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FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

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UNIVERSITY OF VIENNA

FACULTY OF LIFE SCIENCES

Department of Pharmacology and Toxicology

INFLUENCE OF TNF-α ON hENaC SUBUNITS EXPRESSION

DIPLOMA THESIS

Supervisors of the thesis: Prof. Dr. Rosa Lemmens-Gruber

PharmDr. Hana Bártíková, Ph.D.

Hradec Králové 2015

David Martan

Hereby I declare that this thesis is my original authorial work. All literature and other sources which I used were properly cited and listed in complete reference to the due source. The work was specifically used only for one purpose.

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V Hradci Králové David Martan

……...…

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Abstrakt

Universität Wien

Fakultät für Lebenswissenschaften

Department für Pharmakologie und Toxikologie

Univerzita Karlova v Praze

Farmaceutická fakulta v Hradci Králové

Katedra biochemických věd

Kandidát: David Martan

Školitelé: Prof. Dr. Rosa Lemmens-Gruber, PharmDr. Hana Bártíková, Ph.D.

Název diplomové práce: Vliv TNF-α na expresi podjednotek hENaC

Lidský amilorid-senzitivní epiteliální sodíkový kanál (hENaC) je typem iontového kanálu, který má schopnost kontrolovat solnou a vodní homeostázu. Z toho důvodu je hlavní hybnou silou pro reabsorpci vody v alveolárním epitelu. Dysfunkce tohoto kanálu, respektive tohoto mechanismu, vede k velmi vážné chorobě – plicnímu edému.

V předchozích studiích byla testována droga AP301 (nedávno pojmenovaná solnatid). AP301 je cyklický protein zahrnující sekvenci lektinu podobné domény lidského tumor nekrotizujícího faktoru. Tato droga byla nedávno vyvinuta jako potenciální lék proti plicnímu edému. Principem je aktivace lidského epiteliálního sodíkového kanálu pomocí zvýšení pravděpodobnosti jeho otevření. Také bylo dokázáno, že AP301 přechodně zvyšuje expresi tohoto kanálu v buňkách savců.

V této studii jsme použili metodu Western blot k testování vlivu tumor nekrotizujícího faktoru α (TNF-α) na expresi podjednotek kanálu hENaC a tyto výsledky jsme porovnali s výsledky ze studie o AP301.

Zjistili jsme, že TNF-α přechodně signifikantně zvyšuje expresi δ podjednotky a má potenciál zvýšit expresi α podjednotky. Na druhou stranu exprese podjednotek β- a γ-hENaC nebyla signifikantně zvýšena.

Tyto výsledky jsou analogické k těm, které byly zjištěny ve studii s AP301.

Abstract

University of Vienna

Faculty of Life Sciences

Department of Pharmacology and Toxicology

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Biochemical Sciences

Candidate: David Martan

Supervisors: Prof. Dr. Rosa Lemmens-Gruber, PharmDr. Hana Bártíková, Ph.D.

Title of the diploma thesis: Influence of TNF-α on hENaC subunits expression

The human amiloride-sensitive epithelial sodium channel (hENaC) is a type of ion channel which has the ability to control salt and water homeostasis. Therefore it is one of the main driving forces for the reabsorption of water through the alveolar epithelium. A dysfunction of this channel, respectively of this control mechanism, leads to a very severe disease – pulmonary edema and several other pathological conditions.

Previous studies tested a drug called AP301, recently named solnatide. AP301 is a cyclic protein comprising the human tumour necrosis factor lectin-like domain sequence. This drug was recently developed as a potential treatment of pulmonary edema. The principle is that it activates hENaC by increasing the open probability. It was also shown that AP301 transiently increases the expression of hENaC subunits in mammalian cells.

In this study we used the Western blot method to test the influence of tumour necrosis factor α (TNF-α) on hENaC subunits expression and we compared these results with the results from the studies with AP301.

We found that TNF-α transiently significantly increased the expression of δ subunit and it had a potential to increase the expression of α subunit. On the other hand, the expression of β- and γ-hENaC was not significantly increased.

Taken together, these results are analogical to those which were found in the studies with AP301.

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1. Introduction

1.1. Human lungs

Human lungs are one of the most important organs in the human body. Their primary role is to promote exchange of the breathing gases between the airspace and the blood. This substitution happens through alveolar – capillary barrier. The basic entity of the lungs is a thin-walled air sac called alveolus. It is composed mainly of two types of cells – type I (ATI) and type II (ATII) between which are tight junctions. Alveoli are surrounded by a network of capillaries which brings blood into a close proximity with the air inside alveolus. Then carbon dioxide and oxygen are able to move across the membrane by diffusion (Eaton et al. 2010; Folkesson & Matthay 2006; Rhoades & Bell 2012).

Another, but not less important purpose of alveoli is to control the alveolar fluid clearance (AFC). As it was reviewed, the general paradigm says that the net alveolar fluid clearance is driven by an active Na^+ and Cl^- transport (Matthay et al. 1996). The ion transporters, which cause this process, are placed asymmetrically on cell surfaces of the alveolar cells. It is known that ATII cells have the crucial mean for the AFC because they are responsible for the vectorial Na^+ and Cl^- transport (Figure 1) and also for the surfactant secretion (Folkesson & Matthay 2006).

However several mechanisms exist for epithelial fluid transport in the human lung, which are essential for the correct control of the homeostasis. Based on the mechanism, the three groups of channels can be distinguished. The first one is mediated by the amiloride-sensitive sodium channel. We know a few types of these channels which occur in the apical membrane of ATII, nevertheless the most pivotal for the lung liquid balance is the epithelial sodium channel (ENaC). In the second group, where are only mechanisms insensitive to amiloride, are the cyclic nucleotide-gated cation channel (CNG) and Na^+/H^+ exchange. In the third place there is Na^+/K^+-ATPase which is pumping Na^+ out of the cell and K^+ into the cell against their appropriate concentration. And the last important mechanism is the transport of the water through aquaporins (reviewed in Matthay et al. 2002).

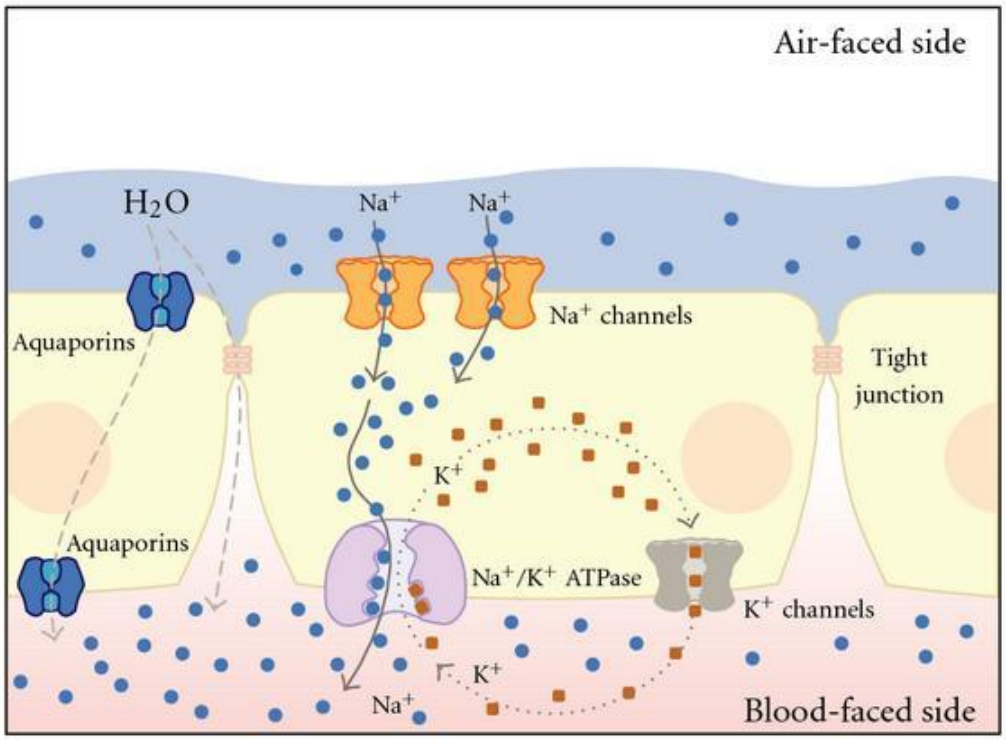

Figure 1: AFC is driven by transepithelial Na^+ transport. Na^+ ions passively enter the cell through a sodium channel (apical membrane) in alveolar cells, following an electrochemical gradient. Then Na^+ ions are actively pumped by Na^+/K^+-ATPase (basolaterally localized) out of the cell, and are exchanged for K^+ ions which then move again out of the cell via potassium channel (basolateral membrane). All these mechanisms together cause a net movement of sodium ions from the apical side of the alveolar epithelium to its basolateral side. Thus osmotic forces are created and water has to move through the epithelium, tight junctions or via aquaporins out from the airspaces (Althaus et al. 2011).

1.2. Pulmonary edema

A collapse of these mechanisms can be one of the ways which lead to a very severe disease – pulmonary edema (Matthay et al. 2002).

A pulmonary edema is a condition when excess fluid accumulates in alveoli and the lung interstitial spaces (Figures 2a, 2b). The fluid exchange in pulmonary capillaries depends on four forces: hydrostatic and colloid osmotic pressures which work against each other and another pair with opposite roles – surface tension and alveolar pressure. The pulmonary hydrostatic pressure is usually lower than the colloid osmotic pressure what causes the net absorption of fluid. On the other hand, the alveolar surface tension works against that, and results in a small continuous flux of fluid out of the capillaries and into the interstitial space.

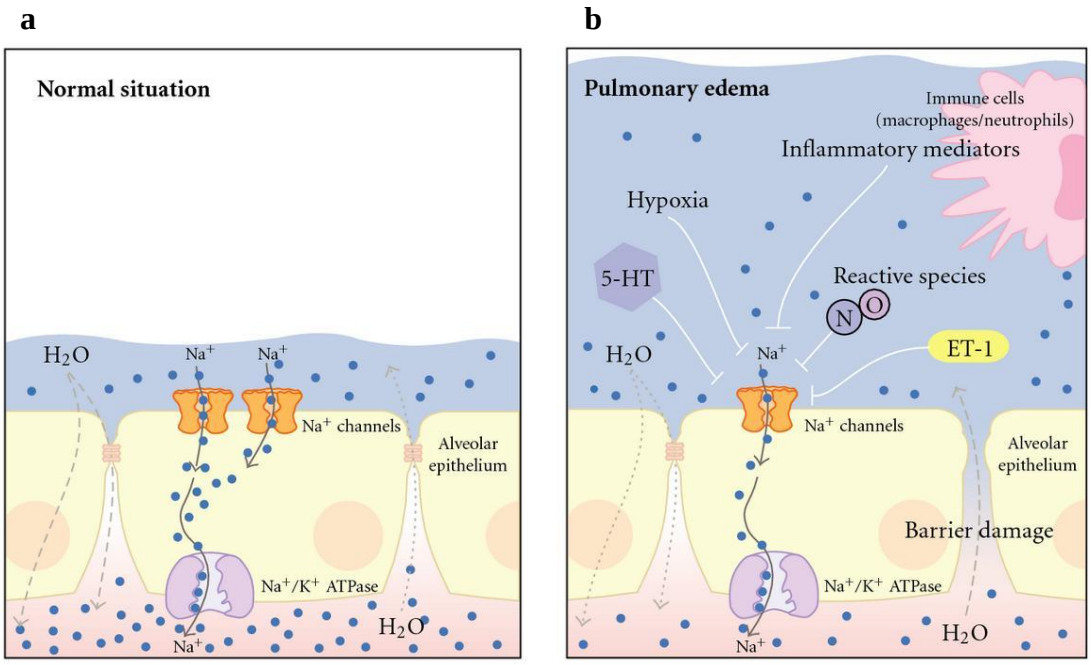

Figure 2a: Normal physiological conditions (Althaus et al. 2011).

Figure 2b: Many and various factors exist which may inhibit the sodium channel, and they are associated with pulmonary edema. Because of the decrease of the channel activity, the water reabsorption also decreases and it accumulates in the airspaces. Another reason can be an increase of fluid filtration into the airspaces – usually caused by an impairment of the epithelial barrier (Althaus et al. 2011).

This disease can be divided into two categories: cardiogenic pulmonary edema and noncardiogenic pulmonary edema.

The causes of cardiogenic pulmonary edema can be the increase in capillary hydrostatic pressure, which is the most frequent impulse because it is usually a consequence of an abnormally high pulmonary venous pressure, or increase in alveolar surface tension, or capillary permeability or by decrease in plasma colloidal osmotic pressure.

One of the ways of possible medication is to use diuretic drugs which have the ability to decrease the blood volume. Or administering digitalis to increase the left ventricular function and a drug which dilates systemic blood vessels (Rhoades & Bell 2012).

The noncardiogenic pulmonary edema is caused by increased permeability of the alveolar-capillary membrane. The fluid together with proteins flood the interstitial spaces and alveoli. Due to the protein leakage this type is more severe because an extra amount of water is absorbed from the capillaries to the alveoli. This type occurs together with pulmonary vascular injury (from inflammatory reaction, oxidative damage, neurogenic shock). Another cause of pulmonary edema may be a high surface tension which is a consequence of a loss of surfactant. And also a decrease in plasma colloidal osmotic pressure can occur when the concentration of plasma protein is reduced. This disease decreases arterial oxygen pressure and increases arterial carbon dioxide pressure – that produce hypoxemia and hypercapnia. It also obstructs airflow and increases airway resistance because of flooding small airways. This data suggests that pulmonary edema decreases lung compliance and therefore the work of breathing is increased.

1.3.ENaC

The epithelial sodium channel (ENaC) or amiloride-sensitive channel is a type of an ion channel that occurs in the apical sides of many kinds of polarised epithelia (lung, renal collecting duct, urinary bladder, distal colon, sweat and salivary glands, taste buds) and regulates Na^+ absorption in them (Garty & Palmer 1997).

There are two main Na^+ transporting systems for transferring those ions. Na^+ ions follow the electrochemical gradient and enter the cell through the already mentioned

ENaC and on the other hand they are pushed out of the cells through the activity of Na^+/K^+-ATPase which is settled in the basolateral side of the cells. All these facts show that the Na^+ transport creates the osmotic pressure which is responsible for the water movement (from the apical to the basolateral side of cells). However, this is not the only way that water overcomes the epithelium – it also uses the tight junctions between the cells and transcellular water channels – aquaporines (Dobbs et al. 2010). As it was written in the Althaus et al. 2011 review, all these facts suggest that ENaC in the lung epithelium plays a key role in the fluid content regulation and therefore also in the alveolar fluid clearance driving.

The channel itself is composed of four homologous subunits called α, β, γ and δ (Figure 3) (Canessa et al. 1994a; Lingueglia et al. 1993; Waldmann et al. 1995). Each of these subunits are basically a polypeptide chain which comprises two transmembrane domains, two short intracellularly located termini (amino terminus and carboxyl terminus) attached to the domains and a large extracellular loop. Each of these polypeptide chains contains a different number of N-linked glycosylation sites, depending on the type of the subunit. α-ENaC has two intracellular sites and five on the extracellular loop, β-ENaC has nine extracellularly, γ-ENaC has three and δ-ENaC has three extracellularly (Althaus et al. 2011; Canessa et al. 1994b; Snyder et al. 1994; UniProt). These sites also influence posttranslational modification since the subunits α, β, γ undergo N-glycosylation on their extracellular loops. Another posttranslational modification, important only for α and γ subunits, is proteolytic cleavage. The proteolytic cleavage is believed to be able to increase the open probability (P_o) of ENaC (Adams et al. 1997; Butterworth et al. 2010; Hughey et al. 2003; Kleyman et al. 2009; Snyder et al. 1994).

The magnitude of whole-cell ion current depends on the number of channels expressed in the plasma membrane, single channel current amplitude and open probability of functional channels. Thus, in principle the mode of action of ENaC activation is based on increased trafficking and expression in the plasma membrane and/or single channel kinetics with enhanced open probability. The latter can be caused by compounds such as S3969 (Lu et al. 2008), by shear force (Fronius et al. 2010) or cross-linking (Collier et al. 2014).

It was shown that even though the combinations of subunits such as α-γ and α-α are able to conduct moderate current, the functional channel has to contain α or δ

or α and δ subunit together with β and γ (Canessa et al. 1994b). In addition, it was proved that around 40% of amiloride-sensitive sodium transport in human primary nasal cells is caused by the last revealed subunit – δ (Bangel-Ruland et al. 2010). This study, as well as the Ji et al. 2012 review, manifests that δ-ENaC is also an essential pathway of Na^+ reabsorption in lung and kidney.

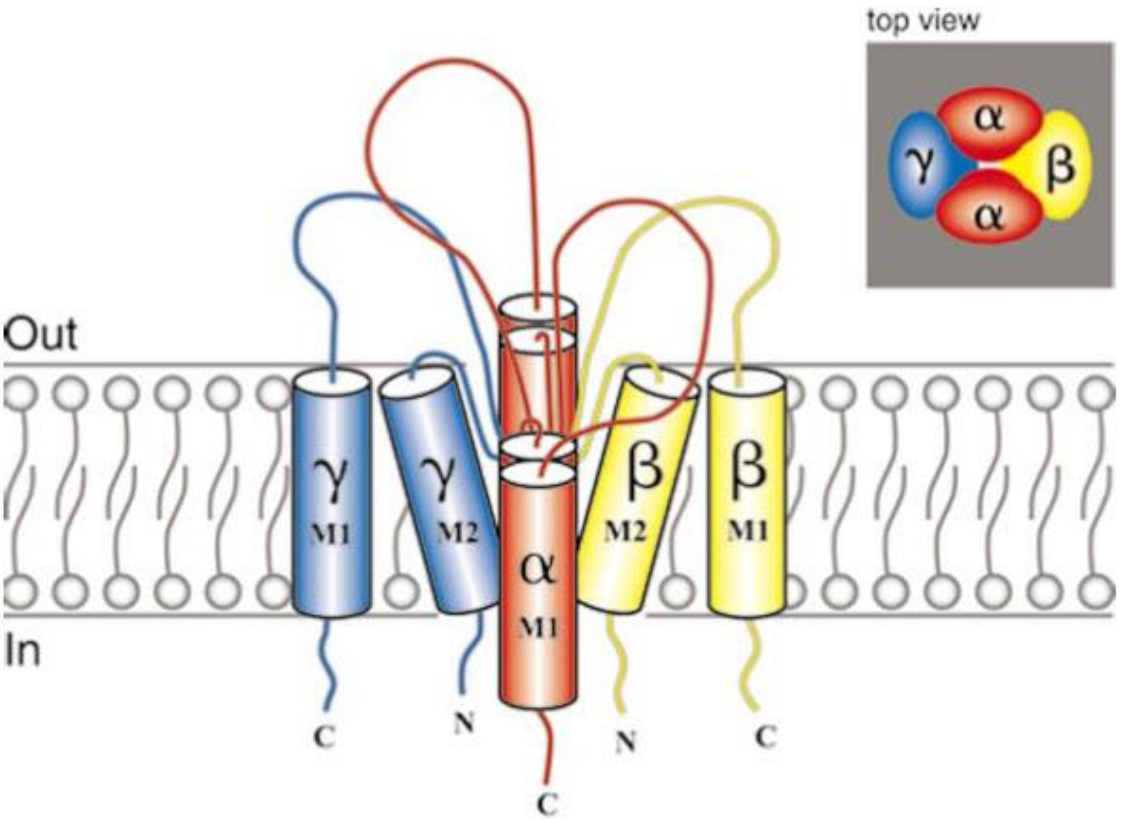

Figure 3: The composition of the epithelial sodium channel as it was described by Gormley et al. 2003. In other experiments it was discovered that δ subunit may replace α subunit. Simultaneously δ subunit undertakes similar functions as α subunit (Waldmann et al. 1995).

1.4. TNF-α

TNF-α, together with TNF-β, CD27 ligands, CD30 and CD40, is a member of a cytokine family. It is expressed in low levels by most of the tissues in a normal physiological condition; however, in the state of an infection or an injury, this expression is increased (Creighton 1999). The major producer of TNF-α in the human blood is the minor population of monocytes – $CD14^+CD16^+$ (Belge et al. 2002). A cell needs a TNF-α receptor to be able to respond to TNF-α cytokine. Two types of them are known,

and both of them form a trimeric structure (as well as TNF-α does) – p55 (TNFR1) and p75 (TNFR2) which bind TNF-α with an equal affinity but they induce different signal pathways (Figure 4) (Creighton 1999; Kishore et al. 2011; Locksley et al. 2001; Rothe et al. 1995).

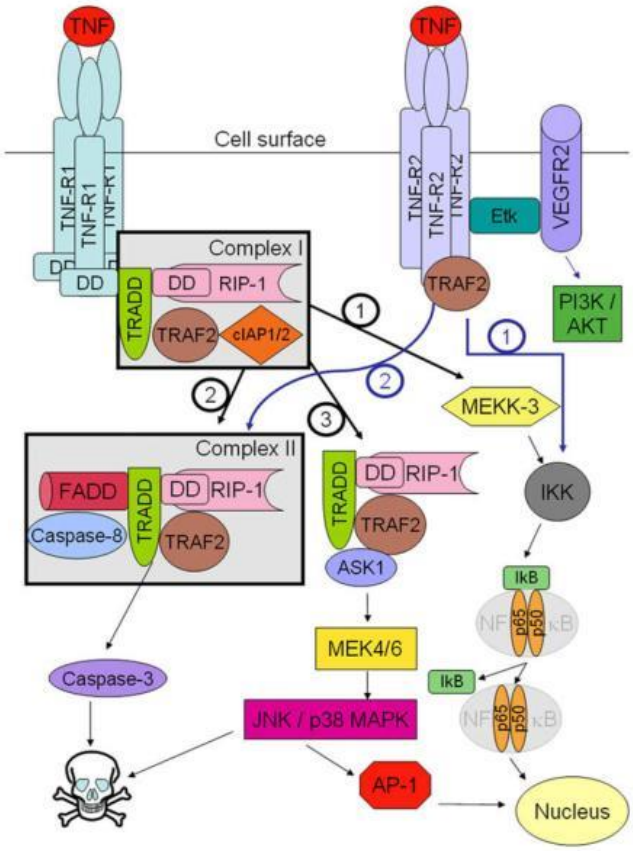

Figure 4: Different signalling pathways mediated through TNFR1 and TNFR2 (Haider & Knöfler 2010).

A consequence of binding to TNFR1 is a kinases activation which leads to phosphorylation of the transcription factor NF-κB (prevents apoptosis), jun N-terminal (JUN) kinase and mitogen activated protein (MAP) kinase and also a liberation of ceramide from sphingomyelin. Moreover, ceramide is a second messenger with an ability to activate NF-κB. The signalling pathway of TNFR2 is not so well known but it is believed that it is also linked to NF-κB activation (Haider & Knöfler 2010). It was also found that TNFR1 is probably the predominant mediator of TNF-α signalling because it occurs in most tissues (Grell et al. 1995). Review by Li et al. 2013 concluded, that the activation of TNF-α receptor superfamily can lead not only to NF-κB activation

but also to apoptosis and programmed necrosis. Nevertheless, as it was mentioned, TNF-α also has a role in physiological conditions. As a result it may be said that TNF-α has diverse biological effects which are represented in Figure 5. Those are the reasons why TNF-α is called a pleiotropic factor (Creighton 1999).

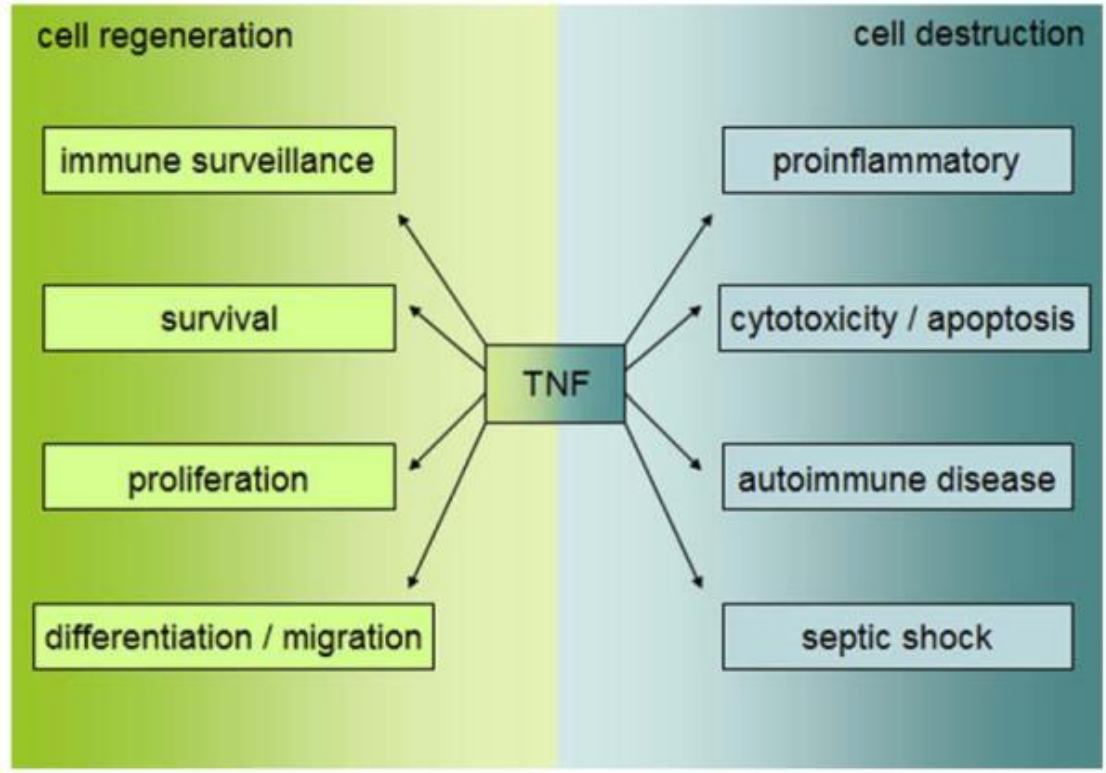

Figure 5: Biological and adverse effects of TNF-α (Haider & Knöfler 2010)

In this work I have focused on the role of TNF-α in pulmonary edema. It was discovered that TNF-α also has a dichotomal role in this disease. The relation between the cytokine and pulmonary edema starts with the fact that TNF-α induces barrier dysfunction and endothelial cell activation (Ward 1996). These two processes are involved in acute lung injury. Moreover TNF-α has three ways of promoting pulmonary edema. The first one is through reactive oxygen intermediates induction. Another is caused by rearrangement of microtubules and because of transendothelial electrical resistance decrease across human pulmonary artery endothelial cells, and the last way is through TNFR-dependent up-regulation of chemokine production and also adhesion molecule expression (Faggioni et al. 1994; Hocking et al. 1991; Koh et al. 1996; Lo et al. 1992; Petrache et al. 2003; Wright et al. 2004; Yi & Ulich 1992).

In previous studies it was discovered that TNF-α downregulates ENaC activity and decreases the expression of α, β and γ ENaC subunits mRNA in alveolar epithelial cells. That can lead to an opinion that TNF-α inhibits edema reabsorption (Dagenais et al. 2004). On the other hand, other studies showed that cytokine has the ability to increase alveolar fluid clearance (AFC) in rodent inflammation models and a blockade of it causes an increase of the amount of edema formation in the pulmonary beds (Börjesson et al. 2000; Fukuda et al. 2001; Guice et al. 1991; Rezaiguia et al. 1997).

The molecule of TNF-α also contains, except for the receptor binding site, a lectin-like domain located at the other tip of the molecule (Figure 6) (Braun et al. 2005; Sherblom et al. 1988). This domain is responsible, among other things, for activating amiloride-sensitive sodium transport (Hribar et al. 1999). Taken together it means that the activity of lectin-like domain is hidden by the effect of TNFRs binding sites (Yang et al. 2010).

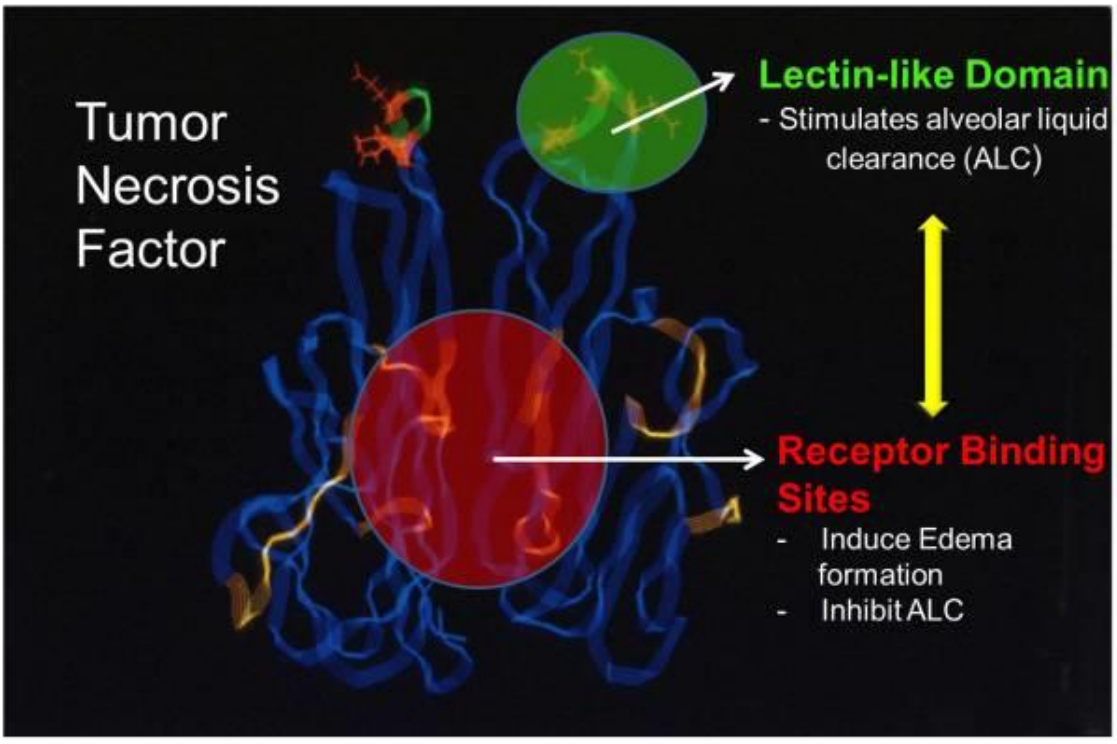

Figure 6: Dichotomal role of TNF-α molecule. Whereas receptor binding sites inhibit ALC and induce edema formation, the lectin-like domains stimulate ALC (Lucas et al. 2013).

1.5. AP301

AP301, so called TIP peptide or TNF-α lectin-like domain derived peptide, is a synthetic cyclic peptide composed of 17 natural amino acids (Figure 7) (Lucas et al. 1994).

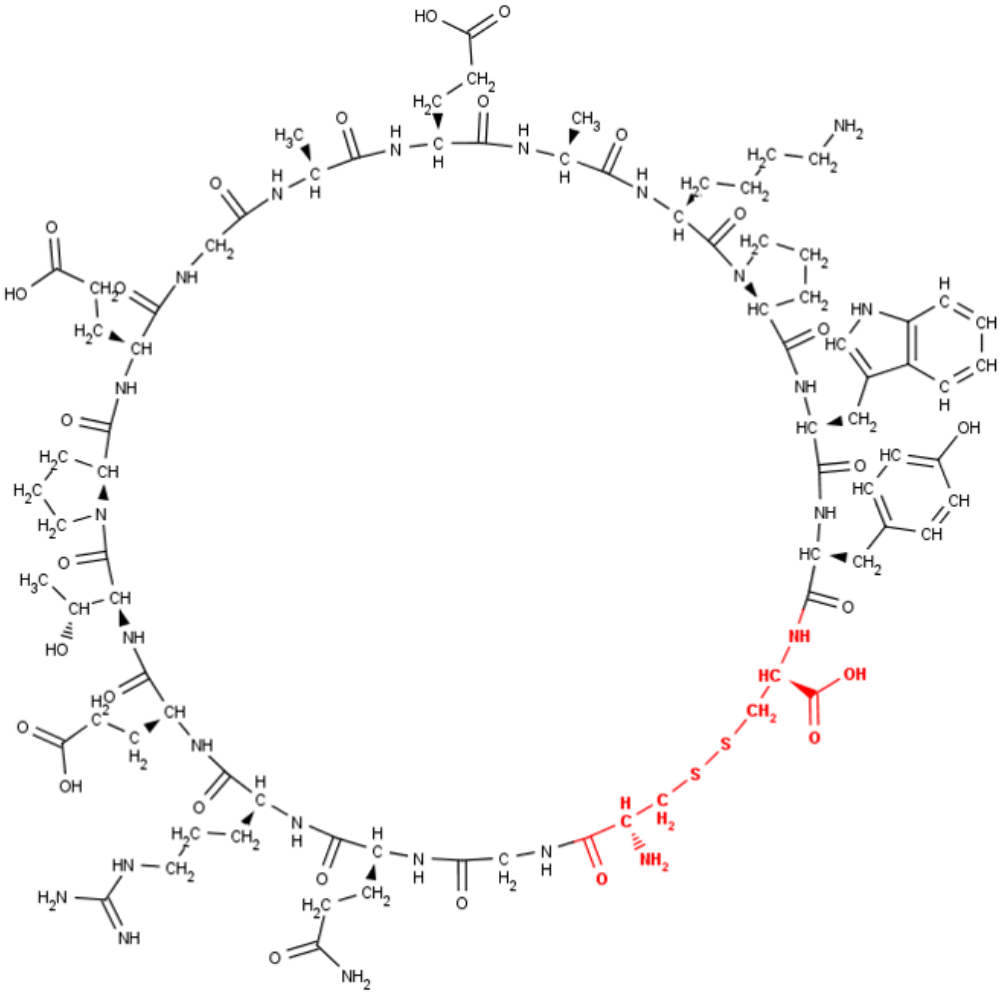

Figure 7: Cyclic structure of amino acids which AP301 is composed of (Hazemi et al. 2010).

It was shown that this molecule activates the Na^+ current through ENaC in dog, pig and rat freshly isolated ATII cells (Tzotzos et al. 2013) and it is also able to improve the lung function during acute lung injury in a pig model (Hartmann et al. 2013). It was also shown that AP301 activated ENaC in A549 cells (Hazemi et al. 2010) and ENaC expressed in HEK-293 cells (Shabbir et al. 2013).

2. Aim of this thesis

The aim of this thesis is to expand the knowledge about TNF-α and its influence on ENaC subunits expression, which has an impact on the severe disease called pulmonary edema. The work follows previous studies which have been done in the Department of Pharmacology and Toxicology at the University of Vienna, Austria.

These following objectives are determined in this thesis:

- Time dependent effect of TNF-α on expression of ENaC subunits

- Comparison the results obtained with TNF-α with the effect caused by TNF-α lectin-like domain derived peptide AP301

3. Materials and methods

3.1. Cell Culture and Transfection

Human embryonic kidney HEK-293 cells in passages 9 – 25 were used in this study. This cell line was seeded into Dubecco's Modified Eagle Medium/F12 nutrient mixture Ham plus L-Glutamine (Gibco™ by Life Technologies, LifeTech Austria) supplemented with 1% penicillin and 10% foetal bovine serum (FBS). Before each splitting the old medium was removed, cells were washed with phosphate-buffered saline (PBS) and they were trypsinised (trypsin/EDTA solution) and homogenised with a new amount of the medium. The cell culture with the medium was kept in a humidified incubator (37°C, 5% CO_2) until another splitting was necessary.

For the experiments the cells were placed in 10 mm^2 plates which were incubated until the confluence reached 70%. Then the cells were transfected with specific combinations of the ENaC wildtype subunits. For this purpose X-tremeGene HP DNA transfection reagent (Roche Diagnostics, Mannheim, Germany) was used. At first a solution of different DNA combinations (the exact volumes depend on particular DNA concentrations) and X-tremeGene HP DNA transfection reagent (8 µl per one plate) in a serum free medium – DMEM/F12 (175 µl per one plate) was prepared. The incubation period till the transfection complex was ready to use took 20 minutes. The medium from the plates was aspirated and the cells were washed with PBS which was also aspirated after washing. Subsequently the transfection complex was pipetted to the dishes and 10 ml of DMEM/F12 with FBS and penicillin was added (Roche 2014).

The transfected cells were put back to the humidified incubator until they were fully confluent in a monolayer which signalled a time for harvesting. The old medium was substituted for a solution of a DMEM/F12 without FBS (control) or a DMEM/F12 without FBS and with TNF-α (Sigma-Aldrich) – solution at the concentration 20 nM. The cell treatment took 5, 10, 30 and 60 minutes. After these periods the medium (with or without TNF-α) was removed and the monolayer was washed twice with PBS. After washing the stuck cells were scrapped and resuspended in an extraction buffer (150 mM sodium acetate, 0.9% NaCl, 0.1% Triton X-100 pH 5.5). An ultrasonication 3×10 seconds and a 30 minutes-long incubation in ice followed. After the 30 minutes all the samples were centrifuged for 10 minutes (4°C) at 13 000 rpm. The total concentration of a pure protein was determined by Bradford assay.

Recipes:

Table 1: List of recipes (part 1)

1× PBS: Phosphate-buffered saline; pH = 7.4	
NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
$CaCl_2 \cdot 2H_2O$	0.13 g
$MgCl_2 \cdot 6H_2O$	0.10 g
HCl	to pH to 7.4
ddH_2O	∑ 1 l
Medium	
Dubecco's Modified Eagle Medium/F12 nutrient mixture Ham plus L-Glutamine (Gibco™ by Life Technologies, LifeTech Austria)	450 ml
FBS	50 ml
Penicillin	5 ml
Extraction buffer	
Lysis buffer (see Western blot analysis)	500 µl
PMSF	10 µl/ml
Complete (protease inhibitor cocktail tablets, Roche)	25 µl/ml

3.2. Bradford assay

Bradford assay is a method of determining a protein concentration in a solution. The principle is that Coomasie brilliant blue binds to the protein and it converts from a protonated red cationic form (A_{max} = 470 nm) to a stable unprotonated blue form (A_{max} = 595 nm). This protein-dye form was detected in a spectrophotometer in 595 nm (Bradford 1976; Compton & Jones 1985; Fazekas De St. Groth et al. 1963; Sedmak & Grossberg 1977).

For the protein standards (diluted in different concentrations) Bovine Serum Albumin (BSA) was chosen, purchased from Sigma-Aldrich.

It was done according to the protocol for the 250 µl microplate assay. The tested proteins were diluted 3 times and 5 times. Each of them was pipetted in the amount of 5 µl

to three wells in the plate as well as the BSA standards and 250 µl of the Quick Start™ Bradford 1x Dye Reagent (Bio-Rad) was added. Then all of the samples were mixed with the pipette tip and whole plate was incubated at room temperature for 5 minutes. After the incubation the absorbances (λ = 595 nm) in a spectrophotometer were measured. The final protein concentrations were calculated using a calibration line (Bio-Rad 2014).

3.3. DNA transformation and extraction

It was essential to have sufficient amounts of the DNAs of ENaC subunits for the transfection. Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen) were used for the DNA transformation, which are basically competent *Escherichia coli* cells recommended for a routine subcloning into vectors. It was done in accordance with the official manual. At first DH5α cells were taken out of −80°C where they had to be stored and they were thawed on ice. 50 µl of them were transferred into a 1.5 ml microcentrifuge tube and 1 – 5 µl (dependent on the certain concentration) of DNA were added and all was mixed gently. A 30 minutes long incubation and afterwards a heat shock for 20 seconds in a 42°C water bath followed. The tubes were placed back on the ice and 950 µl of a prewarmed medium was added. The next step was a 1 hour-long incubation at 37°C at 250 rpm. Then 200 µl from each transformation was spread on prewarmed selective plates. These plates were stored for 16 – 18 hours at 37°C (Invitrogen 2006).

After this period, single colonies, which had grown up on the plates, were picked with a pipette tip and they were placed into glass tubes (filled with Luria-Bertani (LB) medium with a specific antibiotics). All these glass tubes with the picked colonies were incubated for 16 – 18 hours at 37°C and 250 rpm.

The DNA transformation was not always necessary because sufficient abundance of *E. coli* with the required DNA was stored in a glycerol stock. It means that only a cultivation from a glycerol stock was essential which is an almost identical procedure as it is written in the previous paragraph. The only difference is that a small quantity of a suspension from the glycerol stock is used instead of a single colony from the plates.

After the 16 – 18 hours-long incubation (37°C, 250 rpm) of the glass tubes the solution inside of them should be “cloudy”. That means the *E. coli* with the inserted

DNA was sufficiently reproduced. It was also a signal to begin with a DNA extraction. The process of DNA extraction enables a preparation of a high quality plasmid DNA from recombinant *E. coli* cultures. Thermo Scientific GeneJET Plasmid Miniprep Kit (Thermo Scientific) was used for this kind of the DNA extraction. The principle of the extraction was that the bacterial cells were resuspended and subjected to SDS/alkaline lysis and it liberated plasmid DNA. The lysate was neutralised for appropriate binding of plasmid DNA on a silica membrane in a spin column. After a centrifugation a supernatant containing the plasmid DNA was cleaned from cell debris and SDS precipitate. Then the DNA was washed to remove the contaminants and it was eluted with a small volume of the Elution buffer. Concretely, 250 µl of the Resuspension Solution (with added RNase A) was used and the bacteria were resuspended by pipetting and the suspension was transferred to a microcentrifuge tube. For a second step 250 µl of the Lysis Solution was added and all was mixed by inverting the tube. Addition of 350 µl of the Neutralization solution and another inverting followed. Afterwards the tube was centrifuged for 5 minutes and the supernatant was transferred to a GeneJET spin column from the kit. It was again centrifuged for 1 minute and the flow-through was discarded, the membrane was washed twice with 500 µl of the Wash Solution and the flow-through was also discarded in both cases. Another 1 minute of a centrifugation was needed to remove residual Wash Solution. The GeneJET spin column (with the plasmid DNA inside on the membrane) was transferred to a new microcentrifuge tube and 50 µl of the Elution Buffer was added to elute the plasmid DNA. A 2 minutes-long incubation at room temperature followed and then the concentration of the plasmid DNA could be measured. That was accomplished on a NanoDrop 1000 spectophotometer (Thermo Scientific) connected to a PC with NanoDrop programme (Thermo Scientific). The concentrations were measured by three-fold determinations.

Recipes:

Table 2: List of recipes (part 2)

LB medium	
Dry LB	20 g (contains Tryptone, NaCl, Yeast extract)
ddH2O	∑ 1 l

3.4. Western blot analysis

Western blot is one of the most important separation methods for protein analysis. In this study SDS – Polyacrylamide gel electrophoresis (SDS-PAGE) was used. The basis of the method is a separation of proteins in a gel due to an electrophoresis.

Sodium dodecyl sulphate (SDS) is a detergent which has the ability to denature and separate proteins according to their molecular weight. It breaks all native non-covalent intermolecular and intramolecular interactions and also gives a net negative charge to the proteins (Figure 8). That allows them to migrate through the gel in a relation to their charge (Creighton 1999; Hegyi 2013; Magdeldin 2012).

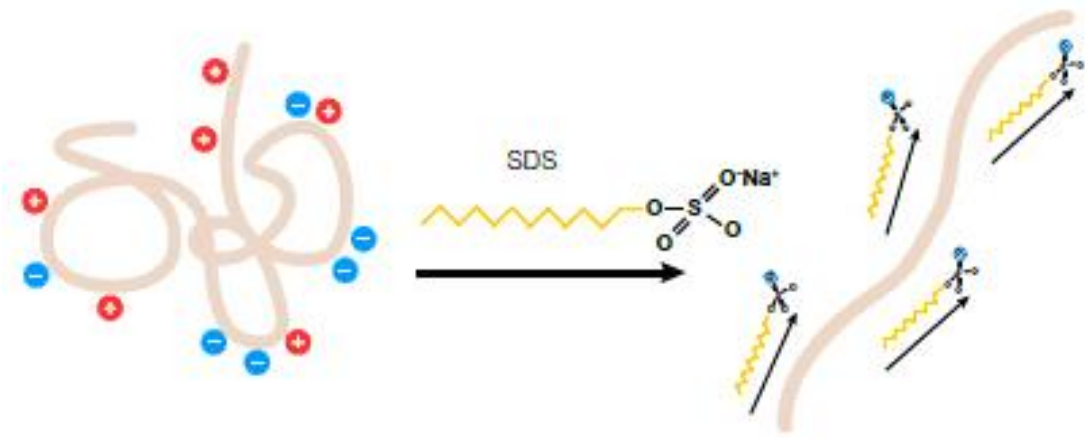

Figure 8: Effect of SDS on the conformation and charge of a protein (Bio-Rad 2015a).

Gel electrophoresis methods can be classified into two types – continuous and discontinuous. For these experiments the discontinuous method was used. In contrast to the continuous method, for discontinuous two types of gels and a running buffer are essential. The difference between the two gels is the size of their pores which is determined by the acrylamide concentration. The size of pores had to be set according to the molecular mass range of the proteins. The gel with a higher concentration of acrylamide (it has a smaller size of pores) is called running or also resolving gel, and the other gel, created from more diluted acrylamide, which is on the top of the running gel, is called stacking gel. Both types of gels (stacking and running) were made almost from the same chemicals but the volumes are different (see recipes below). Only the running gel provides the molecular sieving effect. To create the gel slabs both of them

were poured between two parallel glass sheets. The buffer, which was used for the discontinuous method and the chambers containing the electrodes are filled with, is called running buffer. The samples with a sample buffer (main components were SDS and bromophenol blue) were layered side by side to wells which were made on the top of the stacking gel. Due to the generated electric field the protein ions entered the stacking gel and afterwards the running gel in a sharp band. The proteins were separated in the running gel according to their charge, size and shape (Figure 9). In this case, the larger the molecule the shorter the way which it travelled in the running gel (Hegyi 2013).

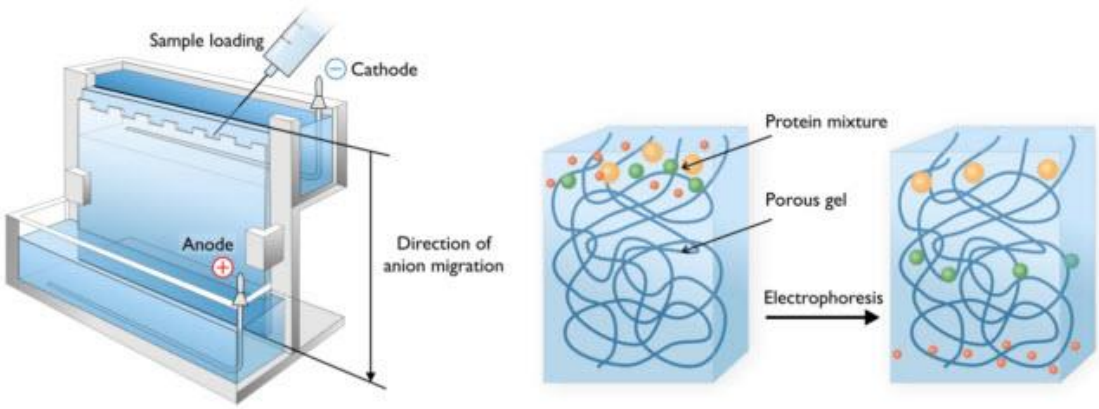

Figure 9: Separation of proteins in a polyacrylamide gel (Hegyi 2013).

When the proteins were spread in the running gel it was a time to transfer them on to a membrane – in this case made of polyvinylidene difluoride (UltraCruz™ PVDF membrane 0.45 μm, Santa Cruz Biotechnology). The membrane should be covered with the gel and it was made into a "sandwich" using two filter papers as it is in Figure 10. This setup was placed to Trans-Blot® Turbo™ Transfer System (Bio-Rad) – a machine performing perpendicular electrophoresis (25 V, 30 minutes, semi-dry blotting). Due to the blotting the proteins were transferred to the PVDF membrane in their spatial arrangement (Hegyi 2013; Magdeldin 2012).

Figure 10: Gel and membrane setup for electrophoretic transfer (Bio-Rad 2015b).

Subsequently the membrane was blocked with a blocking solution to occupy all available spots on the membrane. This quenching minimised the irrelevant background and reduced a nonspecific adsorption of the probe to the PVDF (Creighton 1999).

Thereafter a primary antibody was added. This antibody was specific against the target protein which it bound. To visualise where the primary antibody has bound a secondary antibody was applied. This antibody was specific for the primary antibody and usually conjugated to an enzyme or it was a fluorescent antibody which was detected by a fluorescent scanner (excitation wavelength 778 nm, emission wavelength 795 nm). Enzyme-conjugated secondary antibody (in this study antibodies with horseradish peroxidase were used) could be detected by using enhanced chemiluminescence (ECL) on a autoradiography film – the principle is pictured in Figure 11 (Hegyi 2013; Magdeldin 2012). These films could also be scanned for the purpose of obtaining a digital image.

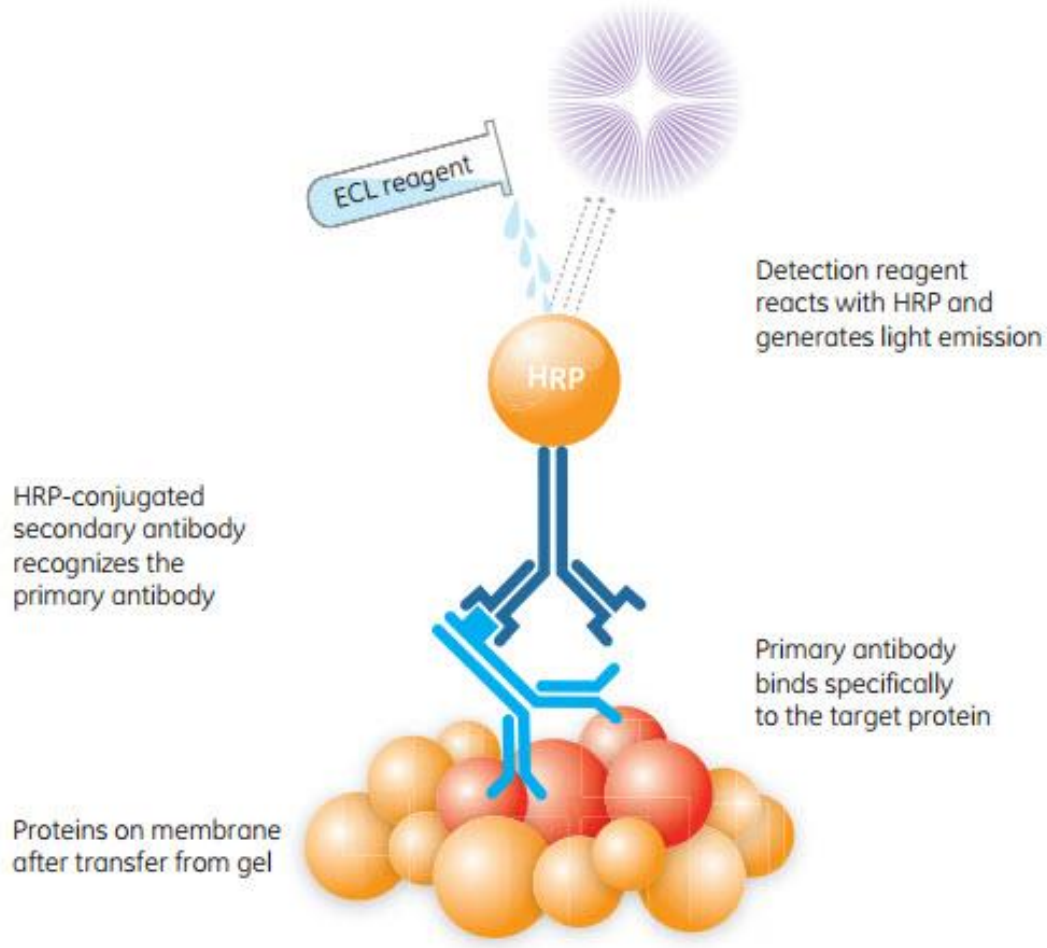

Figure 11: The principle of chemiluminescent Western blotting detection. The emitted light is detected on an autoradiography film (GE Healthcare Life Sciences 2011).

The digital images were analysed using myImageAnalysis™ Software (Thermo Scientific) and the results were calculated in Excel 2013 (Microsoft Corporation) and graphs were designed in Origin 9.0 (OriginLab®).

The whole theoretical process mentioned can be described more concretely as it was performed in this study.

The proteins were thawed and mixed in new vials in a ratio 1:4 with the 4× sample buffer and they were vortexed and centrifuged. The vials were heated (95°C) for 10 minutes and then centrifuged again. A vial with a marker was also prepared – Prism Protein Ladder (10 – 175 kDa) purchased from Abcam® or Color-coded Prestained Protein Marker (43 – 315 kDa) purchased from Cell Signaling Technology®.

Meanwhile, both kinds of gels were poured and polymerised. The electrophoresis machine with the bath (1× running buffer) was set up and the gels between the glasses were put inside the basin.

The small wells created on the top of the stacking gel were filled with the marker and the protein samples (20 μl) and the electrophoresis (200 V) began.

When the electrophoresis had finished the gels were washed in the 1× towbin buffer + MetOH and the "sandwiches" (Figure 10) were made and transferred to Trans-Blot® Turbo™ Transfer System (Bio-Rad) where the blotting took place.

After the blotting the membranes were removed from the "sandwiches" to a shaker and they were washed with TBS (3×5 minutes) and blocked with the blocking solution (for 2 – 3 hours at room temperature or overnight in 4°C). The membranes were subsequently washed with TBS/T (1×5 minutes) and incubated with a solution of a primary antibody for 1 hour. Afterwards they had to be washed again with TBS/T (5×5 minutes) and incubated with a solution of a secondary antibody (90 minutes). The next step was again the washing with TBS/T (3×5 minutes).

Then the membranes were taken from the shaker and incubated for 2 minutes with ECL solution (Cell Signaling Technology) and after this period they were transferred to a plastic foil and into an autoradiography film cassette (Kodak® X-Omatic). The next steps took place in a dark room. A sheet of an autoradiography film (Amersham Hyperfilm ECL was used, purchased from GE Healthcare Life Sciences) was placed on the top of the membrane. The cassette was closed and the film was exposed for 1 minute. The film was removed from the cassette and put into the developer solution (Adefo Chemie) for 3 – 5 minutes. A washing in water and then a bath in the fixing solution (Adefo Chemie) followed. After another washing in water a drying was provided. The duration of the exposure was optimised from the brightness of the first image. The dried images were scanned to obtain digital images which could be analysed.

The other option to taking the pictures was by using fluorescent secondary antibodies (incubation took 1 hour at minimum) and washing twice for 5 minutes with TBS/T. Subsequently the membranes were scanned on Odyssey® CLx (LI-COR, Inc.) and the digital images could be analysed as well.

As it was mentioned the calculations were made in Excel 2013 (Microsoft Corporation). Every result was converted to abbreviation units for the purpose of comparing results from different images. Abbreviation units were obtained from a quotient of the band density of a specific ENaC subunit protein and of the band density of β-actin from the same sample. It means that every membrane was firstly investigated for a specific ENaC subunit protein and secondly for the content of β-actin. β-actin is used as an internal standard and it indicates a total volume of the protein loaded into each well at the top of the stacking gel (GE Healthcare Life Sciences 2011).

It is essential to know the molecular mass of each ENaC subunit protein for the image analysis. The experience, resulted from many studies (Harris et al. 2008; Hughey et al. 2003; Ji et al. 2012; Santa Cruz Biotechnology, Inc.) and from the laboratory where this study was performed the bands of the proteins were detected subsequently: α-ENaC, β-ENaC and γ-ENaC bands are assumed to lie in the interval between 90 – 100 kDa and δ-ENaC around 110 kDa. The band of β-actin occurred every time at 43 kDa.

Recipes:

Table 3: List of recipes (part 3)

Running gel 7.5% (20 ml)	
ddH_2O	9.700 ml
30% Acrylamide/Bis (Bio-Rad)	5.000 ml
1,5 M Tris pH 8,8	5.000 ml
10% SDS (Sigma-Aldrich)	0.200 ml
10% APS (Bio-Rad)	0.100 ml
TEMED (Bio-Rad)	0.010 ml
Stacking gel 4% (12.55 ml)	
ddH_2O	7.460 ml
30% Acrylamide/Bis (Bio-Rad)	1.670 ml
0.5 M Tris pH 6.8	3.150 ml
10% SDS (Sigma-Aldrich)	0.125 ml
10% APS (Bio-Rad)	0.100 ml
TEMED (Bio-Rad)	0.010 ml

4× Sample buffer (10 ml)	
100% glycerol	4.0 ml
0.5 M Tris pH 6.8	4.8 ml
SDS (Sigma-Aldrich)	0.8 g
Bromophenol blue	4.0 mg
β-mercaptoethanol (Sigma-Aldrich)	0.5 ml
ddH_2O	0.7 ml
0.5 M Tris pH 6.8 (50 ml)	
Tris base (Amresco Inc.)	3.0 g
ddH_2O	∑ 50 ml
HCl	to pH 6.8
1.5 M Tris pH 8.8 (50 ml)	
Tris base (Amresco Inc.)	9.1 g
ddH_2O	∑ 50 ml
HCl	to pH 8.8
10× Running buffer (1 l)	
Tris base (Amresco Inc.)	30.0 g
Glycine (Sigma-Aldrich)	144.0 g
SDS (Sigma-Aldrich)	10.0 g
ddH_2O	∑ 1 l
1× Running buffer (1 l)	
10× Running buffer	100 ml
ddH_2O	900 ml
1× Towbin buffer + MetOH (1 l)	
10× Running buffer	100 ml
MetOH (Merck)	200 ml
ddH_2O	700 ml

10× TBS (1 l)	
Tris base	24.2 g
NaCl	80.0 g
HCl	to pH 7.6
ddH_2O	∑ 1 l
1× TBS (1 l)	
10× TBS	100 ml
ddH_2O	900 ml
1× TBS/T (1 l)	
10× TBS	100 ml
Tween 20 (Sigma-Aldrich)	1 ml
ddH_2O	899 ml
Blocking solution 3% (50 ml)	
BSA (Sigma-Aldrich)	1.5 g
10% NaN_3 (Sigma-Aldrich)	0.1 ml
1× TBS	∑ 50 ml
Primary antibody solution (50 ml)	
BSA (Sigma-Aldrich)	1.5 g
10% NaN_3 (Sigma-Aldrich)	0.1 ml
Concentrated antibody	volume depends on a particular type
1× TBS/T	∑ 50 ml
Secondary antobody solution (50 ml)	
FBS	0.25 ml
Concentrated antibody	volume depends on a particular type
1× TBS/T	∑ 50 ml
ECL solution (1 ml)	
SignalFire™ ECL Reagent A (Cell Signaling Technologies)	0.45 ml
SignalFire™ ECL Reagent B (Cell Signaling Technologies)	0.45 ml
SignalFire™ Plus ECL Reagent A (Cell Signaling Technologies)	0.05 ml
SignalFire™ Plus ECL Reagent B (Cell Signaling Technologies)	0.05 ml

Primary antibodies:

Table 3: List of primary antibodies

Antibody	**Animal**	**Dilution**	**Company**
anti-α-ENaC	goat	1:500	Santa Cruz Biotechnology, Inc.
anti-α-ENaC	rabbit	1:500	Thermo Scientific
anti-β-ENaC	goat	1:200	Santa Cruz Biotechnology, Inc.
anti-γ-ENaC	goat	1:500	Santa Cruz Biotechnology, Inc.
anti-δ-ENaC	rabbit	1:500	Santa Cruz Biotechnology, Inc.
anti-β-actin	mouse	1:500	Santa Cruz Biotechnology, Inc.

Secondary antibodies:

Table 5: List of secondary antibodies

Antibody	**Animal**	**Dilution**	**Company**
anti-goat IgG-HRP	donkey	1:5000	Santa Cruz Biotechnology, Inc.
anti-mouse IgG-HRP	rabbit	1:2000	Santa Cruz Biotechnology, Inc.
anti-rabbit IgG-HRP	donkey	1:5000	Santa Cruz Biotechnology, Inc.
anti-rabbit (H+L) IgG-HRP	donkey	1:5000	Promega
fluorescent anti-rabbit IgG (H+L)	donkey	1:5000	LI-COR
fluorescent anti-mouse IgG (H+L)	donkey	1:5000	LI-COR
fluorescent anti-goat IgG (H+L)	donkey	1:5000	LI-COR

3.5. Statistics

Paired t-test was performed using Excel 2013 (Microsoft Corporation) to establish statistical significance. Statistical significance was set at $p < 0.05$.

4. Results

I obtained the following results, which displays the influence of TNF-α on hENaC subunit expression, by using Western blot analysis. The cells were treated for four different time intervals (5, 10, 30 and 60 minutes) in all experiments and for the interval of 5 minutes I also used non-treated cells for a control.

Firstly I aimed to the influence of TNF-α on α-hENaC subunit expression. For this purpose I transfected the HEK-293 cells with the DNA for α subunit expression as well as with the DNA for β and γ subunit expression. The results are pictured in Figure 12. From this figure a transient increase in α subunit expression after 5 minutes treatment is visible, compared to basal levels. Nevertheless, this increase is not significant. However, in other time intervals α subunit is approximately at the same level as the basal one.

Secondly I focussed on δ subunit expression; after the treatment of TNF-α. I transfected the HEK-293 cells with the DNA for δ, β and γ subunit expression. Figure 13 shows an obvious significant transient increase in δ subunit expression after 5 minutes treatment. But as well as in the experiments with α subunit, levels of expression in other time intervals were settled at the similar level as the control.

The experiments with β and γ subunits expression were done concurrently with the previous two because from my experience there is no difference if those subunits are expressed with α or δ subunits.

A graph made from the experiments with β subunit is in Figure 14. Here I established the same conclusion as in the case with γ subunit. The only difference between these two experiments is a small increase in β subunit expression after 10 minutes and 30 minutes TNF-α treatment. But this increase is also not significant.

In Figure 15 the results from experiments with γ subunit expression are visible. It is clear that after the treatment with TNF-α almost no changes in the expression of this subunit occurred.

Figure 16 is a summarising graph of all experiments concerned with the influence of TNF-α on hENaC subunit expression.

The exemplary bands from the experiments, where cellular extracts of HEK-293 cells were subjected to SDS-PAGE and analysed with respective hENaC and β-actin antibodies, are pictured in Figure 16.

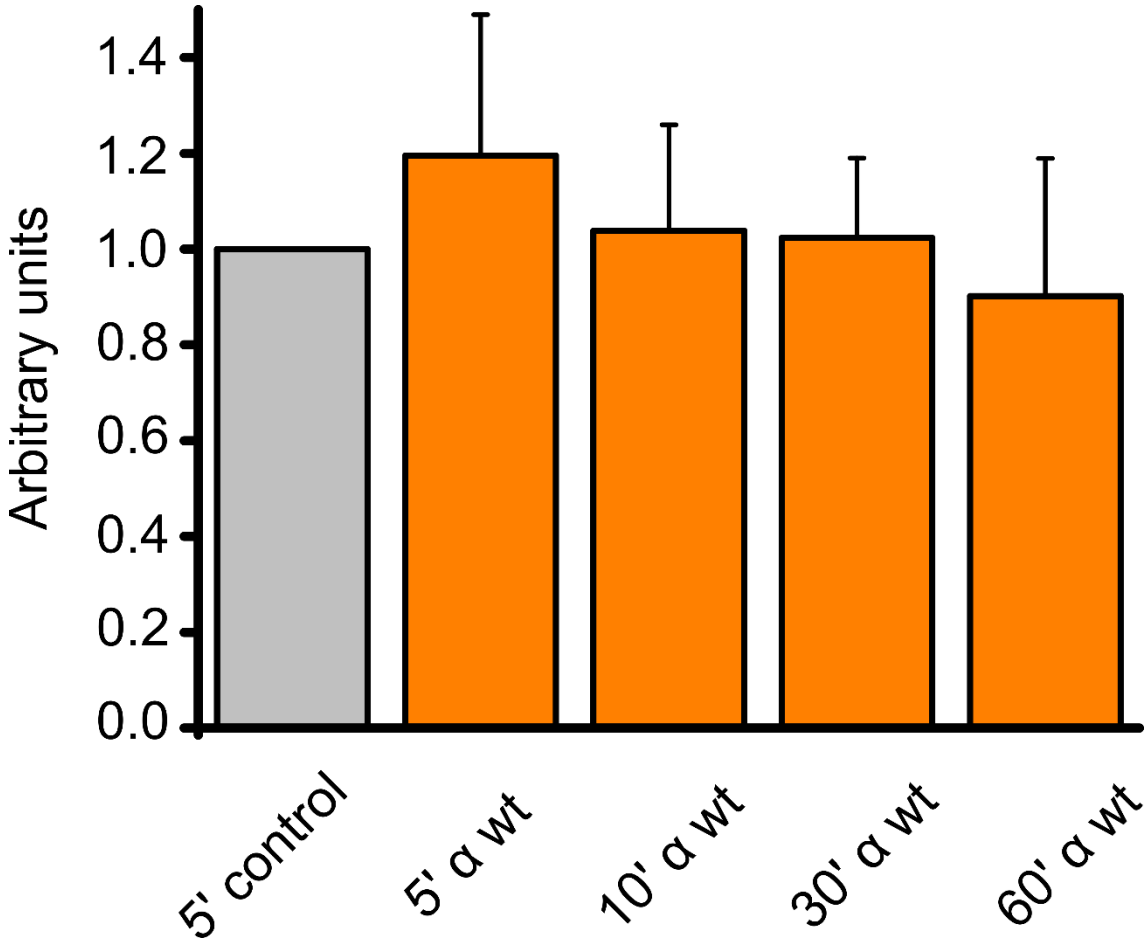

Figure 12: TNF-α does not significantly increase the α-hENaC subunit expression in HEK-293 transfected cells. However it has a potential to transiently increase the α-hENaC subunit expression in 5 minutes after the treatment with TNF-α. This increase was followed by a decrease to approximately a basal level of the expression.

Table 6: Influence of TNF-α on α-hENaC

Time interval and description	**Mean**	**SD**	**N**
5 minutes control	1.00	0.00	4
5 minutes α wt	1.19	0.29	4
10 minutes α wt	1.04	0.22	4
30 minutes α wt	1.02	0.17	4
60 minutes α wt	0.90	0.28	3

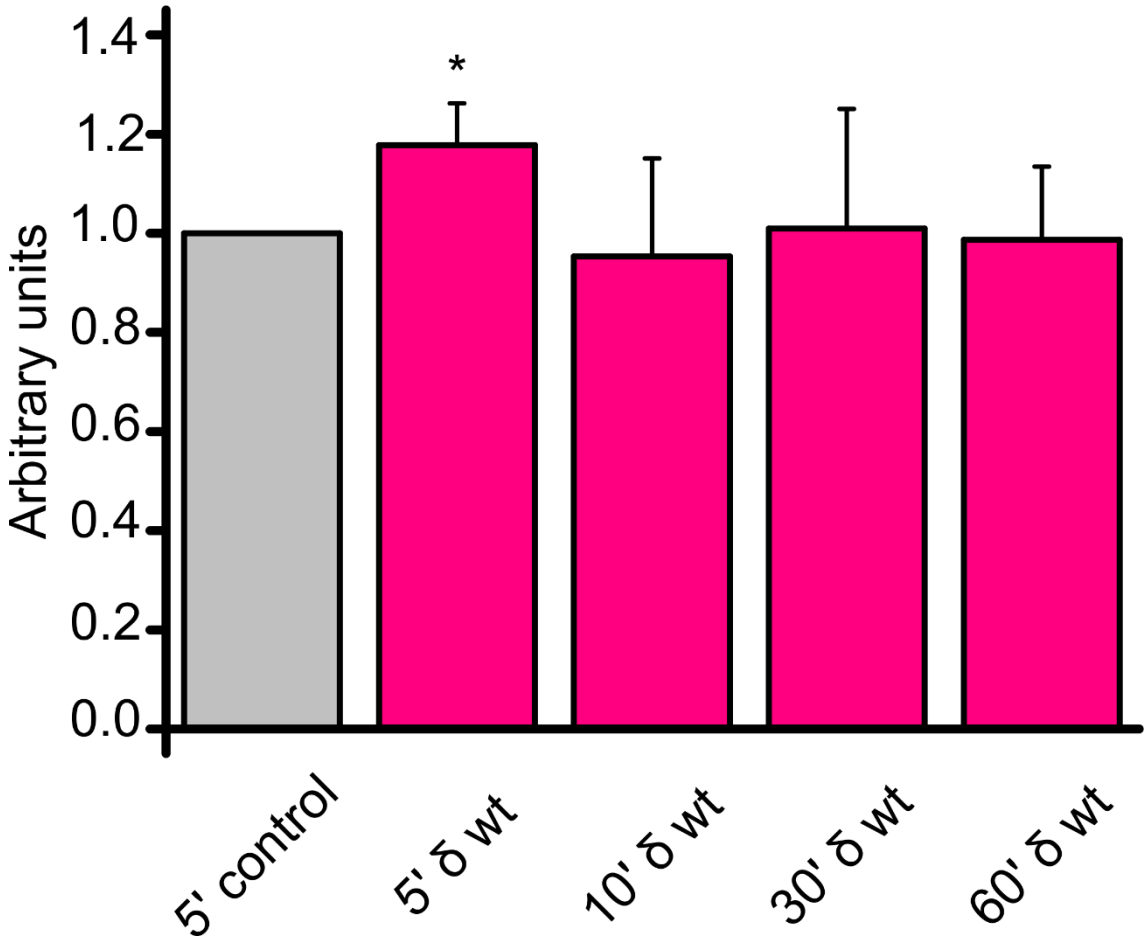

Figure 13: TNF-α significantly transiently increases the expression of δ-hENaC subunit in HEK-293 transfected cells. This significant transient increase is obvious in 5 minutes after the treatment. After this period the expression is settled back to the basal level.

Table 7: Influence of TNF-α on δ-hENaC

Time interval and description	**Mean**	**SD**	**N**
5 minutes control	1.00	0.00	4
5 minutes δ wt	1.18	0.08	4
10 minutes δ wt	0.95	0.20	4
30 minutes δ wt	1.00	0.24	4
60 minutes δ wt	0.99	0.15	4

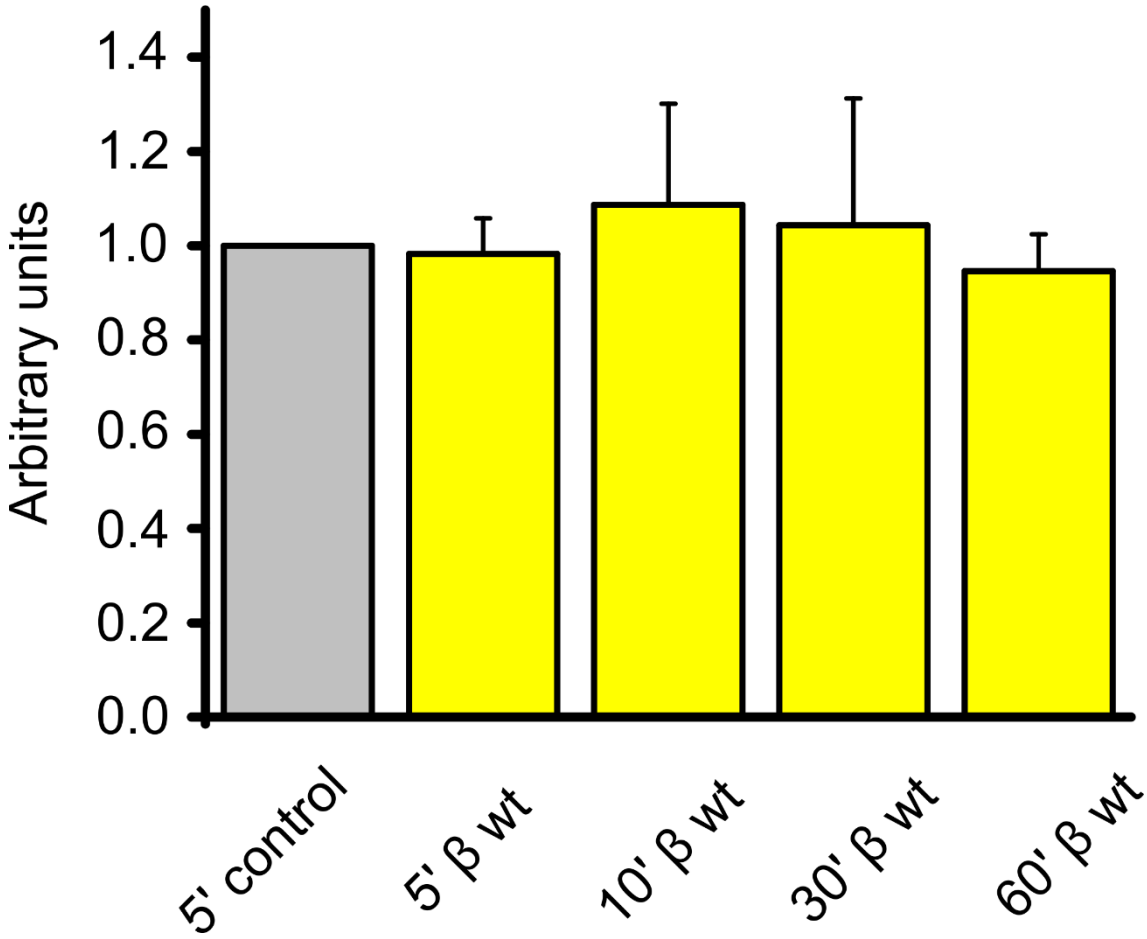

Figure 14: TNF-α has no significant influence to β-hENaC subunit expression in HEK-293 transfected cells. A small transient increase is obvious in 10 minutes and 30 minutes after the treatment; however, this increase is not significant.

Table 8: Influence of TNF-α on β-hENaC

Time interval and description	Mean	SD	N
5 minutes control	1.00	0.00	8
5 minutes β wt	0.98	0.07	8
10 minutes β wt	1.09	0.21	8
30 minutes β wt	1.04	0.27	8
60 minutes β wt	0.95	0.08	7

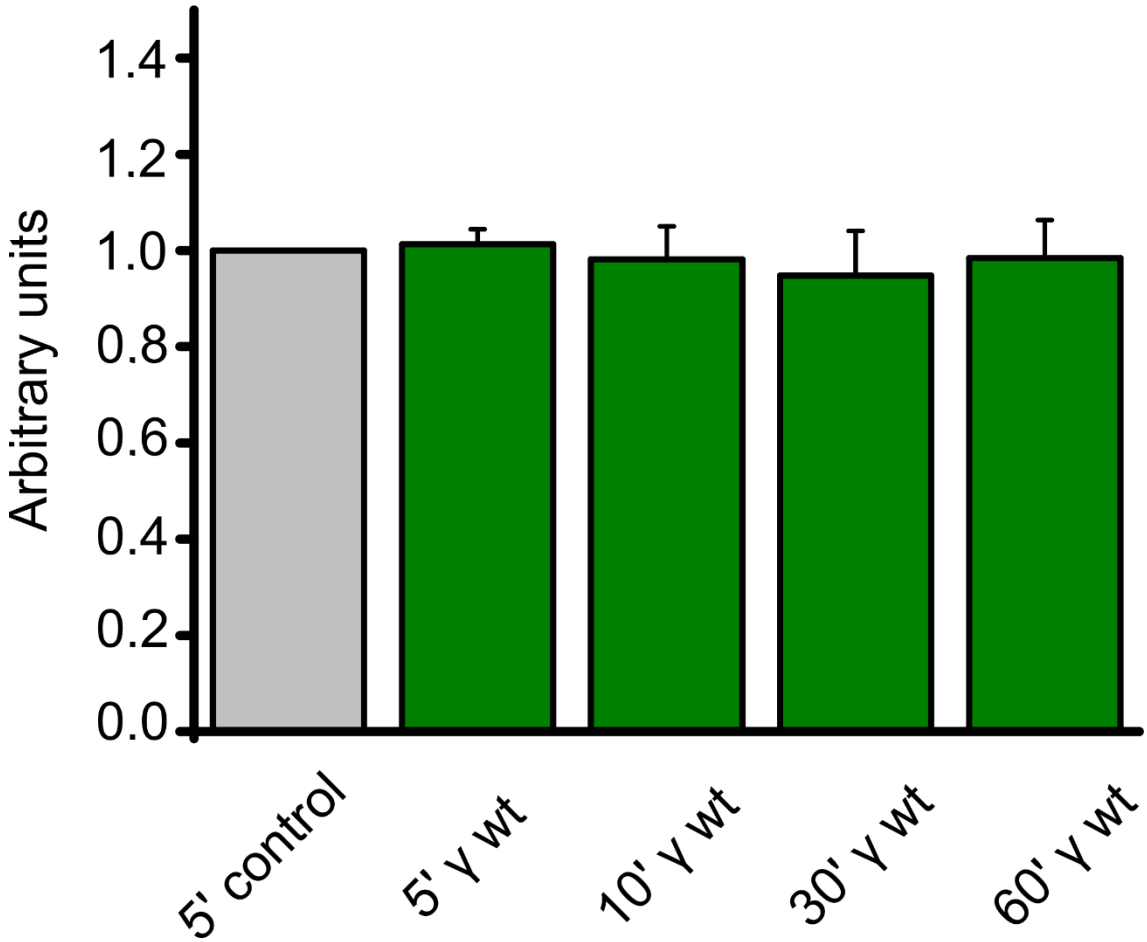

Figure 15: TNF-α has no significant influence on γ-hENaC subunit expression in HEK-293 transfected cells. The expression of this subunit stays at the basal level.

Table 9: Influence of TNF-α on γ-hENaC

Time interval and description	**Mean**	**SD**	**N**
5 minutes control	1.00	0.00	4
5 minutes γ wt	1.01	0.03	4
10 minutes γ wt	0.98	0.07	4
30 minutes γ wt	0.95	0.09	4
60 minutes γ wt	0.98	0.08	4

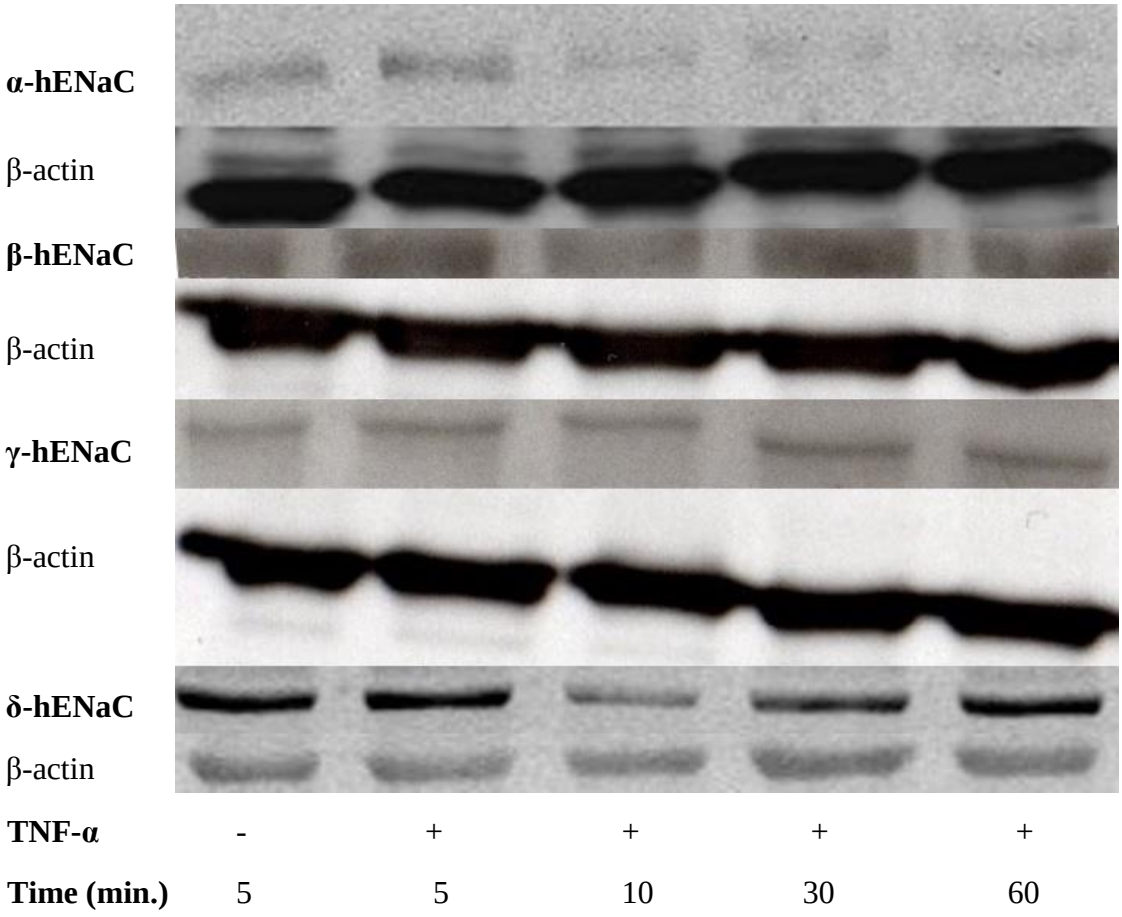

Figure 16: Western blot bands from an experiment. TNF-α transiently increases the expression of δ-hENaC and has a potential to transiently increase the expression of α-hENaC in HEK-293 cells. The expression of β-hENaC and γ-hENaC in the same type of cells remains almost changeless.

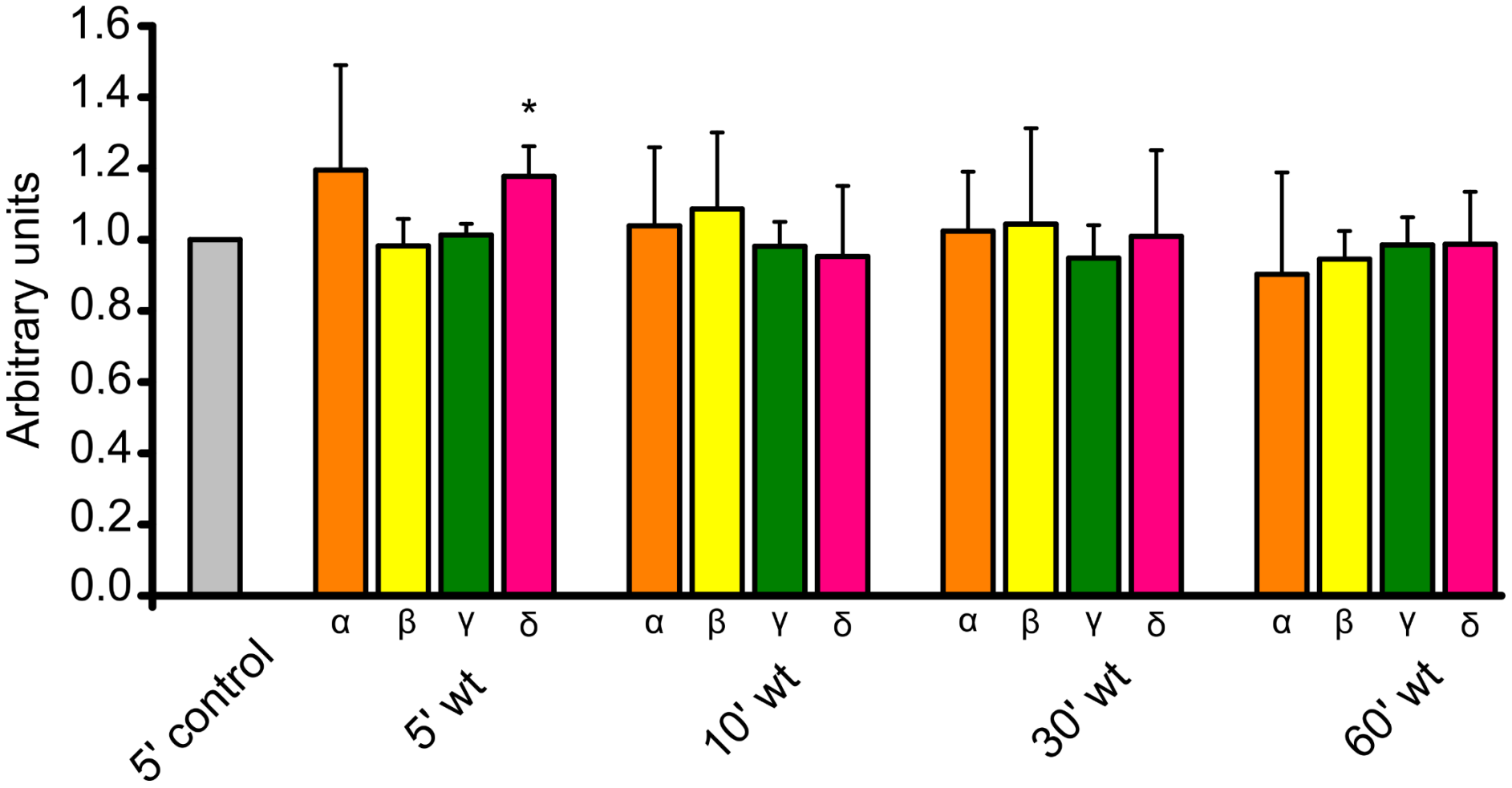

Figure 16: Summarising graph of all the experiments. TNF-α significantly transiently increases only the expression of δ-ENaC subunit in HEK-293 transfected cells.

5. Discussion

Recently published data describes a dichotomal role of TNF-α on pulmonary edema and alveolar liquid clearance (ALC). These effects are caused by two different sites on the TNF-α molecule which have opposite functions – the lectin-like domain and the receptor binding site (Lucas et al. 2013). It is also known that this cytokine downregulates the activity of ENaC and decreases the expression of α, β and γ ENaC subunits mRNA in alveolar epithelial cells (Dagenais et al. 2004).

It was also discovered that a TIP peptide called AP301 (solnatide), which is a synthetic cyclic peptide derived from TNF-α lectin-like domain activates Na^+ current through ENaC in ATII cells (Tzotzos et al. 2013) and an animal models (Hartmann et al. 2013). Recent studies have shown that it is not only because AP301 has an influence on the ion channel kinetics but it also affects the expression of ENaC subunits (Czikora et al. 2014).

The first aim of this study was to reveal the time-dependent influence of TNF-α on the single hENaC subunit expression. I reached this objective by experiments with HEK-293 transfected cells. I transfected the cells with a specific combination of hENaC subunit's DNAs (αβγ or βγδ) and I treated them with 20 nM TNF-α. Afterwards I provided a protein harvesting and western blot analysis. My results suggest that TNF-α has an influence on hENaC α and δ subunits expression. These two subunits are supposed to have the principal responsibility for the channel function (Canessa et al. 1994b; Ji et al. 2012).

The expression of α-hENaC subunit was transiently increased (19%) in 5 minutes after the treatment with TNF-α; however this increase was not significant. Also the expression of δ-hENaC subunit was transiently increased (18%) after 5 minutes treatment with TNF-α. Nevertheless this increase is significant. I have noticed small non-significant transient increases (9% and 4%) in β-hENaC subunit expression in 10 and 30 minutes after the treatment but these deviations are not supposed to be important. In the case of γ-hENaC subunit expression there was no visible increase or decrease in the expression.

These results suggest that TNF-α has an influence on α- and δ-hENaC subunit expression and it has the ability to affect the function of this channel.

The second purpose of this study was to compare the previous results with the results from recent studies with AP301. Results (Shabbir et al. 2015) of experiments which also used Western blot analysis for this part of experiments, showed that endogenously expressed subunits of hENaC are influenceable by AP301 (Figure 17).

I compared these results with the results from my experiments (Figure 16) and a similarity between these two groups of results is obvious.

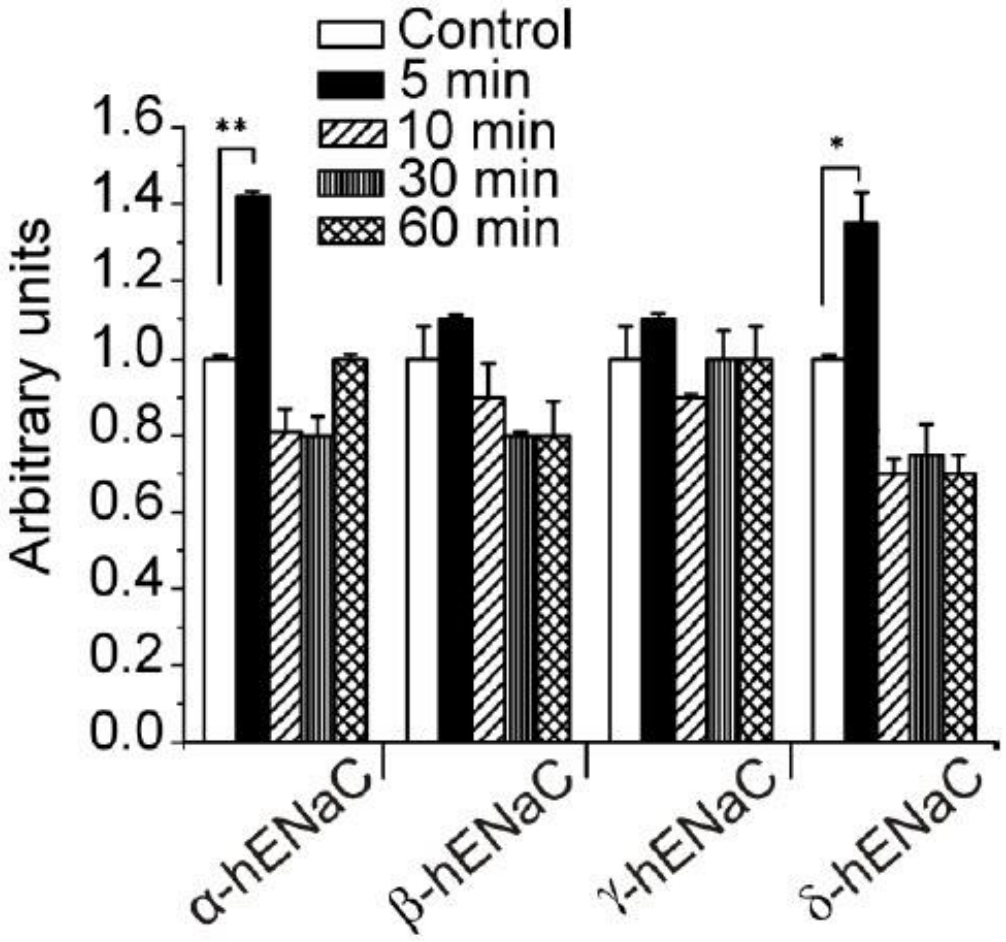

Figure 17: AP301 transiently increases the expression of hENaC in A549 cells. A significant transient increase of the expression of α and δ hENaC subunit in 5 minutes after the treatment, followed by a decrease after 10, 30 and 60 minutes is transparent. On the other hand, the expression of β and γ hENaC subunits are not significantly increased. *$p < 0.05$, **$p < 0.01$ – determined by t-test, n = 3-7 (Shabbir et al. 2015).

However, it has to be said that the results from Figure 17 were obtained from experiments with A549 cells which express all the hENaC subunits endogenously (in contrast with HEK-293). But it was reported that AP301 interacts with ENaC in both cases – when it is expressed endogenously and heterologously (Shabbir et al. 2013).

From Figure 17 is obvious that the expression of α- and δ-hENaC subunits in A549 cells is significantly transiently increased in 5 minutes after the treatment with AP301 and after this period the expression comes back to its basal level. β and γ subunits expression is not increased for whole time of the experiment. These results are analogous to those from my experiments with TNF-α – α and δ subunits expression was transiently increased in 5 minutes after the treatment (even though the expression of α subunit was not increased significantly) and almost no change was noticed in the expression of β and γ subunits.

The deviations between the results comparison could be caused by two different cell lines which were used in each experiment. Moreover, TNF-α might also play the dichotomal role in these experiments; thus one part of the molecule could cause the increase of the expression but simultaneously another part of it might cause a small decrease of the expression. Another explanation of the variety could be that TNF-α, in contrast with AP301, is a much bigger molecule and the lectin-like domain, which is assumed to be the responsible part of TNF-α for the expression increase, could be masked with another part or domain of this molecule. Also it is supposed that AP301 is able to penetrate to the cell.

6. Conclusions

To conclude, my study shows that TNF-α transiently significantly increases δ-hENaC subunit expression and it has a potential to increase the expression of α-hENaC subunit. On the other hand, it has almost no effect on the expression of β-hENaC and γ-hENaC subunits.

These data are in a close relationship with data obtained from similarly designed experiments with AP301, a TNF-α lectin-like domain derived peptide.

7. List of abbreviations

A549 cells	Human lung adenocarcinoma epithelial cells
AFC	Alveolar fluid clearance
ALC	Alveolar liquid clearance
AP301	Solnatide (INN name)
APS	Ammonium persulfate
ATI	Alveolar epithelial type I cell
ATII	Alveolar epithelial type II cell
BSA	Bovine serum albumin
CNG	Cyclic nucleotide-gated cation channel
ddH_2O	Double distilled water
DH5α	Type of competent E. coli
DMEM/F12	Dubecco's Modified Eagle Medium/F12 nutrient mixture Ham plus L-Glutamine
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ENaC	Epithelial sodium channel
FBS	Foetal bovine serum
HEK-293	Human embryonic kidney cell
JUN	Jun N-terminal
hENaC	Human epithelial sodium channel
HRP	Horseradish peroxidase
LB medium	Luria-Bertani medium
MAP	Mitogen activated protein
MetOH	Methanol
N	Sample size
Na^+/K^+-ATPase	Sodium-potassium adenosine triphosphatase

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
mRNA	Messenger ribonucleic acid
PBS	Phosphate-buffered saline
P_o	Open probability
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBS/T	Tris-buffered saline and Tween 20
TEMED	Tetramethylethylenediamine
TIP	TNF-α lectin-like domain derived peptide
TNF-α	Tumour necrosis factor alpha
TNF-β	Tumour necrosis factor beta
TNFR	Tumour necrosis factor receptor
TNFR1	Tumour necrosis factor receptor 1
TNFR2	Tumour necrosis factor receptor 2

8. References

Adams, C.M., Snyder, P.M. & Welsh, M.J., 1997. Interactions between Subunits of the Human Epithelial Sodium Channel. *The Journal of Biological Chemistry*, 272(43), pp.27295–27300.

Althaus, M., Clauss, W.G. & Fronius, M., 2011. Amiloride-sensitive sodium channels and pulmonary edema. *Pulmonary Medicine*, 2011.

Anon, 2003. α ENaC (C-20): sc-22239. Available at: http://datasheets.scbt.com/sc-22239.pdf.

Anon, 2010a. β ENaC (C-20): sc-22242. Available at: http://datasheets.scbt.com/sc-22242.pdf.

Anon, β-Actin (ACTBD11B7): sc-81178. Available at: http://datasheets.scbt.com/sc-81178.pdf.

Anon, 2010b. γ ENaC (F-20): sc-22245. Available at: http://datasheets.scbt.com/sc-22245.pdf.

Anon, δ ENaC (H-230): sc-21015. Available at: http://datasheets.scbt.com/sc-21015.pdf.

Bangel-Ruland, N., Sobczak, K., Christmann, T., Kentrup, D., Langhorst, H., Kusche-Vihrog, K. & Weber, W. M., 2010. Characterization of the epithelial sodium channel delta-subunit in human nasal epithelium. *American Journal of Respiratory Cell and Molecular Biology*, 42(4), pp.498–505.

Belge, K. U., Dayyani, F., Horelt, A., Siedlar, M., Frankenberger, M., Frankenberger, B., Espevik, T. & Ziegler-Heitbrock, L., 2002. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *The Journal of Immunology*, 168(7), pp.3536–3542.

Bio-Rad, 2014. Quick Start Bradford Instruction Manual. pp.1–35.

Bio-Rad, 2015a. Effect of SDS on the conformation and charge of a protein. Available at: http://www.bio-rad.com/en-cz/applications-technologies/protein-electrophoresis-methods#sds_page.

Bio-Rad, 2015b. Protein Blotting Methods. Available at: http://www.bio-rad.com/en-cz/applications-technologies/protein-blotting-methods.

Börjesson, A., Norlin, A., Wang, X., Andersson, R. & Folkesson, H. G., 2000. TNF-alpha stimulates alveolar liquid clearance during intestinal ischemia-reperfusion in rats. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 278(1), pp.L3–L12.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, pp.248–254.

Braun, C., Hamacher, J., Morel, D. R., Wendel, A. & Lucas, R., 2005. Dichotomal role of TNF in experimental pulmonary edema reabsorption. *The Journal of Immunology*, 175(5), pp.3402–3408.

Butterworth, M. B., 2010. Regulation of the epithelial sodium channel (ENaC) by membrane trafficking. *Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease*, 1802(12), pp. 1166–1177.

Canessa, C. M., Merillat, A. M. & Rossier, B. C., 1994a. Membrane topology of the epithelial sodium channel in intact cells. *American Journal of Physiology-Cell Physiology*, 267(6), pp.C1682–1690.

Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J. D. & Rossier, B. C., 1994b. Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits. *Nature*, 367(6462), pp.463–467.

Collier, D. M., Collier, D. M., Tomkovicz, V. R., Peterson, Z. J., Benson, C. J. & Snyder, P. M., 2014. Intersubunit conformational changes mediate epithelial sodium channel gating. *The Journal of General Physiology*, 144(4), pp.337–348.

Compton, S.J. & Jones, C. G., 1985. Mechanism of dye response and interference in the Bradford protein assay. *Analytical Biochemistry*, 151(2), pp.369–374.

Creighton, T. E., 1999. *The encyklopedia of molecular biology*, ISBN 0-471-16661-8.

Czikora, I., Alli, A., Bao, H. F., Kaftan, D., Sridhar, S., Apell, H. J., Gorshkov, B., White, R., Zimmermann, A., Wendel, A., Pauly-Evers, M., Hamacher, J., Garcia-Gabay, I., Fischer, B., Verin, A., Bagi, Z., Pittet, J. F., Shabbir, W., Lemmens-Gruber, R., Chakraborty, T., Lazrak, A., Matthay, M. A., Eaton, D.C. & Lucas, R., 2014. A Novel TNF-mediated Mechanism of Direct Epithelial Sodium Channel Activation. *American Journal of Respiratory and Critical Care Medicine*, 190(5),
pp.1–49.

Dagenais, A., Fréchette, R., Yamagata, Y., Yamagata, T., Carmel, J. F., Clermont, M. E., Brochiero, E., Massé, C. & Berthiaume, Y., 2004. Downregulation of ENaC activity and expression by TNF-α in alveolar epithelial cells. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 286(2), pp.301–311.

Dobbs, L. G., Johnson, M. D., Vanderbilt, J., Allen, L. & Gonzalez, R., 2010. The great big alveolar TI cell: Evolving concepts and paradigms. *Cellular Physiology and Biochemistry*, 25(1), pp.55–62.

Eaton, D. C., Malik, B., Bao, H. F., Yu, L. & Jain, L., 2010. Regulation of epithelial sodium channel trafficking by ubiquitination. *Proceedings of the American Thoracic Society*, 7(1), pp.54–64.

Faggioni, R., Gatti, S., Demitri, M. T., Delgado, R., Echtenacher, B., Gnocchi, P., Heremans, H. & Ghezzi, P., 1994. Role of xanthine oxidase and reactive oxygen intermediates in LPS- and TNF-induced pulmonary edema. *The Journal of Laboratory and Clinical Medicine*, 123(3), pp.394–395.

Fazekas De St. Groth, S. N. E. E., Webster, R.G. & Datyner, A., 1963. Two new staining procedures for quantitative estimation of proteins on electrophoretic strips. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, 71, pp.377–391.

Folkesson, H. C. & Matthay, M. A., 2006. Alveolar epithelial ion and fluid transport: Recent progress. *American Journal of Respiratory Cell and Molecular Biology*, 35, pp.10–19.

Fronius, M., Bogdan, R., Althaus, M., Morty, R. E. & Clauss, W. G., 2010. Epithelial Na+ channels derived from human lung are activated by shear force. *Respiratory Physiology & Neurobiology*, 170(1), pp.113–119.

Fukuda, N., Jayr, C., Lazrak, A., Wang, Y., Lucas, R., Matalon, S. & Matthay, M. A, 2001. Mechanisms of TNF-alpha stimulation of amiloride-sensitive sodium transport across alveolar epithelium. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 280(6), pp.L1258–L1265.

Garty, H. & Palmer, L. G., 1997. Epithelial sodium channels: function, structure, and regulation. *Physiological Reviews*, 77(2), pp.359–396.

GE-Healthcare, 2011. Western Blotting, Principles and Methods. Available at: https://www.gelifesciences.com/gehcls_images/GELS/Related Content/Files/1334667780708/litdoc28999897_20150330003035.pdf.

Gormley, K., Dong, Y. & Sagnella, G. A., 2003. Regulation of the epithelial sodium channel by accessory proteins. *Biochemical Journal*, 371, pp.1–14.

Grell, M., Douni, E., Wajant, H., Löhden, M., Clauss, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K. & Scheurich, P., 1995. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell*, 83(5), pp.793–802.

Guice, K.S. et al., 1991. Anti-tumor necrosis factor antibody augments edema formation in caerulein-induced acute pancreatitis. *The Journal of Surgical Research*, 51(6), pp.495–499.

Haider, S. & Knöfler, M., 2009. Human Tumour Necrosis Factor : Physiological and Pathological Roles in Placenta and Endometrium. *Placenta*, 30(2), pp.111–123.

Harris, M., Garcia-Caballero, A., Stutts, M. J., Firsov, D. & Rossier, B. C., 2008. Preferential assembly of epithelial sodium channel (ENaC) subunits in Xenopus oocytes: Role of furin-mediated endogenous proteolysis. *The Journal of Biological Chemistry*, 283, pp.7455–7463.

Hartmann, E. K., Boehme, S., Duenges, B., Bentley, A., Klein, K. U., Kwiecien, R., Shi, C., Szczyrba, M., David, M. & Markstaller, K., 2013. An inhaled tumor necrosis factor-alpha-derived TIP peptide improves the pulmonary function in experimental lung injury. *Acta Anaesthesiologica Scandinavica*, 57(3), pp.334–341.

Hazemi, P., Tzotzos, S. J., Fischer, B., Andavan, G. S. B., Fischer, H., Pietschmann, H., Lucas, R. & Lemmens-Gruber, R., 2010. Essential structural features of TNF-α lectin-like domain derived peptides for activation of amiloride-sensitive sodium current in A549 cells. *Journal of Medicinal Chemistry*, 53(22), pp.8021–8029.

Hegyi, G., Kardos, J., Kovács, M., Málnási-Csizmadia, A., Nyitray, L., Pál, G., Radnai, L., Reményi, A. & Venekei, I., 2013. Introduction to Practical Biochemistry. Available at: http://elte.prompt.hu/sites/default/files/tananyagok/IntroductionToPracticalBiochemistry/book.pdf.

Hocking, D. C., Ferro, T.J. & Johnson, A., 1991. Dextran sulfate inhibits PMN-dependent hydrostatic pulmonary edema induced by tumor necrosis factor. *Journal of Applied Physiology*, 70(3), pp.1121–1128.

Hribar, M., Bloc, A., Van der Goot, F. G., Fransen, L., De Baetselier, P., Grau, G. E., Bluethmann, H., Matthay, M. A., Dunant, Y. & Pugin, J., 1999. The lectin-like domain of tumor necrosis factor-α increases membrane conductance in microvascular endothelial cells and peritoneal macrophages. *European Journal of Immunology*, 29(10), pp.3105–3111.

Hughey, R. P., Mueller, G. M., Bruns, J. B., Kinlough, C. L., Poland, P. A., Harkleroad, K. L., Carattino, M. D. & Kleyman, T. R., 2003. Maturation of the epithelial Na+ channel involves proteolytic processing of the alpha- and gamma-subunits. *The Journal of Biological Chemistry*, 278(39), pp.37073–82.

Invitrogen, 2006. Subcloning Efficiency ™ DH5α ™ Competent Cells. (18265), pp.5–8.

Ji, H. L., Zhao, R. Z., Chen, Z. X., Shetty, S., Idell, S. & Matalon, S., 2012. δ ENaC: a novel divergent amiloride-inhibitable sodium channel. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 303(12), pp.L1013–26.

Kishore, R., Tkebuchava, T., Sasi, S. P., Silver, M., Gilbert, H. Y., Yoon, Y. S., Park, H. Y., Thorne, T., Losordo, D. W. & Goukassian, D. A., 2011. Tumor necrosis factor-α Signaling via TNFR1/p55 is deleterious whereas TNFR2/p75 signaling is protective in adult infarct myocardium. *Advances in TNF Family Research*. pp. 433–448.

Kleyman, T. R., Carattino, M. D. & Hughey, R. P., 2009. ENaC at the cutting edge: regulation of epithelial sodium channels by proteases. *The Journal of Biological Chemistry*, 284(31), pp.20447–51.

Koh, Y., Hybertson, B. M., Jepson, E. K. & Repine, J. E., 1996. Tumor necrosis factor induced acute lung leak in rats: Less than with interleukin-1. *Inflammation*, 20(5), pp.461–469.

Li, J., Yin, Q. & Wu, H., 2013. Structural Basis of Signal Transduction in the TNF Receptor Superfamily. *Advances in Immunology*, pp.1–17.

Lingueglia, E., Renard, S., Voilley, N., Waldmann, R., Chassande, O., Lazdunski, M. & Barbry, P., 1993. Molecular cloning and functional expression of different molecular forms of rat amiloride-binding proteins. *European Journal of Biochemistry*, 216(2), pp.679–687.

Lo, S. K., Everitt, J., Gu, J. & Malik, A. B., 1992. Tumor Necrosis Factor Mediates Experimental Pulmonary Edema by ICAM-1 and CD18-dependent Mechanisms. *Journal of Clinical Investigation*, 89(3), pp.981–988.

Locksley, R. M., Killeen, N. & Lenardo, M. J., 2001. The TNF and TNF receptor superfamilies: Integrating mammalian biology. *Cell*, 104(4), pp.487–501.

Lu, M., Echeverri, F., Kalabat, D., Laita, B., Dahan, D. S., Smith, R. D., Xu, H., Staszewski, L., Yamamoto, J., Ling, J., Hwang, N., Kimmich, R., Li, P., Patron, E., Keung, W., Patron, A. & Moyer, B. D., 2008. Small molecule activator of the human epithelial sodium channel. *The Journal of Biological Chemistry*, 283(18), pp.11981–11994.

Lucas, R., Magez, S., De Leys, R., Fransen, L., Scheerlinck, J. P., Rampelberg, M., Sablon, E. & De Baetselier, P., 1994. Mapping the lectin-like activity of tumor necrosis factor. *Science*, 263(5148), pp.814–817.

Lucas, R., Czikora, I., Sridhar, S., Zemskov, E., Gorshkov, B., Siddaramappa, U., Oseghale, A., Lawson, J., Verin, A., Rick, F. G., Block, N. L., Pillich, H., Romero, M., Leustik, M., Schally, A. V. & Chakraborty, T., 2013. Mini-review: Novel therapeutic strategies to blunt actions of pneumolysin in the lungs. *Toxins*, 5(7), pp.1244–1260.

Magdeldin, S., 2012. *Gel Electrophoresis – Principles and Basics*, ISBN 978-953-51-0458-2.

Matthay, M. a, Folkesson, H.G. & Clerici, C., 2002. Lung epithelial fluid transport and the resolution of pulmonary edema. *Physiological Reviews*, 82, pp.569–600.

Matthay, M. a, Folkesson, H.G. & Verkman, a S., 1996. Salt and water transport across alveolar and distal airway epithelia in the adult lung. *American Journal of Physiology-Lung Cellular and Molecular Physiology*, 270(4), pp.L487–L503.

Petrache, I., Birukova, A., Ramirez, S. I., Garcia, J. G. & Verin, A. D., 2003. The role of the microtubules in tumor necrosis factor-alpha-induced endothelial cell permeability. *American Journal of Respiratory Cell and Molecular Biology*, 28(5), pp.574–581.

Rezaiguia, S., Garat, C., Delclaux, C., Meignan, M., Fleury, J., Legrand, P., Matthay, M. A. & Jayr, C., 1997. Acute bacterial pneumonia in rats increases alveolar epithelial fluid clearance by a tumor necrosis factor-alpha-dependent mechanism. *Journal of Clinical Investigation*, 99(2), pp.325–335.

Rhoades, R. & Bell, D., 2012. Medical Phisiology: Principles for Clinical Medicine, ISBN 978-1609134273.

Roche, 2014. X-tremeGENE HP DNA Transfection Reagent. pp.1–5.

Rothe, M., Pan, M. G., Henzel, W. J., Ayres, T. M. & Goeddel, D. V., 1995. The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell*, 83(7), pp.1243–1252.

Sedmak, J.J. & Grossberg, S.E., 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Analytical Biochemistry*, 79(1–2), pp.544–552.

Shabbir, W., Scherbaum-Hazemi, P., Tzotzos, S., Fischer, B., Fischer, H., Pietschmann, H., Lucas, R. & Lemmens-Gruber, R., 2013. Mechanism of action of novel lung edema therapeutic AP301 by activation of the epithelial sodium channel. *Molecular Pharmacology*, 84(6), pp.899–910.

Shabbir, W., Tzotzos, S., Bedak, M., Aufy, M., Willam, A., Kraihammer, M., Holzner, A., Czikora, I., Scherbaum-Hazemi, P., Fischer, H., Pietschmann, H., Fischer, B., Lucas, R. & Lemmens-Gruber, R., 2015. Glycosylation-dependent activation of epithelial sodium channel by solnatide.*Biochemical Pharmacology*, Epub ahead of print.

Sherblom, A. P., Decker, J.M. & Muchmore, A. V., 1988. The lectin-like interaction between recombinant tumor necrosis factor and uromodulin. *The Journal of iological Chemistry*, 263(11), pp.5418–5424.

Snyder, P. M., McDonald, F. J., Stokes, J. B. & Welsh, M. J., 1994. Membrane topology of the amiloride-sensitive epithelial sodium channel. *The Journal of Biological Chemistry*, 269(39), pp.24379–83.

Tzotzos, S., Fischer, B., Fischer, H., Pietschmann, H., Lucas, R., Dupré, G., Lemmens-Gruber, R., Hazemi, P., Prymaka, V. & Shabbir, W., 2013. AP301, a synthetic peptide mimicking the lectin-like domain of TNF, enhances amiloride-sensitive Na+ current in primary dog, pig and rat alveolar type II cells. *Pulmonary Pharmacology and Therapeutics*, 26(3), 356–363.

UniProt. Available at: http://www.uniprot.org/uniprot/P37091.

Waldmann, R., Champigny, G., Bassilana, F., Voilley, N. & Lazdunski, M., 1995. Molecular cloning and functional expression of a novel amiloride-sensitive Na+ channel. *The Journal of Biological Chemistry*, 270(46), pp.27411–27414.

Ward, P.A., 1996. Role of complement, chemokines, and regulatory cytokines in acute lung injury. *Annals of the New York Academy of Sciences*, 796(1), pp.104–112.

Wright, T. W., Pryhuber, G. S., Chess, P. R., Wang, Z., Notter, R. H. & Gigliotti, F., 2004. TNF receptor signaling contributes to chemokine secretion, inflammation, and respiratory deficits during Pneumocystis pneumonia. *Journal of immunology*, 172(4), pp.2511–2521.

Yang, G., Gorshkov, B., Sridhar, S., Verin, A., Lucas, R., Hamacher, J., White, R. & Chakraborty, T., 2010. The Dual Role of TNF in Pulmonary Edema. *Journal of Cardiovascular Disease Research*, 1(1), pp.29–36.

Yi, E.S. & Ulich, T.R., 1992. Endotoxin, interleukin-1, and tumor necrosis factor cause neutrophil-dependent microvascular leakage in postcapillary venules. *The American Journal of Pathology*, 140(3), pp.659–663.