

Fig. 3. Dynamic measurement of resistance using electric cell–substrate impedance sensing (ECIS) on Calu-3 epithelial cells using pre-coated ECIS electrodes and subsequent monitoring of barrier function before and after addition of PUFA treatments (dashed line represents time of adding PUFAs to the confluent layer of Calu-3 cells). This figure represents single, representative measurements of each PUFA.

Calu-3 cells, the effect of AA was studied on the transport of salbutamol sulphate [18] across airway epithelial cells. It was reported that transport of SS was significantly higher at the presence of AA. It was claimed that AA increased salbutamol sulphate's transport by changing cell membrane fluidity. In this study, the IC50 of Calu-3 cells treated with AA was reported at 69 μM, which is lower than our experiment. However, in Haghis' study Calu-3 cells were grown in a medium supplemented with growth supplements that could have influenced the different toxicity value.

Although there are many studies supporting the effect of PUFAs on paracellular transport enhancement, there are few studies on the effect of PUFAs on TJ function. For example, Beguin et al. studied the impact of PUFA (with the final concentration ranging from 0 to 150 μM for 7 days) on TJ proteins localisation and on the modulation of epithelial permeability in a model of human intestinal epithelium (Caco-2 cell model). Results from this study indicated that n-3 PUFA did not affect the presence of occludin in TJ complexes, while n-6 PUFA decreased its presence. They also reported that, regardless of PUFAs type, at 30 μM, no distortion of the Caco-2 barrier function was observed. However, 150 μM of docosahexaenoic acid (DHA) affected ZO-1 intensity, but not occludin or the barrier function parameters [38]. The differences between our

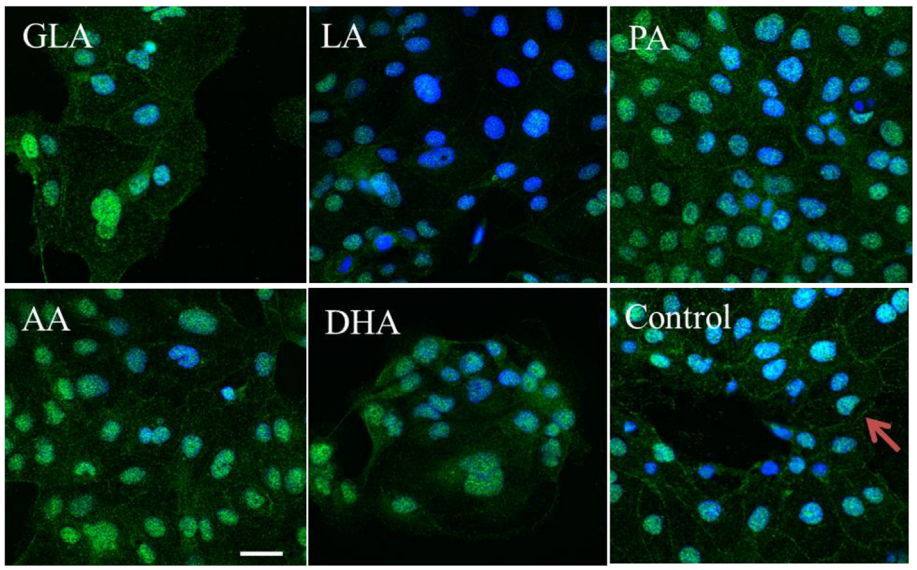

Fig. 4. Immunofluorescence of Calu-3 cells stained with mouse anti ZO-1 Monoclonal Antibody-Alexa Fluor® 488 (Green colour), after treatment for 24 h with 100 μM PUFAs. Arrow is indicating the presence of tight junctions between cells. DNA is counter stained with DAPI (Blue colour). The bar represents 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

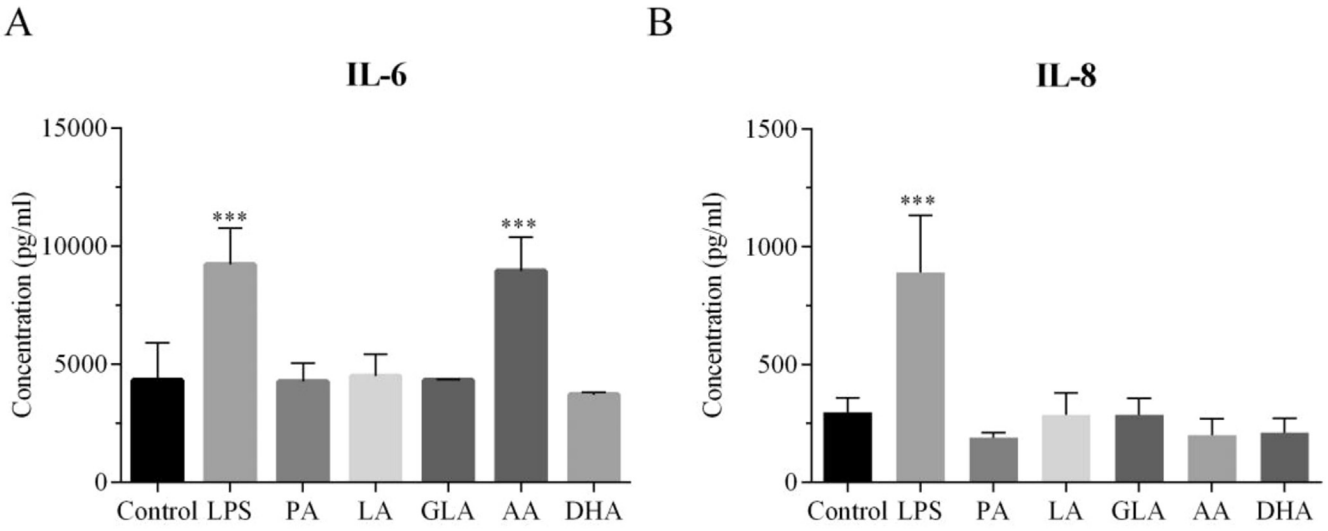

Fig. 5. A- Expression of pro-inflammatory markers IL-6 and, B- IL-8 in Calu-3 cells incubated to 100 μM of PUFAs. Data are means ± SD of three independent replicates. *** ($p < 0.001$) shows statistically significant difference between groups.

study and the previous reports can be related to differences in cell type and experimental procedure, i.e. different concentration of PUFAs or treatment duration.

3.3. Electric cell-substrate impedance sensing (ECIS)

Results from the ECIS experiments on Calu-3 cells incubated with PUFAs are presented in Fig. 3 typical experimental read-out is shown where a growth-phase after cell inoculation occurs (up to 20 h) followed by a plateau-phase when cells reach confluence (20–25 h). After incubating cells with PUFAs (indicated by dashed line in the graph), a gradual decrease in the resistance for all PUFA treatments is observed. The drop in the first 30 min happens after adding the treatment [44]. The resistance value before treatment with medium alone and 30 min after was the same value, indicating there is no change in the resistance due to the addition of medium. However, PA and GLA treatments decreased the resistance transiently in a way that resistance recovered to control level after 1 h. Other PUFAs; AA, LA and DHA resulted in a decrease in the TEER but the resistance level after 4 h did not come back to the control level.

The TEER results obtained using the chopstick method and the ECIS, demonstrated similar trend in reduction of TEER after incubating Calu-3 cells with the PUFAs. It should be stressed out that the absolute TEER values are not comparable in these two techniques [45]. The effect of PUFAs on the TJ's structural integrity was assessed via Immunofluorescence staining of PUFA-treated Calu-3 cells with antibodies against ZO-1. This experiment showed no detectable changes in TJ structural morphology, which is consistent with a non-destructive, sub-microscopic alteration in TJ function. The effect of PUFAs treatment on ZO-1 levels and localisation is illustrated in Fig. 4.

3.4. PUFAs decreased the expression of pro-inflammatory markers in Calu-3 cells

Data from experimental and clinical studies have provided evidence that PUFAs have immunomodulatory effects, specifically, n-3 polyunsaturated fatty acids (PUFA) suppress the production of pro-inflammatory cytokines like tumor necrosis factor (TNF) and interleukin (IL-1β and IL-6) in human and animal models [46,47]. Therefore, in this study the effect of PUFA treatments on expression of pro-inflammatory markers in Calu-3 cells was evaluated (Fig. 5A and B). Results showed that AA, an omega-6, increased the expression of IL-6 in Calu-3 cells, but did not affect IL-8 production. Other PUFAs did not increase inflammatory markers, for both IL-6

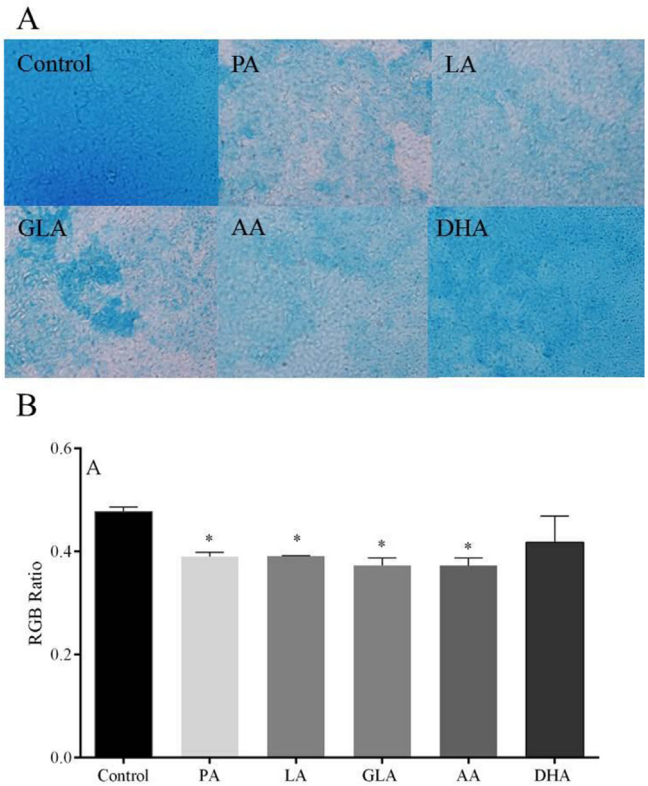

Fig. 6. A- Images of Calu-3 cells treated with PUFAs and stained with alcian blue. B- RGB_b ratio of blue colour in each PUFA treatment. Data are means ± SD of three independent replicates. * ($p < 0.05$) shows statistically significant between groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

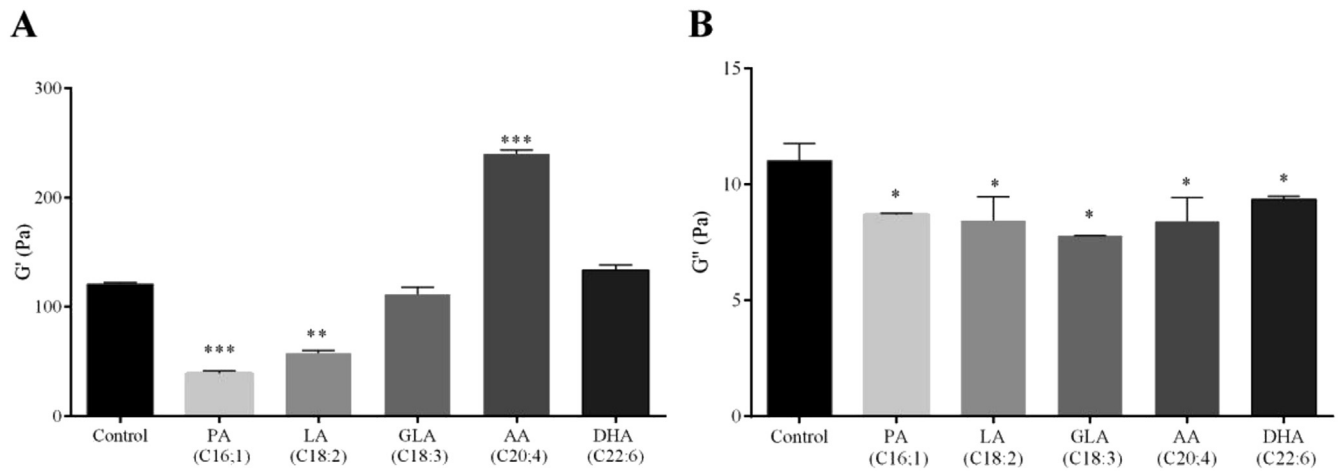

Fig. 7. A- Elastic and B- viscous behaviour of artificial mucus incubated with 100 μM PUFAs for 10 min. Data are means ± SD of three independent replicates. *** ($p < 0.001$), ** ($p < 0.01$) and * ($p < 0.5$) indicate groups are significantly different.

and IL-8. Previous studies on expression of pro-inflammatory markers on a COPD model indicated that two potent inflammatory eicosanoids, prostaglandin E2 and LTB4, are produced from AA (omega-6) and, the metabolism of EPA and docosahexaenoic acid (omega-3) decreased the production of prostaglandin E2 and LTB4 [48]. TNF expression was also evaluated after treating Calu-3 cells with PUFAs, but the readout was below detection limits of the kit, showing that Calu-3 cells did not express detectable amount of TNF, similar to the results by Journeay et al. [49]. In conclusion, the PUFA treatment (except AA) did not induce pro-inflammatory markers expression in Calu-3 cells.

3.5. *Effect of PUFAs on airway mucus production and viscoelasticity*

The ALI Calu-3 cultures produce a layer of mucus [50]. The effect of different drugs on Calu-3 mucus production has been studied previously [30] and it has been reported that PUFA treatment can decrease mucus hypersecretion [21]. However, the effect of PUFAs on mucus secretion has never been studied on Calu-3 cell line. In comparison with control, Calu-3 cells incubated with PUFAs secreted less mucus. Images shown in Fig. 6A depict a visual decrease in the blue colour (alcian blue mucus stain) for all PUFAs studied except DHA. The quantified amount of blue colour (Fig 6B) also demonstrated the decrease in the mucus secretion after Calu-3 cells were treated with PUFAs.

The overall viscoelastic property of AM was characterised using a sensitive stress-controlled cone and plate rheometer. The dynamic response of AM to shear was tested by applying oscillatory deformations of small amplitude. The elastic modulus (G′) of AM was significantly higher (10 times more) than the viscous modulus (G″) over the entire tested range of frequency (data not shown), indicating that the AM used in this study behaves as a viscoelastic solid [51]. Incubating AM with PUFAs decreased the viscoelasticity of AM. It was demonstrated that both viscous and elastic modulus changed significantly compared to the control (Fig. 7A and B) and the effect of PUFAs on elasticity was much greater than viscosity. The viscos modulus (G″) was significantly lower for PA (C16; 1) and LA (C18; 2) treatments ($p < 0.001$ and $p < 0.01$). However, GLA treatment did not change the viscous modulus significantly. In contrast, viscous modulus was significantly higher ($P < 0.001$) with the AA treatment (C20; 4). This change in the elastic modulus of AM had a linear relationship to the PUFAs chain length: the shorter the chain length of PUFA the lower AM elasticity was. Although, DHA has longer chain length (C22) the viscous modulus did not significantly change. The reason for these results could be due to the influence of PUFAs on the surface tension of the AM. It has been reported that surface tension of fatty acids increases as the length of the carbon chain increase [52]. The viscous modulus was significantly lower in PUFA treated AM compared to the control ($P < 0.5$). However, it was not influenced by PUFAs' chemical structure. Subsequently, it can be speculated that PUFAs may reduce the mucus production of Calu-3 cells whilst simultaneously decreasing its viscoelastic property, meaning it could enhance mucus clearance in case of patients with mucus hypersecretion. Further investigation on the effect of PUFAs on mucus rheology and the mechanism of these changes are needed.

4. Conclusions

This study is the first to evaluate the effect of PUFAs on airway epithelial Calu-3 cells in terms of tight junction permeability and mucus properties. Results indicate that PUFAs do not exert structural changes on Calu-3 cells. However, functional changes can be observed, presumably by changing membrane fluidity. Functional effects were mainly on transepithelial resistance of Calu-3 cells, which resulted in decrease of TEER and therefore increase in the paracellular transport of Na-flu. It was also observed that PUFAs could reduce the mucus production of Calu-3 cells and decrease viscoelastic property of mucus.

5. Acknowledgments

This research was supported under Australian Research Council's Linkage Projects Funding Scheme (project number LP 130101123). The authors acknowledge the facilities and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Australian Centre for Microscopy & Microanalysis at the University of Sydney. We also thank Wing Hin Lee for his assistance in inflammation study.

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