

## The Effects of Immunomodulatory Molecule- Lipoxin A<sub>4</sub>- on The Physiology of Cystic Fibrosis Human Bronchial Epithelium

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### ABSTRACT

Cystic fibrosis (CF) is a disease characterised by the depletion of Airway Surface Liquid (ASL) height. Lipoxin A<sub>4</sub> was found to be effective in restoring ASL back to normal by acting on ENaC. The signalling mechanisms involved in lipoxin effect were investigated by testing the action of a calcium chelator (Bapta-AM) and Protein kinase C inhibitor (GF 109203X) on NuLi-1 and CuFi-1 cells. Drug's efficacy was monitored by conducting Ussing chamber experiments and confocal microscopy. Results showed that calcium has a major contribution to the lipoxin A<sub>4</sub> effects while PKC has a limited role. However, our data might lack accuracy due to shortage of time to conduct more experiments.

**Keywords:** CFTR, cystic fibrosis, genetic mutations, human bronchial, Lipoxin A<sub>4</sub>, human bronchial epithelium.

### INTRODUCTION

Cystic fibrosis is a life-threatening autosomal recessive disorder caused by a genetic mutation involving the cystic fibrosis transmembrane regulator (CFTR) that affects ~70,000 individuals worldwide<sup>(1)</sup>. In addition, CFTR is expressed in all epithelial tissue lining tubes and ducts. Thus, a mutant CFTR will give rise to multiple serious conditions such as pancreatitis, cirrhosis, meconium ileus and infertility<sup>(2)</sup>. Among those affected by CF, 90% were at least carriers for one allele of F508Del genotype<sup>(3)</sup>. The condition mainly affects those of European origins, although it has been reported in all races and ethnicities<sup>(4)</sup>. CFTR dysregulation leads to ASL dehydration due to lack of chloride ions secretion. ASL dehydration is further increased by over absorption of sodium ions through epithelial sodium channel (ENaC) on the apical side<sup>(5)</sup>. This disrupts the height of Airway Surface Liquid (ASL) which is kept at 7µm in normal epithelial tissue. This layer is composed of a periciliary liquid layer for lubrication and a mucus layer for dust and pathogens entrapment<sup>(6)</sup>. ASL provides optimal working conditions for cilia that clear mucus. When ASL is damaged, viscous secretions accumulate in the airways of the lungs causing mucosal plugging that prepare favourable conditions for bacteria to cause repeated infections<sup>(7)</sup>. Although the median survival age rose for up to 37 years, the disease is still life shortening with obstructive lung disease being responsible for 80% of mortality caused by CF<sup>(8)</sup>.

Recently, a study carried out by *Alalawi et al.*<sup>(9)</sup> showed a promising curative role for lipoxin. Lipoxin is an immune-modulator that can inhibit ENaC and restore normal physiological height of ASL in low and high concentrations.

### AIM OF THE STUDY

The aim of this study is to further investigate the signalling pathways involved in lipoxin A<sub>4</sub> effects by measuring ion transport in Ussing experiments, ASL height in confocal microscope studies and monitor protein kinase C phosphorylation status by western blotting.

### METHODS

#### Cell culture

NuLi-1 (normal cells) and CuFi-1 (cystic fibrosis cells) were used in all experiments involved in our studies. These cells were cultivated, immortalized and donated by prof. Zabner (USA) to Molecular Medicine research laboratory. In the lab, cells were kept at -180° in liquid nitrogen and when it's time to use them, they get moved to grow in flasks to achieve confluency. Subsequently, cells were placed on inserts which allowed them to further grow and differentiate. Initially, bronchial epithelial growth medium (BEBM) was used for the first week of growing. Confluency is inspected under microscope, and once is reached, medium is switched to DMEM-F12 to enhance cell differentiation. The culture medium was changed under the hood every 3-4 days. The procedure was repeated for up to 4-6 weeks where cells develop confluent monolayers, cilia and mucin secretions with transepithelial electrical resistance of >700 ohms.

#### Ussing chamber experiments

The ringer buffer was prepared using 2.3M of NaCl, KH<sub>2</sub>PO<sub>4</sub> 8mM, NaHCO<sub>3</sub> 0.5M, MgCl<sub>2</sub>\*6H<sub>2</sub>O 24mM, CaCl<sub>2</sub>\*2H<sub>2</sub>O 24mM, Glucose 1M and 400ml of distilled water to make a solution of 500ml. Differentiated cells were moved

to ussing chambers after ruling out any leakage or blockage. Chambers were attached to electrical model amplifiers that serve to measure potential differences, current passed and conductance was measured. Ph level was constant throughout the experiment due to the fact that solution in chambers was gassed with a mixture of 95%O<sub>2</sub>/5%Co<sub>2</sub>. Temperature was checked during experiments using a thermometer and was controlled at 37±1 °C.

#### **Airway surface liquid height measurements**

ASL height was measured by confocal fluorescence microscopy. Dextran red was added apically on cells about 15 hours prior to experiment. On the day of experiment, 30 minutes before examining the cells, calcien green-AM was added to the basolateral side of cells to stain the epithelium. Then, 0.4ml of perfluorocarbon was poured on the apical side of inserts in order to stop ASL from evaporating during transferring and examining processes. Images were obtained using confocal microscopy with the aid of HENE1 and ARGON2 lasers and ASL height was measured in 9 different points in each of the 5 images that were taken from every insert. Analysis of the images was carried out using Carl Zeiss Micro Imaging software.

#### **Western blotting**

The first step is obtaining proteins through cell lysis using a lysing buffer. Then, samples were centrifuged for 10 minutes on 12,000rpm at 4°C. Pipetting the supernatants into new tubes. Proteins

were separated on SDS-PAGE gel, and then they were transferred to a PVDF membrane. A primary antibody was added on the membrane to bind proteins of interest and samples were incubated for approximately 15 hours.

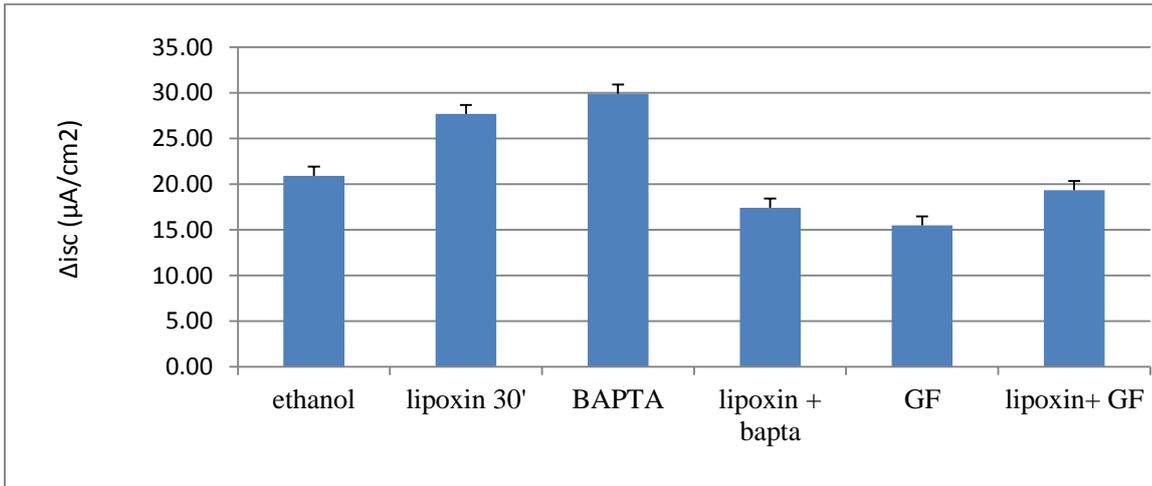
Next, the membrane was put on a shaker immersed in TBST 3-4 times to wash away any unbound antibody. After adding a secondary antibody, the membrane was washed again 3 times in TBST and the bound antibodies were then detected by developing the film enclosed in a cassette using ECL solution. The band thickness corresponds to the amount of protein present and is measured in kDA.

**The study was done after approval of ethical board of King Abdulaziz university.**

#### **RESULTS**

##### **Lipoxin A<sub>4</sub> effect on amiloride and bumetanide-sensitive currents in human bronchial epithelial cells:**

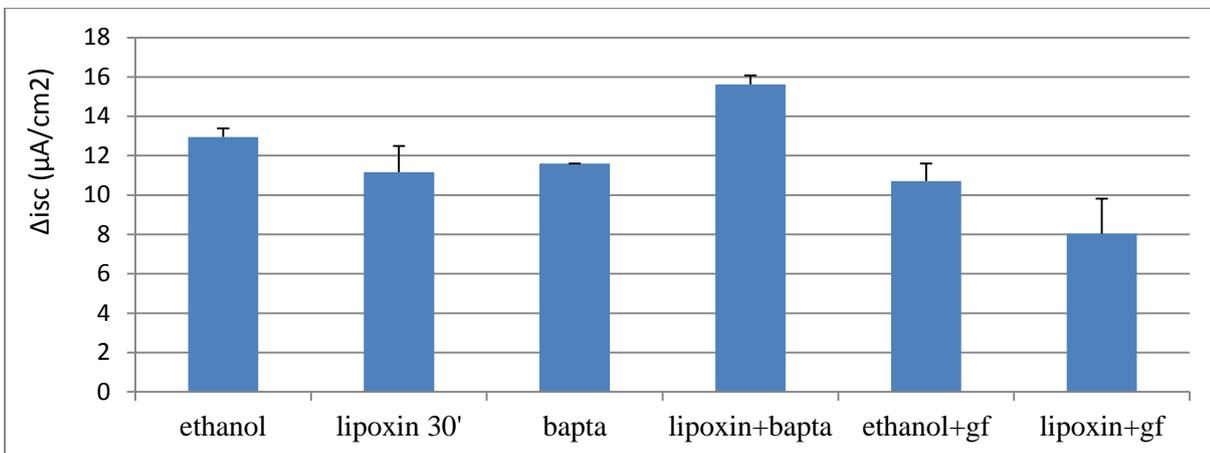
The effect of amiloride on transepithelial currents was tested by placing either CuFi-1 or NuLi-1 cells in Ussing chambers. Cells were treated with 10 μM of either Bapta-AM or 10 μM GF-109203Xinhibitors for 30 minutes. Cells were then exposed to 1 nm of lipoxin for an additional 30 minutes. Amiloride of 1 μm concentration was added on the apical surface for 10 minutes, while 10μm of bumetanide was added on the basolateral side.



**Figure 1.A: Effect of Lipoxin A<sub>4</sub> on Nuli-1 cells.**

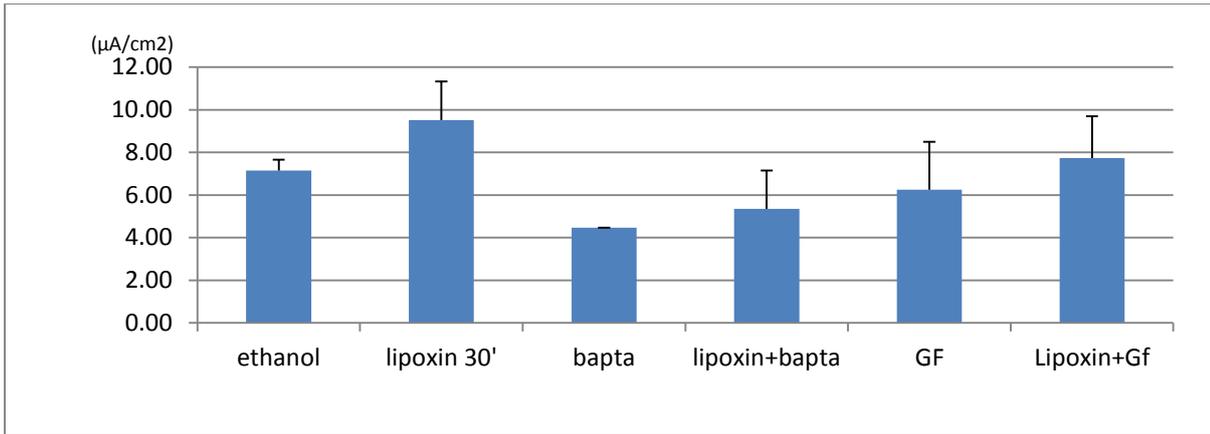
Lipoxin A<sub>4</sub> increased amiloride-sensitive current in NuLi-1 cells. The averaged amiloride-sensitive current in control was 20.92±1.9 μA/cm<sup>2</sup> compared to an average of 27.68±4.49 μA/cm<sup>2</sup> in lipoxin A<sub>4</sub> treated cells (n=3). (Figure 1A) However this increase was not statistically significant. The addition of Bapta-AM abolished lipoxin A<sub>4</sub> induced increase in amiloride-sensitive current to 30±4 μA/cm<sup>2</sup> in control cells compared to 17.81±5.8 μA/cm<sup>2</sup> in lipoxin presence (n=2). GF-109203X slightly decreased amiloride-sensitive currents in both control and lipoxin A<sub>4</sub> treated cells,

which was 15.5±2.54 μA/cm<sup>2</sup> in the control and 19.35± 2.15 μA/cm<sup>2</sup> in the presence of lipoxin (n=3). In CuFi-1 cells, apical exposure to amiloride induced a mean decrease of the transepithelial circuit current by 13 μA/cm<sup>2</sup> in the control compared to 11 μA/cm<sup>2</sup> in lipoxin treated cells. In presence of Bapta-AM, the mean current was increased to 16 μA/cm<sup>2</sup> in lipoxin cells while a drop in the mean by 1 μA/cm<sup>2</sup> was observed in control cells. However, when cells were treated with GF-109203X, a mean of 11 μA/cm<sup>2</sup> was recorded in control cells compared to 8 μA/cm<sup>2</sup> in presence of lipoxin A<sub>4</sub> (n=3).



**Figure 1B: Effects of Lipoxin A<sub>4</sub> on CuFi-1 cells.**

In NuLi-1 cells, treatment with lipoxin resulted in an increase in bumetanide-sensitive currents from 7.14±0.52 μA/cm<sup>2</sup> to 9.52±1.81 μA/cm<sup>2</sup> (n=3). This effect was previously observed by (Al-Alawi *et al.* 2014). When Bapta-AM was added, the current in the control cells was similar to the one in lipoxin A<sub>4</sub> treated cells 4.46±0.0 μA/cm<sup>2</sup> to 5.36±1.73 μA/cm<sup>2</sup> respectively (n=2). This may suggest that Ca<sup>2+</sup> is involved in lipoxin A<sub>4</sub> effect. When GF-109203X was added, bumetanide-sensitive currents were 6.25±2.25 μA/cm<sup>2</sup> and 7.74±1.95 μA/cm<sup>2</sup> in presence of lipoxin (n=3).



**Figure 1C:** Bumetanide-sensitive current on NuLi-1 cells. Varying response in ASL height in NuLi cells to different reagents, most notable when lipoxin was added.

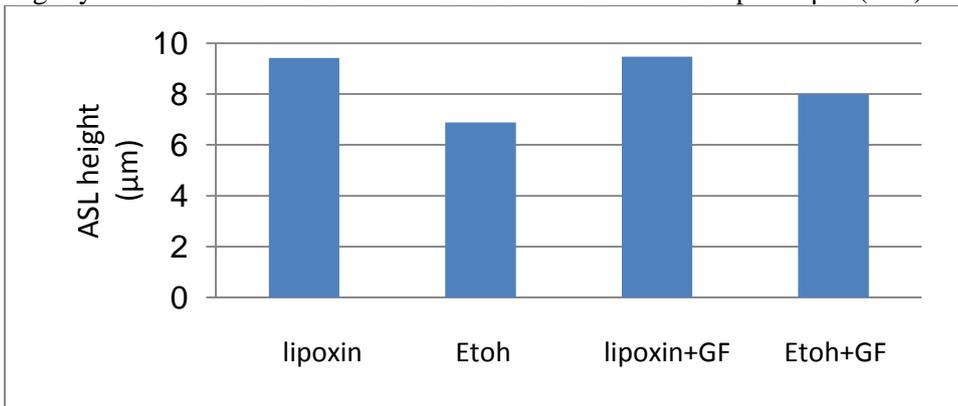
**Effect of Lipoxin A<sub>4</sub> on ASL height in human bronchial epithelial cells**

ASL height was measured in CuFi-1 cells to investigate the potential effects on lipoxin A<sub>4</sub>, both with and without the addition of a protein kinase C inhibitor GF-109203X. Cells were treated 30 minutes prior to examining.

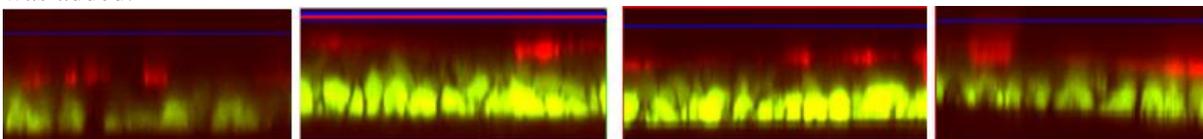
ASL height in cells treated with ethanol was slightly decreased in contrast to an increase in

ASL when treated with lipoxin. The ASL in lipoxin treated cells was 9.45 µm and this is compared to ASL height of 6.9 µm in ethanol treated cells (n=1).

When GF-109203X was added to ethanol, the ASL height remained almost the same as in cells treated with ethanol only, however, LXA<sub>4</sub> in the presence of GF-109203X showed an increase in ASL height for up to 8 µm (n=1).



**Figure 2A:** Marked increase in CuFi cells ASL height when lipoxin is added. No major difference when GF was added.



Control

Lipoxin

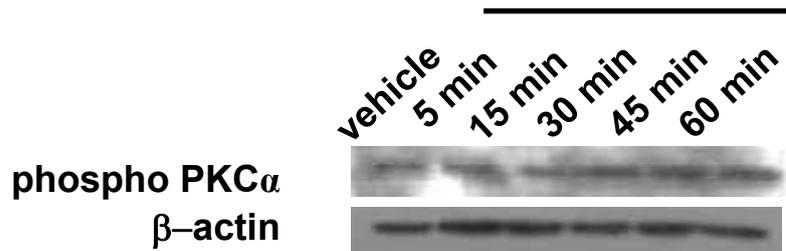
Control+ GF

Lipoxin+ GF

**Figure 2:** A. Comparison of ASL height across control vs intervention cells, B. ASL height of Cufi-1 cells under confocal microscopy imaging. ASL is in red and epithelial cells in green. Blue line is used to measure the height. A trend towards a continuum of ASL height in lipoxin treated cells.

**Effect of Lipoxin A<sub>4</sub> on phosphorylation PKCα**

Western blot presenting phospho PKCα protein expression for samples untreated and treated with Lipoxin A<sub>4</sub> for a period of 5, 15, 30, 45 and 60 minutes. The expression level of phosphorylated PKCα was similar in control and lipoxin A<sub>4</sub> treated cells. (n = 1) (Fig.3). This demonstrates that lipoxin A<sub>4</sub> is unlikely to activate PKCα.



**Figure 3.** Western blot of phospho PKC $\alpha$  protein expression for samples untreated and treated with Lipoxin A<sub>4</sub> for a period of 5, 15, 30, 45 and 60 minutes. No significant difference in band thickness between control and lipoxin treated cells.

## DISCUSSION

Cystic fibrosis (CF) is a lethal disease caused by CFTR gene mutation characterized by ENaC over activation and chloride under secretion. Lipoxin A<sub>4</sub> was suggested as a possible cure for the disease by acting on inhibiting ENaC and activation of CaCC. Investigating lipoxin's mechanism of action is essential to assess the future of its use as a possible drug. Here we investigated the effects of calcium chelator and a PKC on lipoxin A<sub>4</sub> effect on ion transport and ASL height.

We managed to replicate results regarding the effects of lipoxin on bumetanide and amiloride sensitive current in CuFi-1 and NuLi-1 cells. Hence we can confirm that lipoxin function as an ENaC inhibitor and possibly Cl<sup>-</sup> ion secretion stimulator. Moreover we demonstrated that calcium chelator bapta-AM abolished the effect of lipoxin A<sub>4</sub> on Cl<sup>-</sup> secretion in NuLi-1 cells and on ENaC activity in CuFi-1 cells. This means that in order for lipoxin to activate ENaC receptors in CF epithelia, intracellular calcium must be present.

In a previous study done by (Alalawi et al, 2014)<sup>(9)</sup>, lipoxin A<sub>4</sub> was proved to be effective in restoring ASL height in low and high concentrations, we obtained similar results here. Our experiment concluded that PKC had no role in lipoxin effect. However, this is only one experiment. A greater number of experiments are required to be carried out to determine the accuracy of this finding.

Also, we demonstrated that lipoxin A<sub>4</sub> did not affect phosphorylation of PKC $\alpha$  by western blotting, indicating that PKC $\alpha$  is not involved in lipoxin effects.

Finally, our results suggest that calcium has a major contribution to the lipoxin effects in human

bronchial epithelium, while PKC has a limited role. However, this needs to be further investigated.

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