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GENOMIC AND NON- GENOMIC EFFECTS OF A NEW SODIUM-SENSING NETWORK IN RENAL AND LUNG EPITHELIA

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The last sentence first...

*This book is dedicated to
Mattias, Esther and Agnes*

ABSTRACT

Sodium and water homeostasis is crucial for cell function, survival and health of the individual. The kidney is responsible for sodium regulation in the body, balancing sodium secretion and reabsorption, and thereby also regulating the blood pressure, primarily via the Na^+, K^+ -ATPase. The Na^+, K^+ -ATPase is situated in the basolateral part of the polarized cell membrane and drives the vectorial transport of Na^+ over the epithelium and water follows the ionic gradient. Vectorial sodium transport is important also in the lung alveoli, where the epithelium needs to be free from excess fluid for efficient gas exchange. Stimulation of the Na^+, K^+ -ATPase has been shown to increase lung edema clearance necessary for patient survival. The aim of this thesis is to investigate the existence of a cell sodium-sensing network in sodium-transporting epithelia, and the potential role of such network for cell and organ physiology.

In Article I a new network for activation of the Na^+, K^+ -ATPase in response to increased intracellular sodium was discovered. In a cell line originating from the proximal tubules of Opossum kidney (OK), we found that Salt Inducible Kinase 1 (SIK1) was responsible for activating the Na^+, K^+ -ATPase in response to increased intracellular sodium. Briefly, when $[\text{Na}^+]_i$ increases, $[\text{Ca}^{2+}]_i$ increases via the $\text{Na}^+/\text{Ca}^{2+}$ -exchanger which in turn activates calcium-calmodulin kinase 1 (CaMK1). CaMK1 phosphorylates SIK1 that becomes active and subsequently phosphorylates protein phosphatase methylesterase-1 (PME-1). Unphosphorylated PME-1 binds to and inhibits protein phosphatase 2A (PP2A). PME-1 leaves PP2A in its active state and PP2A dephosphorylates the Na^+, K^+ -ATPase that become active and removes the excess Na^+ from the intracellular space. Increased sodium reabsorption via SIK1 activating the Na^+, K^+ -ATPase may cause hypertension.

In Article II we were able to detect all three isoforms of SIK, SIK 1-3, in lung cells from mouse and rat, as well as in cell models from mouse and human. The β -adrenergic receptor (β -AR) agonist Isoproterenol (Iso) has been shown to increase Na^+, K^+ -ATPase activity in lung cells by increasing the number of active Na^+, K^+ -ATPase at the plasma membrane and to increase lung edema clearance. By depletion of SIK1 kinase activity, Na^+, K^+ -ATPase activity did not increase in response to Iso and the transport of Na^+, K^+ -ATPase to the plasma membrane was disrupted. SIK1 may therefor play an important role for lung edema clearance.

In Article III we show that reduction of SIK1 in MLE-12 cells lead to inhibited expression of E-cadherin, the hallmark protein for polarity. This was confirmed in lung tissue from *sik1*^{-/-} mice, and supported further in cells with higher SIK1 levels where more E-cadherin was expressed. We disclosed a regulatory mechanism where SIK1 reduction increased the activity of CREB, leading to increased levels of the transcription factors Snail2 and Twist, which in turn repressed the transcription of E-cadherin. The disruption of E-cadherin expression led to impaired sodium transport and integrity of the polarized epithelium.

In summary, SIK1 is needed for Na^+, K^+ -ATPase activation both in response to increases in $[\text{Na}^+]_i$ in the kidney and in response to β -AR stimulation in lung cells, important for blood pressure control and lung edema clearance, respectively. SIK1 contributes to cell polarity and function via regulation of E-cadherin gene expression.

POPULÄRVETENSKAPLIG SAMMANFATTNING

I kroppen är salt- och vätskebalansen främst reglerad i njurarna där ett protein som heter natrium-kalium-pumpen är ansvarig för återupptaget av natrium från urinen tillbaka till blodet. Natrium drar åt sig vattenmolekyler via osmotisk kraft. Korrekt reglering av natriumupptaget är viktigt, då en förhöjd natriumhalt leder till ett ökat upptag av vatten, en ökad blodvolym och därmed ett ökat blodtryck. Även på cellnivå är salt- och vattenbalansen ytterst viktig för optimal funktion, och om denna inte regleras kommer cellerna att svälla och till slut spricka av vattentrycket. En av de viktigaste regleringsmekanismerna för natriumhalten är just natrium-kalium-pumpen som sitter i cellmembranet och pumpar ut natrium ur cellerna.

I det första delarbetet av denna avhandling upptäckte vi att proteinet Salt-inducible Kinase 1, SIK1, ansvarar för regleringen av natrium-kalium-pumpen vid förhöjda natriumhalter. Kinaser påverkar andra protein genom att modifiera dem med en fosfatgrupp, s.k. fosforylering. Vi kartlade mekanismen hur SIK1 reglerar natrium-kalium-pumpen i en cellmodell från njuren. När natriumhalten ökar i cellerna ökar kalciumhalten, vilket leder till aktivering av en rad specialiserade protein, däribland SIK1, som leder till att natrium-kalium-pumpen aktiveras. Mer natrium pumpas ut ur cellen och natrium- och vattenbalansen återställs. Upptäckten av denna nya regleringsväg för natrium-kalium-pumpen leder till ökade kunskaper om hur salt- och vattenbalansen kan regleras i kroppen och kan eventuellt leda till nya behandlingsmetoder för högt blodtryck.

I det andra delarbetet visar vi att alla varianter av SIK finns i lungorna. Vi visar också att SIK1 är viktigt vid stimulering av s.k β -adrenerga receptorer med hjälp av kemikalien Isoproterenol, vilket leder till en ökad aktivitet av natrium-kalium-pumpen. Isoproterenol används för att öka natriumupptaget från vätskan i lungorna, vilket leder till absorption av vatten och därigenom minskad vätskemängd i lungorna hos patienter med lungödem. Vår upptäckt ökar förståelsen för hur lungödem kan behandlas.

I det tredje delarbetet visar vi att SIK1 reglerar ett protein som heter E-cadherin. E-cadherin finns i epitelceller, en specialiserad sorts celler som bland annat finns i lungorna, njurarna och tarmarna. E-cadherin binder samman en cell med granncellen och gör att epitelcellerna bildar ett tätt, ogenomsläppligt skikt. Det resulterar i att upptag och transport av näringsämnen, salter och vatten kan regleras inne i cellerna, då inget kan läcka däremellan. Vi visar att om mängden SIK1 i cellerna minskar, så minskar även mängden E-cadherin genom att s.k. transkriptionsfaktorer som hindrar bildningen av E-cadherin ökar markant. Epitelcellerna bildar inte ett lika tätt skikt vilket innebär att cellerna inte kan transportera bl. a. natrium på ett korrekt sätt.

Sammantaget visar vi att SIK1 spelar en mycket stor roll i regleringen av natrium-kalium-pumpen och därigenom salt- och vattenbalansen både i njurarna och lungorna. Dessutom behövs SIK1 för etableringen av ett tätt och funktionellt epitelcellslager genom att reglera genuttrycket av E-cadherin.

LIST OF PUBLICATIONS

This thesis is based on the following research articles, referred to in the text by their corresponding roman numerals (I-III).

- I. Sjöström M*, Stenström K*, **Eneling K**, Zwiller J, Katz AI, Takemori H, Bertorello AM. SIK1 is a part of a sodium-sensing network that regulates active sodium transport through a calcium-dependent process.
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- II. **Eneling K**, Chen J, Welch LC, Takemori H, Sznajder JI, Bertorello AM. Salt-inducible kinase 1 is present in lung alveolar epithelial cells and regulates active sodium transport.
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- III. **Eneling K***, Brion L*, Pinto V, Pinho MJ, Sznajder JI, Mochizuki N, Emoto K, Soares-da-Silva P, Bertorello AM. Salt-inducible kinase 1 regulates E-cadherin expression and intercellular junction stability.
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LIST OF ABBREVIATIONS

AEC	Alveolar epithelial cell
AFR	Alveolar fluid reabsorption
AJ	Adherens junction
ALI	Acute lung injury
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AngII	Angiotensin II
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
CaMK	Calcium Calmodulin-dependent protein kinase
cAMP	Cyclic AMP
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CREB	Cyclic AMP-responsive element binding protein
DA	Dopamine
DNA	Deoxyribonucleic acid
E-cadherin	Epithelial cadherin
ECM	Extracellular matrix
EGTA	Ethylen glycol tetraacetic acid
EMT	Epithelial to mesenchymal transition
ENaC	Epithelial Na ⁺ channel
ER	Endoplasmatic reticulum
GFP	Green fluorescent protein
GPRC	G-protein coupled receptor
GST	Glutathione S-transferase
H.T.	Hypertensive
HepG2	Human hepatoma-derived cell line
HK-2	Human kidney cell line
IPF	Idiopathic pulmonary fibrosis
MDCK	Madin-Darby Canine Kidney Cells
MLE-12	Murine lung epithelial cell line
mRNA	Messenger ribonucleic acid
N.T.	Normotensive
Na ⁺ ,K ⁺ -ATPase	Sodium potassium adenosine triphosphatase
NCCT	Sodium chloride co-transporter
NCX	Na ⁺ /Ca ²⁺ exchanger
NHE	Na ⁺ /H ⁺ exchanger
OK	Opossum kidney
OLF	Ouabain-like factor
PCR	Polymerase chain reaction
PKA	cAMP-dependent kinase
PKC	Protein kinase C
PME-1	Protein phosphatase methylesterase-1
PP2A	Protein phosphatase 2A

PTH	Parathyroid hormone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
sc-shRNA	Scrambled control shRNA
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
SIK	Salt-inducible kinase
siRNA	Small interfering RNA
Snflk	Sucrose non-fermenting like kinase
SOS	Sodium overly sensitive
TER	Transepithelial electric resistance
TJ	Tight junction
TORC2	Transducer of regulated CREB activity coactivator 2
W.T.	Wild type
ZO-1	Zona Occludens 1

1 INTRODUCTION

The sodium and water balance (cell homeostasis) in all living cells is crucial for the cell functionality and survival. The main regulator of total sodium in the body is the kidney, where the amount of sodium that is secreted with the urine is tightly regulated by a complex system of sodium transporters under the control of circulating hormones. In the kidney, the nephron is covered by epithelial cells, with a polarized distribution of the sodium transporters. The so called lateral side of the cell is facing the urine side of the nephron, and the basolateral part is attached to the basement membrane and extracellular matrix. In the lung, sodium and water homeostasis is a prerequisite for efficient gas exchange over the alveolar epithelium, where the apical side is directed toward the lumen of the alveolus.

1.1 CELL SODIUM AND WATER HOMEOSTASIS

During development, starting from plant cells, the ability to cope with high sodium load or changes thereof, has led to sophisticated sodium regulating mechanisms within the cells. The more sophisticated and advanced cell the more proteins and metabolites are present within. These particles have a negative net charge during physiological pH. The negative charges attracts positively charged ions, mainly sodium and potassium, balancing the negative charge (Donnan effect) (Leaf 1959). In turn, positively charged ions attract water. Sodium is the ion attracting most water molecules, due to the electron shell properties, and therefore sodium regulation is of highest importance for controlling cell volume. Uncontrolled water influx leads to swelling and subsequent bursting of the cell.

Plant cells, supported not only by the cell membrane but also by the cell wall, withstands higher sodium load than mammalian cells, due to the possibility to store excess sodium within vacuoles in the cytoplasm. Even so, plant cells suffer from *chemical salt stress* induced by high sodium concentration in the soil. High salinity affects cellular processes, such as metabolic enzymes that, can cause oxidative stress. Also, the uptake of potassium and water absorption by the roots is impaired by high levels of sodium in the soil (Katiyar-Agarwal, Zhu et al. 2006). As the plant cells cannot escape the high salinity, a number of salt tolerance systems have evolved. Salt overly sensitive (SOS) is a family of salt stress protective proteins. The Na^+/H^+ -antiporter SOS1 in the plasma membrane is responsible for removing excess sodium out from the cell, whereas other Na^+/H^+ -antiporters transport sodium into the vacuoles for storage (Gaxiola, Rao et al. 1999). SOS2 is a serine/threonine protein kinase that is activated by SOS3, a Ca^{2+} responsive kinase homologous to the Ca^{2+} Calmodulin-dependent kinase (CaMK) in mammalian cells (Liu and Zhu 1998; Liu, Ishitani et al. 2000).

By a mutation, ending up in a single amino acid change, a H^+ -pump was converted to the Na^+ , K^+ -adenosine triphosphatase (Na^+ , K^+ -ATPase), which is the main regulator of sodium in mammalian cells (Stein 1995). High sodium concentrations in the extracellular milieu cause *osmotic stress* and the ability to cope with the osmotic stress is crucial for cell function and survival.

By the appearance of the Na^+, K^+ -ATPase, mammalian cells developed the possibility to control the cell volume and to use the salinity, i.e. for building up membrane potential necessary for the excitable function of neurons, contraction of muscle cells, vectorial transport of nutrients over the plasma membrane, and secretion. A wide variety of sodium transporters have evolved, which will be described in the following section.

1.2 SODIUM TRANSPORTERS IN MAMMALIAN CELLS

The cell membrane is highly permeable to water but almost totally impermeable to sodium ions. The intracellular level of sodium is dependent on a network of co-operating sodium transporters.

1.2.1 Sodium channels

Sodium channels (ENaC) are constitutively active, allowing primary Na^+ , but also Li^+ and H^+ , into the cells. The activity of the channels can be increased by aldosterone, and the amiloride sensitive subtype can be inhibited by amiloride or triamterene (Garty and Palmer 1997; Soundararajan, Pearce et al. 2010).

1.2.2 Na^+/H^+ exchanger and $\text{Na}^+/\text{HCO}_3^-$

Sodium antiporters (Na^+/H^+ exchangers, NHE) and symporters ($\text{Na}^+/\text{HCO}_3^-$, NBC) are both pH regulating systems important for regulating the pH in the whole body. The two groups of transporters collaborate to extrude acidic residuals produced under metabolic processes. The CO_2 produced by these processes form HCO_3^- in the blood. In the kidney NHE secrete H^+ ions into the urine and HCO_3^- is reabsorbed via the NBCs, and in the lung the gas exchange process removes CO_2 from the blood into the exhaled air. This together, regulate cellular and whole-body pH (Sterling and Casey 2002).

1.2.2.1 Na^+/H^+ exchanger

The NHE extrude hydrogen ions from the cells, to increase the intracellular pH. Meanwhile, sodium ions are transported into the cells. The activity of the well-studied and highly conserved family of NHE's is regulated by both phosphorylation/de-phosphorylation events or via G-protein coupled receptor (GPCR) stimulation (Pedrosa, Gomes et al. 2004) and depending on isoform they can be highly sensitive to amiloride (Koliakos, Paletas et al. 2008). The NHE's are involved in many other cell functions than just pH_i and cell volume control such as; cell adhesion, migration, proliferation and apoptosis. The most widely expressed isoform is the NHE1, although in the kidney the NHE3 isoform is the dominating variant. The cellular localization differs between the isoforms as well, with all isoforms expressed in the cell membrane except the NHE-6 and -7, which are localized in intracellular membranes (Koliakos, Paletas et al. 2008).

1.2.2.2 $\text{Na}^+/\text{HCO}_3^-$ symporter

The sodium dependent family of NBC are almost ubiquitously expressed, and to a very high level in the kidney where it serves as a pH regulator (Aalkjaer, Frische et al. 2004; Bernardo, Bernardo et al. 2006). The NBCs transport can be either electrogenic with a $\text{HCO}_3^-:\text{Na}^+$ stoichiometry 3:1 as for transport out over the basolateral part of the

membrane in proximal tubules, 2:1 as in astrocytes and leech glia cells, or electro-neutral (1:1) as in small arteries and in the heart, favoring influx of HCO_3^- and Na^+ into the cells (Aalkjaer, Frische et al. 2004).

1.2.3 Sodium Calcium exchangers

The family of Sodium Calcium exchangers (NCX) constitutes electrogenic antiporters that can work in both directions, i.e. transport sodium ions both in and out of the cell. Three sodium ions are transported in one direction whereas one calcium ions is transported in the other, and the direction is dependent on the electrochemical ion concentration. Other stoichiometric ratios have also been reported. NCX1 is expressed almost ubiquitously at low levels, but highly abundant in heart, kidney and brain. NCX2 and NCX3 expression is limited to brain and skeletal muscle (Blaustein and Lederer 1999). The exchangers are located in the basolateral part of the cell membrane in kidney, but in olfactory epithelium it has been reported to be expressed in the apical part (Kwon, Koo et al. 2009). Calcium *per se* is a very important signaling molecule regulating various cascade reactions in the cells, moving both in and out of the cells and in between intracellular compartments (Clapham 2007; Lytton 2007).

1.2.4 Na^+ /glucose co-transport system

The Na^+ /glucose co-transport system (SGLT) takes advantage of the sodium gradient in order to transport glucose into the cells at the apical side (Scheepers, Joost et al. 2004). Since glucose is the main source for the production of ATP, cellular metabolism and homeostasis is highly dependent on glucose uptake, mainly in the intestine by SGLT1. Also in the kidney, glucose reabsorption takes place but primary (90%) via the SGLT2 in the first segment and to a lesser extent by SGLT1 in the third segment of the proximal tubule. The kidney reabsorbs 99% of the plasma glucose and at the same time, sodium (Bakris, Fonseca et al. 2009; Neumiller, White et al. 2010).

1.2.5 Na^+ /phosphate (Pi) co-transport system

The Na^+ /phosphate (Pi) co-transport systems (NPT1 and 2) are located in the brush border membrane of the renal proximal tubule cells, where they reabsorb most of the phosphate from the primary urine. While regulating the phosphate homeostasis, sodium is co-transported into the cell. The NPTs function is under the regulation of parathyroid hormone (PTH), dopamine (DA) and dietary phosphate levels (Murer and Biber 2010).

1.2.6 Na^+ /amino acid co-transport system

The Na^+ /amino acid co-transport systems are driven by the Na^+ gradient across the plasma membrane. There are different system for the transport of amino acids depending on charge and polarity of the amino acid and whether the transporter is sodium dependent or not. Amino acid transport over the membrane was shown to cause depolarization of the membrane as well as decrease the membrane resistance (Gonska, Hirsch et al. 2000). In lung alveolar epithelium, the Na^+ /amino acid co-transport system accounts for up to 13% of the sodium absorption from the alveolar fluid (Brown, Kim et al. 1985). Also, the system contributes to the electrical current over the plasma membrane (Brown, Kim et al. 1985; Jiang, Ingbar et al. 2000).

1.2.7 Sodium Chloride co-transporter

The sodium chloride co-transporter (NCCT) is, together with the potassium chloride and the sodium-potassium-chloride co-transporters, a member of the solute carrier transporter 12 family, and transports sodium from apical side into the cells. NCCTs are electroneutral and regulated by aldosterone, angiotensin II (AngII), insulin and vasopressin (Song, Hu et al. 2006; Pedersen, Hofmeister et al. 2010; van der Lubbe, Lim et al. 2011). Inactivation of NCCT by mutations causes low blood pressure, whereas over-activity leads to hypertension (Hoorn, Nelson et al. 2011). The function is inhibited by thiazide, one of the most potent antihypertensive drugs (Wright and Musini 2009).

1.2.8 Na⁺,K⁺-ATPase

The energy dependent Na⁺,K⁺-ATPase drives sodium out of the cell against the concentration gradient by pumping three sodium ions out and two potassium ions in. It utilizes one ATP per cycle (Skou 1957; Skou 1998) and is the main regulator of cell volume. The Na⁺,K⁺-ATPase is electrogenic, creating an electrical potential over the cell membrane, with a negative net charge on the inside compared with the outside of the cell, normally ranging from -30 to -70mV in mammalian cells (Morth, Pedersen et al. 2011). In both excitable and non-excitable cells, this electrical potential is necessary for the regulation of opening and closure of certain ion channels and transporters. The Na⁺,K⁺-ATPase consumes up to 60 % of the ATP in the cells, still only working on a third of its theoretical maximal capacity (Liang, Tian et al. 2007). The Na⁺,K⁺-ATPase is expressed in all mammalian cells, and in polarized cells, such as epithelial cells, it is located in the basolateral part of the membrane. The Na⁺,K⁺-ATPase consist of 2 to 3 subunits; the catalytic α -subunit (α_{1-4}), the β -subunit (β_{1-3}) with regulatory features and in some tissues also the γ -subunit interacts with the first two (Floyd, Wray et al. 2009). The γ -subunit belongs to the FXD family of proteins that serves as mediators of stability in a tissue specific manner (Geering 2006). The catalytic α -subunit has 10 transmembrane domains and holds the binding sites for Na⁺, K⁺, ATP and ouabain (a cardiotonic steroid), as well as numerous regulatory phosphorylation sites (Chibalin, Pedemonte et al. 1998; Done, Leibiger et al. 2002; Khundmiri, Bertorello et al. 2004). The β -subunit is required for proper folding, stability and insertion into the plasma membrane (Geering 1991; Geering 2001) but also important for the formation and integrity of cell polarity via the formation of tight junctions (TJ) and for suppression of cell motility and invasion of cancer cells (Rajasekaran, Palmer et al. 2001). The α - and β -subunits assemble in the endoplasmic reticulum (ER), and only correct formed dimers are transported to the basolateral part of the plasma membrane (Beguin, Hasler et al. 2000). The Na⁺,K⁺-ATPase lacks the possibility to enter “lipid rafts” and can therefore not be transported to the apical side of the membrane (Hammerton, Krzeminski et al. 1991; Mays, Siemers et al. 1995). In renal cells, the α_1 - and β_1 -subunits are expressed whereas lung epithelial cells also express the α_2 -subunit (Shyjan and Levenson 1989; Barquin, Ciccolella et al. 1997; Ridge, Rutschman et al. 1997).

The Na⁺,K⁺-ATPase is a p-type ATPase, changing its conformation in response to phosphorylation. The two main conformational stages have different affinities for the ions to be transported across the plasma membrane; E1 binding sodium and E2 binding potassium (Ogawa, Shinoda et al. 2009; Morth, Pedersen et al. 2011). Besides its role

in sodium transport, the Na^+, K^+ -ATPase has emerged as a signal transducer. When exposed to very low doses of ouabain the Na^+, K^+ -ATPase mediates a number of cellular responses including increase in intracellular calcium acting as a second messenger, activation of Src kinases, Ras, NF κ B, and production of reactive oxygen species (ROS) by the mitochondria (Xie and Askari 2002; Aperia 2007).

1.3 RENAL AND LUNG PHYSIOLOGY

In sodium transporting epithelia, simplified, the apical part of the cell membrane contains channels and exchangers letting sodium into the cells, while Na^+, K^+ -ATPases at the basolateral part actively pumps sodium out. The intracellular sodium concentration is kept relatively constant while ensuring a directed transport of sodium over the epithelial barrier, reabsorbed by the ECM and the blood stream and/or surrounding tissues.

1.3.1 The kidney

The kidneys are the key determinants of fluid volume in the body. The blood volume filtered by the kidney is approximately 180 L per day, producing 1 to 1.5 L of excreted urine. They are surrounded by the renal capsule and consist of the cortex, the medulla and the renal pelvis (Figure 1 A). The functional part of the kidney, the nephron (Figure 1 B), is built up by several segments spanning the cortex and medulla, and the urine produced is collected in the collecting tubes leading into the pelvis. The blood enters the kidney via the afferent arteriole into the glomerulus surrounded by the Bowman's capsule. The Bowman's capsule is the actual filtering unit, and the starting point of the nephron. The fluid is driven by hydrodynamic forces through the layer of endothelial cells of the capillaries, via the basement membrane and further through the epithelial layer of the capsule. Large molecules, like proteins, are physically hindered to pass through this filtering barrier (Gaudin 1989).

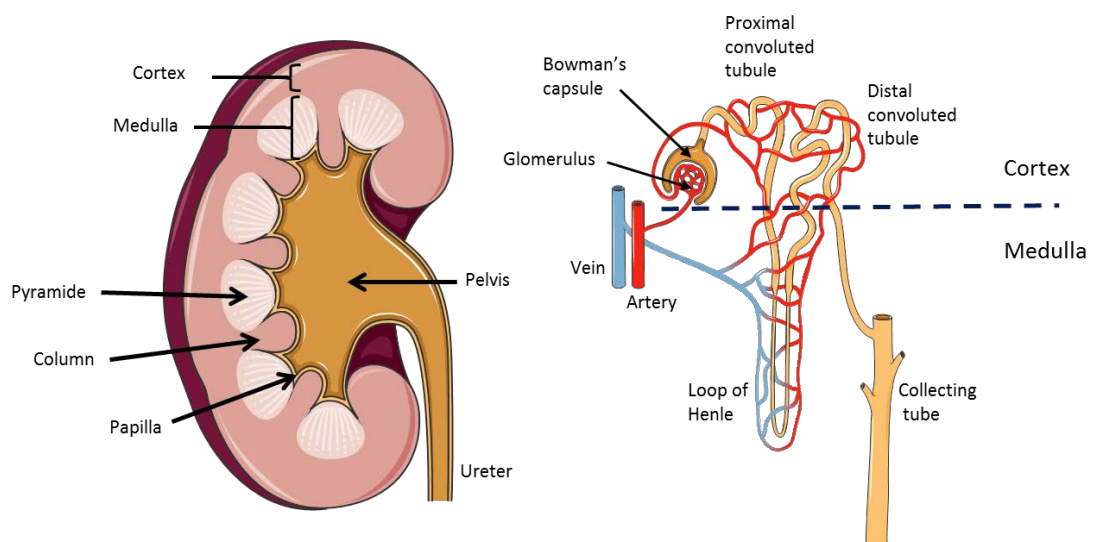


Figure 1. Kidney and nephron schematics. The kidney consists of the renal cortex, in which the Bowman's capsule and the proximal and distal convoluted tubules are situated with extremely high abundance of Na^+, K^+ -ATPases. The Loop of Henle descends into the medulla, also consisting of the collecting ducts, leading the urine to the pelvis.

After the capsule the tubular systems begins. The cells in the tubules are highly polarized epithelial cells, bound more or less tightly together via the TJ throughout the length of the nephron. The tighter the junctions, the tighter the membrane layer is, resulting in less ions and water passing over the epithelial layer by paracellular diffusion, but instead being actively transported through the cells. Sodium homeostasis in the cell is maintained via the Na^+, K^+ -ATPase at the basolateral part of the membrane that, together with sodium channels at the apical part of the membrane, account for the vectorial transport of Na^+ and subsequently H_2O . Up to 50 million Na^+, K^+ -ATPases are present in each cell (Feraille and Doucet 2001).

In the proximal convoluted tubules (PCT) the apical part of the cell is covered by brush borders consisting of numerous microvilli and thus, the surface area of the apical part of each cell is very large. The membrane is highly permeable to sodium, and 60-70% of the sodium in the filtrate is reabsorbed in the proximal tubule and further transported out of the cells via active sodium transport by the Na^+, K^+ -ATPase, with water following the osmotic gradient. The proximal tubule consists of a leaky, less tight epithelium, enabling water to pass through easily, renders up to total sodium reabsorption without changing the sodium concentration in the urine. DA has been shown to inactivate Na^+, K^+ -ATPases in the proximal tubule by endocytosis, thereby acting as a diuretic (Bertorello, Hokfelt et al. 1988). AngII on the other hand, affects the proximal tubules by recruitment of active Na^+, K^+ -ATPases to the basolateral membrane, thereby contribute to sodium reabsorption (Zhang, Guo et al. 2010).

The primary urine then reaches the Loop of Henle, consisting of a descending and an ascending segment. In the highly permeable descending part, water is passively moved by osmotic forces into the highly hypertonic medulla. The ascending part, on the other hand, has a very low permeability to water, although active sodium transport occurs, causing the medulla to be hypertonic. The Na^+, K^+ -ATPases at the basolateral part create an electrochemical gradient for sodium, serving as the driving force for the $\text{Na}^+/\text{K}^+/\text{Cl}^{2+}$ co-transport system to move sodium and chloride. Loop diuretics are used to block the movements of sodium and calcium in the ascending part of the loop, leading to a decreased osmolality in the medulla and thereby a decreased reabsorption of water from the descending part of the loop. Loop diuretics hence cause more sodium and water to retain in the filtrate, resulting in more water being excreted as urine and a decrease of the blood volume (Rang 1999).

Next part of the nephron is the early distal tubule where the epithelial layer is kept highly impermeable by TJs. At the apical side of the cell, NCCTs transport sodium into the cells while the Na^+, K^+ -ATPases at the basolateral part extrude sodium into the interstitium. Thiazide diuretics act on the NCCT, reducing the sodium uptake into the body (Rang 1999). At this part of the nephron, potassium and hydrogen ions are added to the filtrate. Also the calcium excretion is regulated in this segment (Gaudin 1989).

The filtrate is then collected from several distal tubules into collecting tubules, which join to form collecting ducts. The collecting tubules consist of two different cell types, the principal cells and the intercalated cells, and the epithelial layer is kept highly impermeable to both water and ions by TJs. The main function of principal cells is to reabsorb sodium and secrete potassium, whereas the intercalated cells mainly secrete

hydrogen ions. In this part of the nephron, the sodium and water regulation can be affected by hormones. Aldosterone stimulates sodium reabsorption by three mechanisms. Either rapidly by stimulation of NHEs via membrane aldosterone receptors, more long-term by binding to receptors within the cells leading to an upregulation of a mediator protein, which in turn activates sodium channels in the apical part of the membrane, or by increasing the number of basolateral Na^+, K^+ -ATPases (Welling, Caplan et al. 1993; Summa, Mordasini et al. 2001). Amiloride is used to inhibit sodium reabsorption via the ENaC in the distal convoluted tubule and collecting ducts in order to increase the excretion of sodium and water from the body. Anti-diuretic peptide (ANP) also blocks the ENaC (Rang 1999).

In summary, sodium reabsorption from the primary urine may occur in several compartments of the kidney and to various degrees. Accumulation of sodium in the renal medulla causes water retention and increased plasma volume, which in turn contributes to elevated blood pressure (Blaustein, Leenen et al. 2012). A fine-tuned regulation of sodium reabsorption is hence for sufficient blood pressure control.

1.3.2 The lung

The lung consists of the proximal conducting part, including the trachea, bronchi and bronchioles, and the distal part, which is built up by the respiratory bronchioles ending in the alveolus (Figure 2) (Gaudin 1989). The alveolus is where the gas exchange takes place, i.e. oxygen is taken up and carbon dioxide leaves the blood by diffusion. The alveolar wall consists of the endothelial cells in the capillaries, the interstitium containing smooth muscle cells, collagen and elastic fibers as well as macrophages, and the alveolar epithelial cell (AEC)s type 1 and type 2.

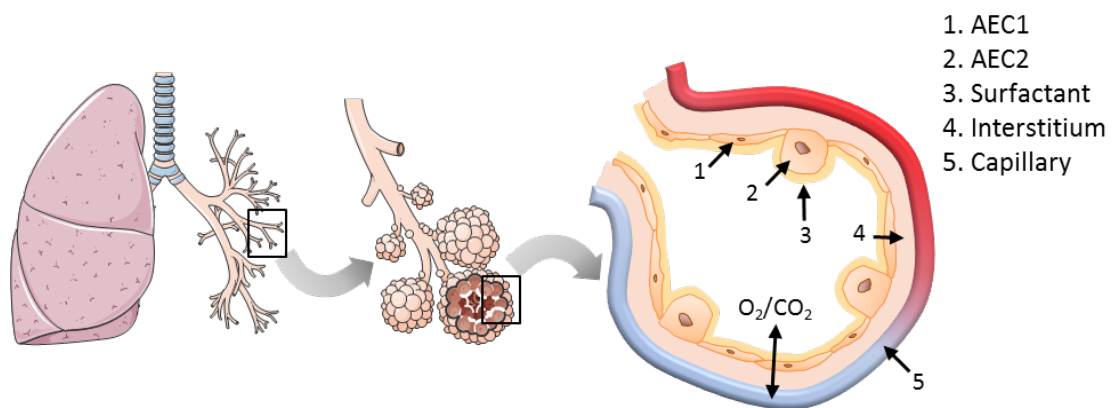


Figure 2. Lung structure and cell types. The respiratory tree branches from the trachea into the main stem bronchi, the conducting bronchioles and the respiratory bronchioles ending up in the alveolus. The alveolar epithelium consists of thin alveolar epithelial cells (AEC) type 1 and the cuboidal type 2.

The AEC1 and AEC2 are present in equal numbers, but the thin AEC1 cover more than 90 % of the surface. The cubical AEC2 might differentiate into type 1 cells (Johnson, Widdicombe et al. 2002). The AEC2 produces surfactant lipids and proteins that cover the epithelial cells and prevent the alveolar cavity to collapse due to the surface tension created by the thin film of water covering the epithelial cells in the alveolus (Herzog, Brody et al. 2008).

The surface tension of the alveolar epithelium, as well as proper O₂/CO₂ gas exchange over the epithelial-capillary barrier, is highly dependent on an efficient alveolar fluid reabsorption (AFR), i.e. absorption of excess fluid. The reabsorption is mainly driven by active sodium transport across the AEC1 and AEC2 via Amiloride sensitive- and insensitive epithelial sodium channels (ENaC) and cGMP gated cation channels at the apical part of the membrane (Yue, Russell et al. 1995; Jain, Chen et al. 1999). The Na⁺,K⁺-ATPase at the basolateral part of the membrane pump the Na⁺ into the lung interstitium and capillaries (Factor, Senne et al. 1998; Sznajder, Factor et al. 2002), creating a sodium gradient across the lung-capillary barrier. Water follows the sodium gradient isosmotically by entering the AECs via aquaporins at the apical side or via paracellular transport, generating an efficient AFR (Matthay, Folkesson et al. 2002).

1.4 REGULATION OF Na⁺,K⁺-ATPASE

As the Na⁺,K⁺-ATPase is the key regulator of Na⁺ concentration within the cell, its regulation needs to be tightly controlled. The short-term regulation of the catalytic activity occur primarily either via changes in substrate abundance (e.g. Na⁺, ATP, cAMP), increased Na⁺ affinity or antagonist binding inhibiting the pump (e.g. ouabain). Reversible phosphorylations are a cornerstone in Na⁺,K⁺-ATPase regulation. Generally, signaling pathways stimulating kinase activity correlates with an inhibition of Na⁺,K⁺-ATPase, whereas signaling pathways known to activate phosphatases correlates with increased Na⁺,K⁺-ATPase activity (Ewart and Klip 1995). Different circulating hormones may also regulate the abundance of active Na⁺,K⁺-ATPases at the plasma membrane, either by endocytosis of active Na⁺,K⁺-ATPases or recruitment of Na⁺,K⁺-ATPases from intracellular compartments. Prolonged exposure to hormones leads to regulation of Na⁺,K⁺-ATPase abundance either by increased *de novo* synthesis or degradation. The amount of Na⁺,K⁺-ATPase α - and β -subunits are affected via increased transcription of the ATP1A1 and ATP1B1 genes respectively (Therien and Blostein 2000).

The hormonal regulation of the Na⁺,K⁺-ATPases is complex, acting in a tissue specific manner. Hormones often exert their effect via GPCRs, where the binding G protein variant determines the response (Therien and Blostein 2000). DA for example, binds to and signals via both D₁-like and D₂-like DA receptors in the renal epithelial cells. Binding to D₁-like receptors leads to G α_s and G α_q activation, where G α_s activates adenylyl cyclase (cAMP) and subsequently cAMP-dependent kinase (PKA), and G α_q activation leads to phospholipase C (PLC) and protein kinase C (PKC) activation. D₂-like receptor stimulation activates G α_i which instead inhibits adenylyl cyclase and cAMP (Feraille and Doucet 2001). In PCT, DA treatment was shown to induce endocytosis of active Na⁺,K⁺-ATPases in a PKC ζ dependent manner via clathrin coated vesicles (Aperia, Bertorello et al. 1987). DA is used to decrease sodium reabsorption and acts as diuretics. A high salt intake could trigger the local (non-neuronal) DA production in the renal PCT cells and thereby serve as a regulator of sodium reabsorption and sodium homeostasis (Bertorello, Hokfelt et al. 1988; Soares-da-Silva, Fernandes et al. 1993). The DA system accounts for up to 50% of the regulation of sodium excretion when subjected to high sodium diet (Zeng, Sanada et al. 2004) and plays an important role for salt sensitivity and the development of essential hypertension (Zhang, Yao et al. 2011; Harris 2012).

In lung epithelium, almost the opposite takes place. DA increases Na⁺,K⁺-ATPase activity by stimulating recruitment of Na⁺,K⁺-ATPases from intracellular compartments via the D1 receptor (Bertorello, Komarova et al. 2003). By insertion of active Na⁺,K⁺-ATPases, the net flow of sodium over the epithelial layer increases, leading to improved AFR (Bertorello and Sznajder 2005; Vadasz, Raviv et al. 2007).

AngII, on the other hand, increases the number of active Na⁺,K⁺-ATPases at the plasma membrane of PCT by stimulation of the type I and type II receptors (AT1R and AT2R) if applied at picomolar concentration, whereas micromolar levels of AngII decreases the activity (Bharatula, Hussain et al. 1998; Efendiev, Budu et al. 2003). Yingst et al reported that AngII directly stimulates the activity and alters the phosphorylation of Na⁺,K⁺-ATPase in rat proximal tubule within minutes (Yingst, Massey et al. 2004).

Insulin binding to the insulin receptor acts as an antinatriuretic hormone by translocation of Na⁺,K⁺-ATPases to the plasma membrane of the PCT via tyrosine phosphorylation (Feraille, Carranza et al. 1994; Feraille, Carranza et al. 1999). In alveolar epithelial cells, insulin increases Na⁺,K⁺-ATPase activity by translocation of Na⁺,K⁺-ATPases to the plasma membrane in a Akt dependent manner (Sweeney and Klip 1998; Comellas, Kelly et al. 2010).

β₂-adrenergic receptor (β₂-AR) stimulation by e.g. isoproterenol (Iso) induces an increase of Na⁺,K⁺-ATPases at the plasma membrane in the alveolar epithelium via Rho dependent actin cytoskeleton rearrangements (Bertorello, Ridge et al. 1999; Lecuona, Ridge et al. 2003). In the kidney medulla and COS-7 cells isoproterenol has been shown to inhibit the Na⁺,K⁺-ATPase via PKC dependent phosphorylation (Giesen, Imbs et al. 1984; Cheng, Fisone et al. 1997) and in the tubular cells Iso sensitizes D1 receptors (Brismar, Agren et al. 2002).

In renal epithelia PTH inhibits Na⁺,K⁺-ATPase activity (Ribeiro, Dubay et al. 1994; Derrickson and Mandel 1997). PTH activates PKC, extracellular signal-regulated kinase (ERK) 1 and 2, and Src leading to phosphorylation and endocytosis of the Na⁺,K⁺-ATPase α-subunit in clathrin coated vesicles (Khundmiri, Bertorello et al. 2004; Khundmiri, Ameen et al. 2008).

Corticosteroids, such as aldosterone and dexamethasone regulate the Na⁺,K⁺-ATPase activity in both long- and short-term fashion, by increased transcription and localization of active Na⁺,K⁺-ATPases to the plasma membrane in both kidney and lung (Welling, Caplan et al. 1993; Olivera, Ciccolella et al. 2000).

Not only activity, cellular localization and expression levels of the Na⁺,K⁺-ATPase affect the sodium transport, desensitization of GPCRs and turnover of Na⁺,K⁺-ATPases also contributes to the regulation of sodium homeostasis. The Na⁺,K⁺-ATPase is ubiquitinated in renal cells (Coppi and Guidotti 1997) as well as in lung cells (Vadasz, Weiss et al. 2012), which serves as a signal for degradation by the proteosomal or lysosomal systems (Comellas, Dada et al. 2006).

1.5 CELL POLARITY AND VECTORIAL TRANSPORT

As mentioned earlier, epithelial cells are polarized in their architecture, with an apical and a basolateral side. The apical side faces the lumen of the tissue, i.e. the airspace in the alveolus, the lumen of the nephron or the interior of the colon, whereas the basolateral part of the cell is anchored in the extracellular matrix (ECM) and/or the basement membrane. Integrins are primarily responsible for the ECM binding (Berman, Kozlova et al. 2003) and the integrity of the epithelial layer is maintained by junctional structures in the lateral part of the membrane, starting from the apical part with the tight junctions (TJ), adherens junctions (AJ) and desmosomes (Farquhar and Palade 1963). The intracellular communication takes place via the gap junctions. This structural arrangement ensures that the epithelial layer can act as a diffusion barrier by regulating the paracellular diffusion of ions and small non-charged solutes. To assess the integrity of an epithelial layer, transepithelial electrical resistance (TER) can be measured in cells grown on permeable support. The tighter the epithelium, the higher resistance is over the cell monolayer (Perkins and Handler 1981).

TJs are mainly built up by claudin and members of the tight junction-associated MARVEL protein (TAMP) family (including occludin, tricellulin, and MARVELD3) (Schulzke, Gunzel et al. 2012) connecting one cell to the neighboring by homo- or heterodimerization. Intracellularly, Zona Occludens (ZO) 1 and other scaffolding proteins connect the TJ proteins to the cytoskeleton. The complexity of TJs has been investigated by proteomic approaches and bioinformatics, suggesting up to 100 different proteins interacting (Tang 2006; Yamazaki, Okawa et al. 2008). Although TJs form a sealed structure, it can also act as a pore for paracellular transport, i.e. in the lung epithelium (Flynn, Itani et al. 2009) and in intestinal wall (Shen 2012).

AJs consist of cadherin and nectin proteins that form homodimers with corresponding protein on the neighboring cell. AJs are calcium dependent due to the calcium binding properties of the cadherin extra-cellular domains. The cadherin superfamily consist of both classical and non-classical cadherins, where E-cadherin is present in epithelial cells (Meng and Takeichi 2009). The catenin p120CAS binds to the juxtamembrane domain of the intracellular tail of E-cadherin, and β -catenin and α -catenin mediates the connection to the cytoskeleton (actin and microtubules). The cadherin-catenin complex is crucial for the regulation of cell to cell adhesion (Ireton, Davis et al. 2002; Nishimura and Takeichi 2009).

The desmosomes contribute to the cell-cell interactions and stabilize the epithelial layer against shear forces. The desmosomes consist of five major components; desmogleins and desmocollins forming dimers between the cells, plakoglobin and plakophilins binding the intracellular parts and the plakin linker protein desmoplakin linking the desmosome to the intermediate keratin filaments (Brooke, Nitoiu et al. 2012).

The Na^+, K^+ -ATPase β -subunit and sodium homeostasis has been shown to play a central role in the formation of the intercellular junctions via association with both TJ, AJ and desmosomal proteins as well as epithelial phenotype development (Rajasekaran, Palmer et al. 2001; Rajasekaran, Palmer et al. 2001; Vogelmann and Nelson 2005; Vagin, Tokhtaeva et al. 2006).

1.5.1 Polarized distribution of sodium transporters

As mentioned earlier, the distribution of sodium transporters is of highest importance for vectorial sodium transport and body sodium and water homeostasis. The majority of the sodium transporters are situated in the apical part of the plasma membrane letting sodium in from the lumen of the organ (e.g. the nephron, alveolus, intestine), as the Na^+, K^+ -ATPase is situated in the basolateral part of the membrane pumping sodium out of the cell towards the basolateral milieu. The intracellular sodium concentration is kept relatively constant. The localization of the transporters is regulated by sorting and selective transport, trafficking pathways determined by information embedded in the structure. The Na^+, K^+ -ATPase is targeted to the basolateral part directly from the trans golgi network, whereas other proteins are sorted via the ER and recycling endosomes with or without adaptor protein involvement (Farr, Hull et al. 2009). As mentioned earlier, the Na^+, K^+ -ATPase also lack the possibility to enter “lipid rafts” and therefore it cannot be transported to the apical side of the membrane (Hammerton, Krzeminski et al. 1991; Mays, Siemers et al. 1995). Selective stabilization also seems to regulate the differentiated localization of the transporters, but is far less common (Matter 2000). In the junctional structures, the β -subunits form bridges to the adjacent cell that prevents apical translocation (Tokhtaeva, Sachs et al. 2011) whereas the α -subunit binds to ankyrin, which in turn binds to the actin cytoskeleton (Zhang, Devarajan et al. 1998).

1.5.2 Polarity in development and tissue repair

The ability of cells to convert from a highly polarized epithelial phenotype towards a nonpolarized phenotype without intracellular junctions is a process referred to as epithelial-mesenchymal transition (EMT). EMT is necessary for embryonic organogenesis, wound and tissue healing, and is also a key regulator in metastasis (Acloque, Adams et al. 2009). Cells transformed into a mesenchymal phenotype possess migratory and invasive features and a higher resistance to anoikis, i.e. cell death induced by lack of growth support (Frisch and Francis 1994). The transformation is however reversible, enabling the mesenchymal cells to revert into epithelial cells when reaching the new location. The EMT process is associated with loss of E-cadherin whereas it is re-expressed in cells converting into an epithelial phenotype (Acloque, Adams et al. 2009). During EMT, different transcription factors known to inhibit E-cadherin are upregulated, i. e. Snail1 and 2, Twist, Zeb 1 and 2 (Dave, Guaita-Esteruelas et al. 2011).

During the healing process of a damaged epithelium, provisional EMT takes place, ensuring regenerative proliferation restoring the normal tissue architecture. This is followed by a phase of fibrosis, in which connective tissue replaces the normal tissue in a reversible process. If the fibrotic process is not controlled appropriately, myofibroblasts will accumulate, excessive collagen will be deposited and the extracellular matrix will be remodeled into a pathogenic stage, eventually leading to organ function failure. Fibrosis commonly occur in kidney disease, ALI, liver cirrhosis among other (Wynn 2008; Chapman 2011; Wynn and Ramalingam 2012).

1.6 SODIUM HOMEOSTASIS: IMPACT FOR DISEASE DEVELOPMENT

The capacity of sodium handling is of high importance for health. The regulation of ionic concentrations within the body is dependent on the activity of the transporters as well as the epithelial cell polarity characteristics.

1.6.1 Kidney

Increased sodium reabsorption in the kidneys and subsequent water restraint is one of many causes of hypertension (Guyton 1991). The body controls blood pressure by regulating natriuretic and anti-natriuretic functions via hormonal actions on the Na^+, K^+ -ATPase (See section 1.4). Increased dietary sodium increases the blood pressure, and when reducing the sodium, the blood pressure decreases (Melander, von Wöhrn et al. 2007). An increase in sodium load stimulates the local renal production of DA (Bertorello, Hokfelt et al. 1988) as well as ouabain-like factors (OLFs) from the adrenal cortex (Goto, Yamada et al. 1996). The role of OLFs in the kidney is not fully clear. Contributing to the pathology of hypertension, OLFs cause vasoconstriction by inhibition of Na^+, K^+ -ATPases in the vascular smooth muscle cells (Blaustein, Zhang et al. 2009).

Individuals with hypertension, independently of etiology, bear an increased risk of cardiovascular, cerebrovascular and renal disease, causing high risk for morbidity and mortality (He, Jenner et al. 2010). One complication from hypertension is cardiac hypertrophy. The cardiomyocytes grow in size in response to sustained mechanical stress causing a pathological enlargement of the heart. Animal studies have shown that a high salt intake contribute to the development of cardiac hypertrophy, both before and after development of hypertension (Schmieder, Messerli et al. 1988; Frohlich, Chien et al. 1993; Heineke and Molkentin 2006). Sodium homeostasis and intracellular signaling mechanisms may therefore be of importance for growth of cardiac myocytes (Popov, Venetsanou et al. 2012).

Independently of blood pressure, sodium intake has been associated with urinary albumin excretion, a sign of kidney damage. Dietary sodium load can cause an increased blood pressure in individuals with an underlying kidney disease, as the malfunctioning kidney cannot maintain normal sodium homeostasis. The increased blood pressure will further accelerate the damage to the kidneys and decrease the renal function (He, Jenner et al. 2010). Acute kidney injury is common and often leads to the development of chronic kidney disease with interstitial fibrosis, infiltration of inflammatory cells and glomerulosclerosis. This in turn leads to poor filtration capacity and eventually end stage renal failure (Prunotto, Budd et al. 2012).

1.6.2 Lung

If the epithelial barrier loses the polarized phenotype, the tightly regulated ion- and water balance will be impaired. Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) can be induced by several reasons; systemic infection/inflammation (sepsis), ventilated-induced lung injury, hypoxia due to high altitude climbing among other. When the lung epithelium is damaged it loses its impermeable properties and fluid leak into the alveolar space. Due to this, AFR is impaired, leading to development

of life threatening lung edema. The edema fluid, as well as the damaged epithelium interferes with the gas exchange, resulting in oxygen deprivation and acidification of the blood via increasing levels of H^+CO_3^- . Resolving the edema is of great importance for the survival of the patient, and many sodium transport systems have been addressed for increasing the AFR, for example, the use of diuretics (primary loop diuretics as Furosemide (Lasix) blocking the $\text{Na}^+\text{-K}^+\text{-Cl}^{2-}$ symporter) (Johnson and Matthay 2010).

During the hypoxic condition that arises following injury, the $\text{Na}^+\text{,K}^+\text{-ATPase}$ is downregulated to save energy. Upon injury, ROS are formed and the $\text{Na}^+\text{,K}^+\text{-ATPase}$ is downregulated via a calcium dependent pathway, including AMP-activated protein kinase (AMPK) activation (Dada, Chandel et al. 2003; Dada and Sznajder 2003; Gusarova, Trejo et al. 2011). Ubiquitination of $\text{Na}^+\text{,K}^+\text{-ATPase}$ and ENaC has recently been suggested to effect the alveolar epithelial barrier with regards to function and integrity (Helenius, Dada et al. 2010; Vadasz, Weiss et al. 2012). DA, aldosterone and β -AR agonists have been shown to accelerate lung edema clearance in animal experiments (Olivera, Ciccolella et al. 2000; Adir and Sznajder 2003), and studies of the β -AR agonist Salbutamol IV have indicated that β -AR agonist could also accelerate lung edema clearance and improve survival in humans (Perkins, McAuley et al. 2006). The mechanism of action of the β -AR agonists is in part due to stimulation of sodium transport by increasing the number of actively pumping $\text{Na}^+\text{,K}^+\text{-ATPases}$ at the basolateral plasma membrane (Bertorello, Ridge et al. 1999), but also via stimulation of sodium and calcium channels at the apical part (Saldias, Comellas et al. 1999; Matthay, Folkesson et al. 2002; Mutlu, Adir et al. 2005). β -AR agonists are also anti-inflammatory and used as treatment for sustained bronchitis and asthma (e.g. Ventoline), which is beneficial for treating the inflammatory component of lung edema. *In vitro* studies suggest that salbutamol also help the regeneration and healing of epithelial monolayers (Perkins, Gao et al. 2008). The $\text{Na}^+\text{,K}^+\text{-ATPase}$ also play a role in keeping the epithelial barrier intact to avoid edema formation by dimerization in the TJ formations (Rajasekaran, Palmer et al. 2001).

When a damaged alveolar epithelium heals, scarring may occur leading to accumulation of fibrotic tissue and development of pulmonary fibrosis (Chapman 2011). The etiology of pulmonary fibrosis varies, and in the case of idiopathic pulmonary fibrosis (IPF) is still unclear. After ALI, the wound-healing process is initiated by inflammation followed by the repair phase. Myofibroblasts are differentiated from fibroblasts, AEC2, or as suggested recently, from lung stem cells (Wynn 2008; Kajstura, Rota et al. 2011). The fibrotic lung loses flexibility and the oxygen diffusion capacity is reduced by the fibrotic lesions.

2 HYPOTHESIS AND AIMS

2.1 HYPOTHESIS

The overall hypothesis of this thesis is that sodium load in a short- and long-term perspective demand an effective regulation, capable to detect and adjust for small changes in the sodium concentration, and that such a system could be used by the cell for diverse functions involving sodium reabsorption and maintenance of the polarity of epithelial cells.

2.2 AIMS

The aim of this thesis is to investigate the existence of a cell sodium sensing network in transporting epithelia, and the potential role of such network for cell and organ physiology.

2.2.1 Specific objectives

- I. To investigate if there exist a sensor of small elevations in intracellular sodium leading to a rapid increase in the catalytic activity of the Na^+ , K^+ -ATPase.
- II. To elucidate the role of an activator of sodium and water transport in lung epithelial cells.
- III. To examine the role of sodium homeostasis regulation for intercellular junction stability.

3 COMMENTS ON METHODOLOGY

This chapter comments on the material and methods used in the articles included in this thesis. Review the Material and Method sections in each article for further details.

3.1 CELL LINES AND SPECIMENS:

The first study is based on work using a cell model of renal proximal tubule cells originating from the kidney of Opossum, OK cells. These cells are well characterized (Malstrom, Stange et al. 1987) and used extensively for studying the regulation of the Na^+, K^+ -ATPase (Silva and Soares-da-Silva 2009). OK cells stably expressing rat Na^+, K^+ -ATPase α -subunit with green fluorescent protein tag (GFP) were selected with the addition of 13 μM ouabain as previously described in (Efendiev, Bertorello et al. 2000), as the rat Na^+, K^+ -ATPase is less sensitive to ouabain inhibition. (Article I and II)

HepG2 cells (American Type Culture Collection, Manassas, CA) were used to investigate the role of SIK1 in cells from the liver as well as in a cell line of human origin. Using a human cell line made it possible to silence SIK1 by small interfering RNA (siRNA), which was not possible for the Opossum derived cells. (Article I)

MLE-12 (Murine lung epithelial cells) (ATCC), were used as a model of AEC2. Stable clones of SIK1-shRNA and sc-shRNA MLE-12 cells were selected and maintained with Hygromycin B (100 $\mu\text{g}/\text{ml}$). (Article II and III)

A549 cells and NCI-H441 cells, both cell lines exhibiting a number of AEC2 characteristics, originating from human lung carcinoma were also used (Lieber, Smith et al. 1976). (Article II)

An epithelial cell lines originating from human proximal tubules, HK-2 (Ryan MJ 1994), modified by stable transfections with either human α -adducin WT (N.T.) or the α -adducin bearing the hypertensive mutation, G460W (H.T.) (Ferrandi, Molinari et al. 2010) were used as the hypertensive variant of α -adducin has been reported to activate SIK1 (Stenstrom, Takemori et al. 2009). (Article III)

Madin-Darby Canine Kidney (MDCK) cells originate from the distal tubules in dog kidney and were used to study the effect of SIK1-siRNA on epithelial integrity by transepithelial electrical resistance (see section 3.3.16), TER. These cells exhibit a natural ability to form very tight intercellular junctions with high TER. T84 cells originating from human colon carcinoma were also used for this purpose. (Article III)

In order to investigate SIK isoform expression *in vivo*, lung sections from rats (Sprague-Dawley) and mice (C57Bl/6) were used, as well as isolated AEC1 and AEC2 from rat. (Article II)

sik1^{-/-} mice were used to study the effect of SIK1 on E-cadherin expression in the lungs *in vivo*. The *sik1*^{-/-} line was based on C57BL/6J mice and did not show any difference in neither phenotype, reproduction nor life span when compared with the *sik1*^{+/+} mice. The mice had free access to chow diet and water. (Article III)

3.2 PLASMIDS AND TRANSFECTION (ARTICLE I-III)

All transfections of expression plasmids were performed with LipofectAMINE 2000 (Invitrogen) in serum free media without Penicillin/Streptomycin, according to manufacturer's protocol. The expression time was 24 to 48h for transient transfections. Stable transfection of rat Na⁺,K⁺-ATPase α -subunit was maintained by addition of ouabain in culture media. Stable clones of shRNA SIK1 or a scramble control sequence were selected and maintained by addition of Hygromycin B. For siRNA transfections, the transfection reagent supplied from the manufacturer was used and the expression time was between 24 and 72 h.

3.2.1 Plasmids and siRNAs

SIK1 wild type (WT) in a pIRES vector, as described in (Lin, Takemori et al. 2001) were used in Article I, II and III.

The kinase inactive SIK1 mutant K56M (Lysine \rightarrow Methionine) was constructed by site-directed mutagenesis using a QuickChange mutagenesis kit (Stratagene, La Jolla, CA) as previously reported (Lin, Takemori et al. 2001) and used in Article I and II.

The SIK1 mutant lacking the CaMK regulatory residue was generated by exchanging Threonine at position 322 by Alanine (T322A). (Article I and III)

SIK1-GST (Glutathione S-transferase) (Lin, Takemori et al. 2001) was used for SIK1 activity assays. (Article I and II)

SIK1 was also silenced by establishment of stable clones using short hairpin RNA (shRNA). pSilencer 3.1-H1-hygro containing shRNA for SIK1

(Forward 5' \rightarrow 3'

GATCCGGGAGTACGAGGGTCCCCAGTTCAAGAGACTGGGGACCCTCGTAC
TCCTTTTTTGGAAA;

Reverse 5' \rightarrow 3'

AGCTTTTCCAAAAAAGGAGTACGAGGGTCCCCAGTCTCTTGAAGTGGGGA
CCCTCGTACTCCCG)

or a negative control containing a scramble sequence

(Forward 5' \rightarrow 3' GATCCGTTACTTTTTTGGAAA;

Reverse 5' \rightarrow 3' CTAGGCAATGTGAAAAACCTTT). (Article II and III)

An expression plasmid of pCMVSPORT6-mouse PME-1 (IMAGE 5062326) was purchased from Invitrogen. (Article I)

cDNAs for PP2A wild type and the dominant negative mutant L199P were kindly provided by B. A. Hemmings (Friedrich Miescher Institute, Basel, Switzerland). (Article I)

LKB1, MO25, STRAD and SIK1 T182A vectors were provided by Dr. Hiroshi Takemori (National Institute of Biomedical Innovation, Osaka, Japan). (Article III)

E-Cadherin luciferase promoter construct (-108 to +125) Plasmid 19290:pGL2Basic-EcadK1 from Addgene (Hajra, Ji et al. 1999). (Article III)

pTAL-Cre luciferase vector (Lin, Takemori et al. 2001). (Article III)

Renilla luciferase vector from Promega. (Article III)

All constructs were verified by DNA sequence analysis and the expression validated by western blot.

For transient silencing experiments, siRNA transfection system for CREB1, CREB2, C/EBPa, SIK1 (h), SIK1 (dog) were bought from Santa Cruz Biotechnology.

3.3 METHODS

3.3.1 Gel electrophoresis and Western blot (Article I-III)

Central for the work in this thesis is the investigation of protein amount, modification and interactions. By sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins can be separated based on size on a gel. The gel can either be stained with silver staining (Article I) or by colouring methods (i.e. coomassie brilliant blue), or the separated proteins can be transferred to a protein binding membrane (e.g. Polyvinylidene fluoride, PVDF) and subjected to Western blot (WB) (as in Article I-III).

Western blot is a method where the protein of interest is detected by using a high affinity antibody recognizing an epitope with high specificity. The antibody is then detected by another, secondary antibody, conjugated to a visualization system, e.g. horse-radish peroxidase (HRP) that can be detected by adding a substrate solution. The chemiluminescent signal that is produced is detected by light sensitive film or charge-coupled device (CCD) camera. The amount of protein, degree of phosphorylation etc. is quantified by image software, in this thesis by Image J software.

3.3.2 Immunoprecipitation (Article I and II)

Immunoprecipitation is a method to enrich the protein of interest from the total pool of proteins in a sample using an antibody directed towards the selected protein. Further, SDS-PAGE and Western blot can be used to study e.g. the degree of phosphorylation of the protein.

To investigate if two, or more, proteins interact within a cell, and if the degree of interaction changes upon stimulus, co-immunoprecipitation can be used accordingly. The protein of interest is targeted with an antibody, and any interacting protein will be co-immunoprecipitated. By denaturation of the sample, the interacting proteins will detach from each other and can be separated by SDS-PAGE. WB analysis of the proteins of interest reveal if the proteins were co-immunoprecipitated, and to which extent.

3.3.3 Mass Spectrometry (Article I)

To identify unknown proteins, mass spectrometry (MS) can be used. The methodology is based on usage of the molecular mass and charge of peptide fragments of the unknown protein, acquiring a mass spectrum thereof. The procedure in short; a protein of interest is extracted from a separation gel, digested into fragments using e.g. Trypsin, loaded into the MS machine where the sample is vaporized, ionized by an electrical beam, separated by the mass-to-charge ratio in an electromagnetic field and recorded

by a detector. The information from the detector is processed and renders the mass spectrum, which can be compared with databases of known proteins. In our study, MALDI-TOF was used, which stands for Matrix-Assisted Laser Desorption/Ionization – Time of Flight. The ions are created by laser, and the time ions fly within the magnetic field before reaching the detector is measured and used for spectrum acquisition and analysis.

3.3.4 Determination of Na⁺,K⁺-ATPase activity (Article I-III)

The catalytic activity of the Na⁺,K⁺-ATPase and the regulation thereof is fundamental for this thesis. To determine the activity of the Na⁺,K⁺-ATPase, ouabain-sensitive transport of radioactive rubidium (⁸⁶Rb⁺) was used. The method utilizes the potassium-transporting function of the Na⁺,K⁺-ATPase, where two potassium ions are transported into the cell for each three sodium ions and for each cycle, exchanging the potassium for radioactive Rubidium by addition of trace amounts of ⁸⁶Rb⁺ into the media. Rubidium transport into the cells can then be analyzed by a scintillation counter, and the ouabain sensitive transport is considered to be specific for the Na⁺,K⁺-ATPase mediated transport (Efendiev, Bertorello et al. 2002).

3.3.5 Cell-Surface Biotinylation (Article I and II)

To study proteins situated in the plasma membrane, with some part of the protein on the extracellular side, and if the amount of the protein of interest changes upon stimulation of the cells, Cell-Surface Biotinylation can be used. The methodology is based on the strong interactions between biotin and proteins on the cell surface. Adding biotin to intact cells will cause biotin to bind to any protein exposed on the cell surface, and this interaction is stable throughout lysis of the cell. The biotinylated proteins are then extracted and purified by adding streptavidin, which binds to the biotin with extremely high affinity and specificity. The extracted and purified proteins, originating only from the cell surface, can then be separated by SDS-PAGE and analyzed by WB.

3.3.6 Phosphorylation of Na⁺,K⁺-ATPase α-Subunit (Article I)

Investigating the degree of phosphorylation of the highly abundant Na⁺,K⁺-ATPase requires special considerations, taking into account the localization of the proteins to be analyzed. We were exclusively interested in the Na⁺,K⁺-ATPases situated in the plasma membrane, responsible for controlling the intracellular sodium concentration. We decided to perform isolation of crude membranes, using a mannitol based centrifugation separation technique (Khundmiri, Bertorello et al. 2004). From the crude membranes, the Na⁺,K⁺-ATPases originally in the plasma membrane were immunoprecipitated and the degree of phosphorylation was determined by Western blot using an antiphosphoserine antibody and compared to the total amount of Na⁺,K⁺-ATPase determined after stripping the membrane and retesting with an antibody towards the Na⁺,K⁺-ATPase α-subunit.

3.3.7 Back Phosphorylation (Article I)

To investigate if PME-1 constitutes a target for SIK1 phosphorylation, and if PME-1 is phosphorylated in response to increases in intracellular sodium, the so called Back Phosphorylation Technique was used (Chibalin, Ogimoto et al. 1999).

Immunoprecipitated PME-1 from untreated and monensin treated cells was mixed with purified GST-SIK1 in the presence of radiolabeled [γ - 32 P]ATP. The reaction was stopped by the addition of sample buffer, and proteins were separated on SDS/PAGE and transferred to PVDF membranes. The amount of radioactively labeled phosphate incorporated in the PME-1 was analyzed by autoradiography and correlated to the amount of PME-1, as detected by Western blot. The more radioactive signal within a sample, the more phosphorylation has taken place during the assay. When comparing two samples, e.g. controls and monensin treated, the obtained radioactive signal is inversely proportional to the degree of phosphorylation before the Back Phosphorylation assay. If less radioactive signal is obtained in the monensin treated sample, this means that monensin treatment of the cells resulted in phosphorylation of PME-1, leaving fewer residues free for 32 P-phosphorylation.

3.3.8 In Vitro Phosphorylation of SIK1 by CaMK1 (Article I)

To prepare an SIK1-SNH (sucrose non-fermenting homologue) peptide, a cDNA fragment of the SIK1-SNH domain (amino acids 301–354) was amplified by PCR and ligated into a pGEX-6P3 vector containing the sequence for GST-fusion protein. An active CaMK was prepared by using an expression plasmid (pSport6-CaMK1, IMAGE 4483612; Invitrogen). To convert a native CaMK1 into a constitutive active form, a stop codon was inserted at the amino acid 296 position by site-directed mutagenesis and ligated into a pEBG vector. The active GST-CaMK1 was expressed in COS-7 cells, extracted and purified by GST-purification columns. Purified GST-CaMK1 was mixed with GST, GST-SNH (wild type), or GST-SNH (T322A mutant) and incubated with or without ATP. The reaction was terminated by the addition of sample buffer and an aliquot was subjected to SDS/PAGE, followed by WB analysis using an anti-pT322 antibody.

3.3.9 Determination of Protein Phosphatase Activity (Article I)

Phosphatase activity was assayed using a fluorescence based method where dephosphorylation of 3-*O*-methylfluorescein phosphate was monitored by measuring the fluorescence of the methylfluorescein product over time in a plate reader.

3.3.10 Determination of SIK1 activity (Article I and II)

SIK1 activity can be assayed in different ways. In the articles included in this thesis, two methods were used. Both methods are based on transient overexpression of GST-SIK1, treatment of the cells according to specific protocol, followed by GST purification of SIK1. The first method is based on an assay where radiolabeled ATP ([γ - 32 P]ATP) is used to determine the degree of autophosphorylation of SIK1 or its substrate Transducer of Regulated CREB activity 2 (TORC2) by SDS-PAGE and subsequent autoradiography and WB. The second method is based on SDS-PAGE and Western blot with an antibody towards SIK1 phosphorylated at Tyrosine at position 182. Phosphorylation of T182 has been shown to be dependent of the autophosphorylation of 186, and required for kinase activity (Hashimoto, Satoh et al. 2008). The signal from SIK1 phosphorylated at T182 is compared with the total amount of SIK1.

3.3.11 Immunofluorescence microscopy (Article I-III)

Immunofluorescence microscopy was used in all three Articles of this thesis. The method is used to examine the expression and localization pattern of proteins within cells and tissue sections. Fluorescently labeled antibodies directed towards the protein of interest are visualized by fluorescent or confocal microscopy.

In Article I, the association of SIK1 with the Na⁺,K⁺-ATPase in cells stably expressing the Na⁺,K⁺-ATPase α -subunit tagged with GFP was examined using confocal microscopy (Efendiev, Krmar et al. 2004) together with an antibody directed towards SIK1.

In Article II, paraffin-embedded lung tissue sections from mouse and rat were devoid of paraffin with xylene and rehydrated before analyzed using SIK isoform specific antibodies, with FITC-conjugated secondary antibody. Antibodies for T1 α and LB180 was used for detection of AEC1 and 2 respectively, and detected with Texas Red-labeled secondary antibody. The slides were examined using a Nikon Eclipse E800 fluorescence microscope, and the images were processed by MetaMorph software (Molecular Devices, Inc.).

In Article III, confocal microscopy was used to detect the expression and localization of proteins in both cell-to-cell interactions and the cytoskeletal structures of cultured cells expressing normal or decreased levels of SIK1. F-actin was visualized by rhodamine-labeled phalloidin, whereas all other proteins were detected with antibodies and species matched fluorescence-labeled secondary antibodies.

3.3.12 Isolation of rat AEC1 and AEC2 cells (Article II)

AEC1 and AEC2 were isolated from male Sprague-Dawley rats as described (Chen, Chen et al. 2004). The Northwestern University Animal Use and Care Committee approved all animal procedures performed in this study. Rats were anaesthetized and a tracheotomy was performed while rats were ventilated with a rodent ventilator. The heart was transected and a catheter placed in the pulmonary artery. After perfusion, the lungs were removed for isolation of AEC1 and AEC2. The purities of the final AEC1 and AEC2 preparations were >90% and >98%, respectively (assessed by immunocytochemistry using anti-T1 α antibodies anti-LB180 antibodies, respectively). Cross-contaminations of AEC1 and AEC2 were less than 0.5%. The viabilities of both cell preparations were >95%.

3.3.13 Reverse transcription-PCR (Article II)

To study the mRNA expression of SIK isoforms in lung tissue and cell models, total RNA was isolated using RNEasy Mini kit (Qiagen, Valencia, CA). mRNA was reversely transcribed into cDNA and polymerase chain reactions (PCR) were performed using specific primers for SIK1, 2 and 3 (as described in Table 1 of Article I). Agarose-gel electrophoresis and ethidium bromide staining were used to visualize PCR bands.

3.3.14 Real-time PCR (Article II and III):

To detect changes in mRNA expression, TaqMan real-time PCR was used. Total RNA was extracted, any genomic DNA was digested, followed by reverse transcription of the purified mRNA to complementary DNA (cDNA). The cDNA of the gene of interest was then investigated using gene specific assays consisting of a primer pair for transcription and a fluorescent probe binding to the cDNA of the gene of interest. Each time the cDNA strand is amplified, the probe is cleaved by Taq-polymerase and the fluorescent dye emits light, detected by the TaqMan machine in real time. The exponential increase of cDNA per cycle during the PCR reaction causes an exponential increase of light emitting dye. At a certain cycle, the emitted light will increase dramatically, reaching a threshold level denoted as Ct. Differences in Ct values are used to compare mRNA levels between different samples, either by using the comparative Δ Ct method or by using a standard curve, giving quantitative assessments of the mRNA levels in the samples.

3.3.15 Promoter activity measurements (Article III)

To determine the promoter activity of E-cadherin and CREB, we used luciferase reporter vectors. The general principle used is that production of firefly (*Photinus pyralis*) luciferase is driven by the promoter for the gene of interest, and thereby used as a measure of the promoter activity. For normalizing the transfection efficiency, the cells are co-transfected with a vector expressing Renilla (*Renilla reniformis*) luciferase, and optimized to avoid trans-effects. In short, the cell lysate containing luciferase is mixed with the Luciferase Assay Reagent provided in the Promega Dual luciferase kit, consisting of the other components of the reaction. The light emission is measured for 10 s using a luminometer. The reaction is quenched by addition of Stop & Glo reagent, and subsequently, the renilla luciferase is measured in the same sample. The ratio of emission from Firefly Luciferase/Renilla Luciferase denotes the promoter activity of the gene of interest.

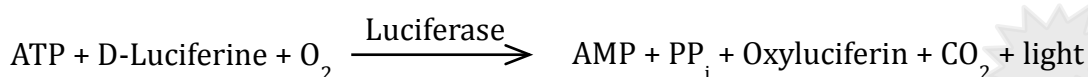


Figure 3. The Luciferase reaction. The luciferase formed in the cells catalyzes the light producing reaction.

3.3.16 Transepithelial Electric Resistance (Article III)

Epithelial cells form tight monolayers by the junction structures at the cell-to-cell interaction points. A tight monolayer is a necessity for regulated transport of solutes and water over the epithelium, otherwise, passive diffusion takes place in between the cells. The sealing function of the monolayer can be assessed by measure the Transepithelial Electric Resistance (TER). Cells were grown on a permeable support, and the TER over the monolayer was measured with an epithelial volt-ohm meter with chopstick electrodes. The importance of SIK1 for cells to form a tight epithelium was

studied in cells transiently or stably repressed in SIK1 expression compared with control cells.

Calcium switch experiments were carried out using the TER experimental setup. Cells were grown to confluency on a permeable support. Using addition of EGTA into the media, low-calcium media was obtained, yielding a decrease in TER. The cells ability to recover the tightness of the epithelial layer can then be studied when calcium is added to the media. By SIK1 reduction, the role of SIK1 in recovering of the epithelial function was investigated.

3.3.17 Membrane potential (Article III)

The membrane potential was studied in order to investigate the function of the cells with reduced levels of SIK1 expression. The Na^+, K^+ -ATPase creates a membrane potential by the active sodium transport over the plasma membrane towards the concentration gradient. Cells should depolarize by ouabain as this treatment inhibits the Na^+, K^+ -ATPase, and increase by addition of KCl, by increasing substrate availability. The methodology applied uses the voltage-sensitive fluorescent dye DiBAC4(3) (bis-(1,3-dibutylbarbituric acid)-trimethine oxonol) that changes slowly in fluorescence upon changes in membrane potential. The changes in fluorescence were monitored by sampling every 5 s at excitation and emission wavelengths of 488 and 520 nm, respectively.

3.3.18 Intracellular pH (Article III)

To study Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchanger activity, intracellular pH was measured by a fluorescence based method where the pH-dependent variation of BCECF (2',7'-bis (carboxyethyl)-5,6-carboxyfluorescein) excitation wavelength is used. At acidic pH, BCECF is excited at $\lambda 490$ nm and at basic pH, the optimal wavelength is 440 nm. The emission was measured at 535 nm, and the emission ratio was used for pH calculation, by comparison with values from an intracellular calibration curve obtained by using nigericin and high- K^+ method.

For assessment of Na^+/H^+ exchanger activity, pH_i recovery after an acidic load and subsequent Na^+ removal was measured under $\text{CO}_2/\text{HCO}_3^-$ free conditions. NaCl is exchanged by ethylammonium chloride (Gomes and Soares-da-Silva 2006).

There is no specific assay for $\text{Cl}^-/\text{HCO}_3^-$ exchanger activity, but it is reflected by pH_i recovery after removal of $\text{CO}_2/\text{HCO}_3^-$ under Na^+ free conditions where NaHCO_3 is replaced by choline.

3.3.19 ATP assay (Article III)

Intercellular junction stability is highly dependent on the energy level within the cells, which can be studied by analysis of the intracellular ATP concentration (Fish and Molitoris 1994). In our study, ATPLite assay from PerkinElmer was used. The assay is based on the ATP-dependent light emitting conversion of D-Luciferin to Oxyluciferin by Firefly Luciferase, where the emitted light is proportional to the ATP concentration in the cell sample, the same reaction as described in Figure 3. In short, cell were grown in 96-well plates and lysed within. The added lysis solution also stabilized the ATP. A substrate solution was added, and following dark adaptation, the luminescence was measured. ATP standard curves and blank samples were included in each plate.

3.4 STATISTICAL ANALYSIS (ARTICLE I-III)

Statistical analyses of quantitative data were performed using GraphPad Prism, GraphPad Software (San Diego, CA, USA). To estimate the significance of the differences between the samples, the unpaired Student's t-test or Mann-Whitney test were used as appropriate. P-values ≤ 0.05 were considered statistically significant.

4 RESULTS

This section highlights the main findings of each Article. For detailed descriptions of the results, see Result section for Article I-III, respectively.

4.1 SIK1 IS A PART OF A CELL SODIUM-SENSING NETWORK THAT REGULATES ACTIVE SODIUM TRANSPORT THROUGH A CALCIUM-DEPENDENT PROCESS

We sought to investigate if there exists a modulator of Na^+, K^+ -ATPase activity, able to sense small, but physiologically relevant, increases in intracellular sodium. We used a cell model of renal proximal tubule cells originating from the kidney of Opossum, OK cells. These cells are well characterized (Malstrom, Stange et al. 1987) and used extensively for studying the regulation of the Na^+, K^+ -ATPase (Silva and Soares-da-Silva 2009).

4.1.1 Salt-Inducible Kinase 1

The Na^+, K^+ -ATPase activity is controlled by phosphorylation/de-phosphorylation processes, regulating the actual catalytic activity of the Na^+, K^+ -ATPase or the transport of active Na^+, K^+ -ATPases to and from the plasma membrane (Ewart and Klip 1995). These phosphorylation/de-phosphorylation processes require physical interaction of the Na^+, K^+ -ATPase and the responsible kinase or phosphatase (Ogimoto, Yudowski et al. 2000; Yudowski, Efendiev et al. 2000; Dada, Chandel et al. 2003). To find new potential regulators of the Na^+, K^+ -ATPase, we performed immunoprecipitation of the Na^+, K^+ -ATPase complex in cells overexpressing rat Na^+, K^+ -ATPase bearing a GFP tag. The proteins interacting with the Na^+, K^+ -ATPase were co-immunoprecipitated and separated via SDS-PAGE and subsequently identified by mass spectrometry. One protein caught our special interest, the salt-inducible kinase-1 (SIK1) [NP_067725].

SIK1 was first identified in by Ruiz and colleagues in 1994 (Ruiz, Conlon et al. 1994) in cardiomyocytes in the developing mouse heart. At that stage, SIK1 was however designated *msk*, myocardial SNFI-like kinase, due to the high sequence homology with the yeast sucrose non-fermenting like kinase (*snflk*). A few years later, SIK1 was identified and subsequently cloned in rats fed with a high salt diet (Wang, Takemori et al. 1999), and the *msk* was renamed accordingly. The family of SIKs (SIK1, SIK2 also named QIK2, and SIK3 also named QSK) belong to the AMPK family of Serine/Threonine kinases.

We focused on SIK1 for several reasons. Firstly, it is a kinase, able to phosphorylate, and thereby theoretically possess the ability to regulate the activity and localization of the Na^+, K^+ -ATPase (Carranza, Rousselot et al. 1998; Chibalin, Ogimoto et al. 1999). Secondly, it has been shown to be upregulated in response to high salt diet (Okamoto, Takemori et al. 2004), and thirdly, SIK1 has been shown to be expressed in several transporting epithelia (Feldman, Vician et al. 2000) and may thus act as mediator of changes in the intracellular levels of sodium and the response in Na^+, K^+ -ATPase activity.

The interaction of Na⁺,K⁺-ATPase with SIK1 was further confirmed by immunoprecipitation of the endogenous Na⁺,K⁺-ATPase α -subunit followed by WB. In addition, co-localization studies by immunofluorescence, with Na⁺,K⁺-ATPase α -subunit tagged with Green fluorescent protein (GFP) and an antibody towards SIK1 (Lin, Takemori et al. 2001), support this finding. The nature of the interaction, i.e. if it is direct or indirect, and which domains are responsible for the interactions etc. were however not investigated and remains to be elucidated.

4.1.2 Increased [Na⁺]_i increases the Na⁺,K⁺-ATPase activity via SIK1

To increase intracellular sodium in the cells, the ionophore monensin was used. Monensin has previously been shown to specifically and rapidly increase the intracellular sodium concentration to a physiologically relevant level without disturbing the cellular architecture and morphology of the OK cells (Efendiev, Bertorello et al. 2002). Treatment of the OK cells with 5 μ M monensin corresponds to an increase in [Na⁺]_i from ~8 mM to ~20 mM, reaching a steady state level within 30 minutes. Treatment of the OK cells with 5 μ M monensin resulted in a time-dependent increase in the Na⁺,K⁺-ATPase activity, as investigated by rubidium transport assay. No change in the abundance of Na⁺,K⁺-ATPase at the plasma membrane was however observed, as determined by cell surface biotinylation, suggesting that the increase in transport efficiency relate to a true increase in catalytic activity of the Na⁺,K⁺-ATPase.

We then investigated if increased intracellular sodium levels activate SIK1 within the same time-frame. The enzymatic activity of SIK1 was determined by the degree of autophosphorylation of Tyrosine at position 182 and phosphorylation of the substrate TORC2. Stimulation of cells with monensin increased the SIK1 activity in a time dependent manner, but neither sodium nor monensin *per se* could increase the SIK1 activity *in vitro* investigated by using a cell free system.

To investigate the relevance of SIK1 activity in the regulation of Na⁺,K⁺-ATPase activity and sodium transport the wild type (W.T.) form of SIK1 and a mutated form of SIK1, Lysine at position 56 replaced with Methionine (K56M) lacking catalytic activity, were transiently overexpressed in OK cells. In the cells overexpressing the inactive K56M-SIK1, the increase in Na⁺,K⁺-ATPase activity was no longer significant, indicating a functional role of SIK1 in the stimulation of Na⁺,K⁺-ATPase activity. The role of SIK1 was then further investigated in HepG2 cells by the introduction of a plasmid with SIK1-siRNA. By transiently silencing the SIK1 expression, the increase in Na⁺,K⁺-ATPase activity was almost fully blocked, supporting the hypothesis that SIK1 is needed for controlling the sodium transport. As the same results were obtained using both a human liver cell line and cells from the kidney of Opossum, we speculate that the role of SIK1 reported is conserved in different species and that SIK1 play a role in controlling active sodium transport in different tissues.

4.1.3 Calcium and CaMK is needed for SIK1 activation

As neither sodium nor monensin *per se* were responsible for the activation of SIK1, we sought for an additional signaling molecule, converting the increased intracellular sodium into SIK1 activation. We know from previous studies that monensin increases

the intracellular Ca^{2+} in OK cells (Efendiev, Bertorello et al. 2002). Calcium is both a signal transducer (via influx from ion channels) and a second messenger (via e.g. GPRCs), known to stimulate various cellular pathways (Clapham 2007). The level of calcium influx following Monensin treatment is dependent on the extracellular calcium concentration (Efendiev, Bertorello et al. 2002). Therefore, the monensin treatment was preceded by EGTA and BAPTA treatment in order to quench both the extracellular and intracellular calcium ions. By removing the calcium ions, the increase in Na^+, K^+ -ATPase activity in response to increased intracellular sodium was no longer detected.

The NCX is present in OK cells, and by blocking the influx of calcium ions by two different NXC inhibitors, KB-R7943 and SEA0400 (Matsuda, Arakawa et al. 2001) the increase in Na^+, K^+ -ATPase activity in response to monensin was significantly lower. We conclude that calcium enters the cells via the NCX working in reverse mode in response to increased intracellular sodium, and that this increase in calcium mediates the effect on the Na^+, K^+ -ATPase. However, calcium *per se* cannot activate SIK1. Some of the most abundant and primary targets for Ca^{2+} signaling are the Calmodulin-dependent protein kinases that might act as a mediator of the sodium induced calcium dependent activation of the Na^+, K^+ -ATPase. Indeed, we found within the SIK1 sequence a CaMK phosphorylation site, and in the presence of a CaMK inhibitor (KN-93), SIK1 was no longer activated or able to increase the Na^+, K^+ -ATPase activity in response to Monensin treatment. To further confirm that CaMK is responsible for the activation of SIK1, we made a construct modulating the SIK1 sequence in the CaMK phosphorylation site. The resulting mutated peptide could not be phosphorylated by CaMK. Using a full length SIK1 construct mutated in the T322A site in cells significantly decreased the degree of activation of the Na^+, K^+ -ATPase in response to monensin.

The increase of intracellular sodium leads to an influx of calcium, which in turn activates CaMK that is responsible for the activation of SIK1.

4.1.4 SIK1 regulates PP2A activity via PME-1

Activation of SIK1 led to dephosphorylation of the Na^+, K^+ -ATPase α -subunit in a time-dependent manner. This dephosphorylation by PP2A was responsible for the increase in Na^+, K^+ -ATPase activity, as investigated by an inactive mutant of PP2A (Leucine at position 199 exchange to a Proline).

The de-phosphorylation of the Na^+, K^+ -ATPase could however not take place when transiently overexpressing the kinase inactive form of SIK1 (K56M). Protein phosphatase activity increased in response to increased intracellular sodium, and was inhibited when pre-treating the cells with the kinase inhibitor Staurosporin at 5 nM, a concentration inhibiting SIK1 (K_d 2,2 nM) but not interfering with e.g. PKC (K_d 9,6 nM) (Kato, Takemori et al. 2006; Karaman, Herrgard et al. 2008). Also, when introducing the kinase inactive SIK1 mutant, the increase in PPase activity was inhibited.

Previous reports show that a direct phosphorylation of PPases leads to a decrease in PPase activity (Janssens and Goris 2001), so we focused on methylation of PPase catalytic subunits as a regulatory pathway. PP2A form complexes of a catalytic subunit

called C or PR65 α/β , a regulatory A subunit and sometimes a B subunit (Chen and Gingras 2007). PP2A is normally inactive when in complex with protein phosphatase methylesterase-1 (PME-1), as PME-1 catalyzes the demethylation of PP2A catalytic subunits and thereby inhibits the activity of PP2A. When PME-1 demethylates PP2A, PME-1 leaves the PP2A complex in its active state. It has been reported previously that PP2A is associated with the Na^+, K^+ -ATPase (Efendiev, Bertorello et al. 2002), and our hypothesis was that in order for PP2A not to dephosphorylate (and thereby activate) the Na^+, K^+ -ATPase permanently, PP2A is inactivated under normal conditions. When immunoprecipitating the Na^+, K^+ -ATPase, both $\text{PP2A}_{\text{PR65}}$ and PME-1 were co-immunoprecipitated, and the amount of PME-1 decreased following monensin treatment, indicating that it leaves the complex. This relationship was also supported by the mirror experiment, where PME-1 was immunoprecipitated and less PP2A is co-immunoprecipitated following monensin treatment.

When transiently overexpressing the SIK1 kinase inactive mutant (K56M), PME-1 does no longer leave the Na^+, K^+ -ATPase complex in response to increased intracellular sodium. Interestingly, PME-1 bears a SIK1 consensus phosphorylation site and might be a direct target for SIK1. Using purified SIK1, together with immunoprecipitated PME-1 in an *in vitro* assay known as backphosphorylation, we could verify that PME-1 can be phosphorylated in the presence of SIK1, as detected by autoradiography. The immunoprecipitated PME-1 samples were pre-treated with or without monensin, and the monensin treated cells had a higher degree of phosphorylation originally, before the *in vitro* assay took place.

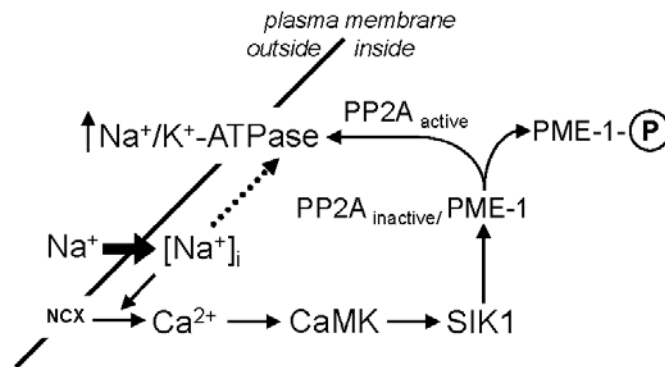


Figure 5. The proposed SIK1 network. Increases in intracellular sodium levels lead to a calcium dependent activation of SIK1 that phosphorylates PME-1. PME-1 then leaves the PP2A complex in its active state and PP2A dephosphorylates and thereby activates the Na^+, K^+ -ATPase α -subunit.

In summary, our results suggest a new regulatory pathway where physiological increases in intracellular sodium leads to an influx of calcium ions, in turn activating CaMK. CaMK phosphorylates SIK1, which becomes active and phosphorylates PME-1. PME-1 then leaves the PP2A complex in its active state and the Na^+, K^+ -ATPase situated in the plasma membrane is dephosphorylated and activated, extruding the extra sodium.

Our finding, that it is not sodium *per se* that is responsible for the increase in Na⁺,K⁺-ATPase activity, is contrary to prediction. This may reflect a possibility for cells to regulate sodium transport in a very fine-tuned manner, with several intermediate control steps that need to be fulfilled.

Under normal sodium load, SIK1 only associates with a limited pool of Na⁺,K⁺-ATPases, which do not increase in amount when subjected to higher sodium load. The dormant pool of Na⁺,K⁺-ATPases seems to be phosphorylated, but not be subjected to endocytosis. The constitutively associated PP2A is inactivated by PME-1, which may serve as a determinant for SIK1 dependent activation. Only the Na⁺,K⁺-ATPases associated with PP2A and PME-1 can be regulated by SIK1 in response to increasing sodium concentrations.

4.2 SALT-INDUCIBLE KINASE 1 IS PRESENT IN LUNG ALVEOLAR EPITHELIAL CELLS AND REGULATES ACTIVE SODIUM TRANSPORT

We hypothesized that as sodium regulation is crucial for the water reabsorption and thereby for keeping the alveolar space free of excess fluid, a sodium handling network of proteins might exist also in lung epithelia, thereby regulating the Na⁺,K⁺-ATPase activity and sodium homeostasis. In the light of our previous findings (Article I) we wanted to investigate if SIK1 is present in lung epithelial tissue, and if so, it's role in sodium transport.

4.2.1 SIK isoform expression

We started by investigating the presence of different SIK isoforms in alveolar epithelial cells and cell lines thereof. By reverse transcription PCR we detected mRNA from all three SIK isoforms in cell lines originating from human alveolar epithelium (A549 and H441 cells) as well as in AEC1 and AEC2 cells obtained from mouse and rat lungs. Using immunofluorescence in lung sections from rat and mouse, we showed the presence of all three SIK isoforms at protein level. The presence of the SIK1 isoform in both AEC1 and AEC2 suggests a possible role of SIK1 as a regulator of Na⁺,K⁺-ATPase activity and sodium transport in both cell types. The Na⁺,K⁺-ATPase has been ascribed a crucial role for resolving lung edema, based on functional studies performed in isolated AEC2 or cell line models thereof, mainly because of the difficulty to isolate and propagate the thin AEC1. AEC1 covers 95% of the surface of the alveolar cavity, and theoretically, sodium transport could take place also in these cells (Chen, Chen et al. 2004).

In the present study, we chose a model of AEC2 from mouse, MLE-12, where all three SIK isoforms were detected at both mRNA and protein level by RT-PCR and Western blot, respectively. As SIK1 associates with the Na⁺,K⁺-ATPase α -subunit in renal proximal tubule cells (OK cells) and regulates the Na⁺,K⁺-ATPase activity in response to increases in intracellular sodium, as shown in Article I (Sjostrom, Stenstrom et al. 2007), we performed immunoprecipitation of Na⁺,K⁺-ATPase in MLE-12 cells. This showed that also in the lung cells, SIK1 co-immunoprecipitated with the Na⁺,K⁺-ATPase and might participate in the regulation.

4.2.2 SIK1 mediates Na⁺,K⁺-ATPase activation in response to Iso

β -AR agonists are known to activate the Na⁺,K⁺-ATPase activity in lung and thereby increase AFR (Bertorello, Ridge et al. 1999; Vadasz, Raviv et al. 2007). This has led to intense research for drug development aiming at increasing lung edema clearance based on β -AR stimulation (Perkins, McAuley et al. 2006; Perkins, Gao et al. 2008; Johnson and Matthay 2010). We investigated whether the β -adrenergic receptor agonists Iso could regulate SIK1 activity in MLE-12 cells by determining the state of phosphorylation of residue Thr-182, which reflects the degree of autophosphorylation and activity of SIK1 (Katoh, Takemori et al. 2006). This revealed that SIK1 activity was indeed increased by Iso. The role of SIK1 as a mediator for the increases in Na⁺,K⁺-ATPase activity was investigated by ouabain sensitive rubidium transport in cells transiently over-expressing a kinase deficient mutant of SIK1, K56M, compared to wild type SIK1. In cells having a functional SIK1, Na⁺,K⁺-ATPase activity increased in response to Iso treatment, whilst this effect was absent in cells expressing the mutated form of SIK1 (K56M). This suggests a role of SIK1 in the network of proteins responsible for the stimulation of Na⁺,K⁺-ATPase activity in response to Iso.

To further investigate the role of SIK1, we created a cell line stably expressing shRNA directed towards SIK1 mRNA (SIK1-shRNA). A randomized, scramble sequence, not targeting any known mouse mRNA, was used as control (sc-shRNA). The shRNA reduced SIK1 by 40 % at both mRNA and protein level, as determined by TaqMan RT-PCR and Western blot respectively. SIK1 was reduced without changing the expression of the other SIK isoforms. As previously shown, Iso increases the Na⁺,K⁺-ATPase activity in lung alveolar cells by increasing the number of molecules at the plasma membrane (Bertorello, Ridge et al. 1999). By cell surface biotinylation experiments, we could detect an increased number of Na⁺,K⁺-ATPase α -subunits at the plasma membrane in response to isoproterenol in the control cell line (sc-shRNA), but this effect was abolished in the cell line with reduced levels of SIK1. This finding suggests a possible role for SIK1 in regulation of intracellular trafficking of Na⁺,K⁺-ATPase-containing vesicles. Motor proteins, such as kinesin, that propel vesicles to the plasma membrane are regulated by hormones (Verhey and Hammond 2009). Kinesin contain SIK1 phosphorylation sites, and we speculate that SIK1 takes part in a signaling network regulating the transport of active Na⁺,K⁺-ATPase molecules to the plasma membrane in response to stimulation of β -adrenergic receptors by Iso.

In Article I of this thesis, we describe a signaling network, with SIK1 at its core, responsible for the activation of the catalytic properties of the Na⁺,K⁺-ATPase already in the plasma membrane in response to increases in intracellular sodium. The findings in Article II suggest that SIK1 could mediate also the trafficking of active molecules to the plasma membrane, or act in combination to increase the Na⁺,K⁺-ATPase activity, sodium transport and alveolar fluid reabsorption and lung edema clearance.

4.3 SALT-INDUCIBLE KINASE 1 REGULATES E-CADHERIN EXPRESSION AND INTERCELLULAR JUNCTION STABILITY

It is well established that the polarized characteristics of epithelial cells are a prerequisite for vectorial transport of sodium, water and nutrients over the epithelial layer in e.g. kidney, lung and colon. Liver kinase 1B (LKB1) is central for cell polarity via E-cadherin expression (Roy, Kohno et al. 2010) and a known activator of SIK1 activity (Lizcano, Goransson et al. 2004; Hashimoto, Satoh et al. 2008). To investigate if SIK1 may serve as a molecular link in the regulation of the expression of polarity associated proteins, we used the cell line described in Article II, where SIK1 is stably repressed by shRNA directed towards SIK1 mRNA (SIK1-shRNA). A corresponding cell line stably transfected with a scramble sequence was used as control (sc-shRNA). As SIK1 decreased, the cells changed their phenotype acquiring a more rounded shape with less lamellipodia, the growth rate decreased and the cells grew in isolated island formations instead of forming a monolayer. Only SIK1 was reduced by the shRNA, not SIK2 and SIK3.

4.3.1 Reduced SIK1 levels lead to decreased E-cadherin expression

Interestingly, the reduced level of SIK1 was associated with an almost complete abolishment of E-cadherin expression. To determine if this was an effect of SIK1 reduction *per se*, we investigated the protein expression of known upstream regulators of SIK1 (LKB1, GSK3 β), as well as another member of the AMPK family of kinases, the AMPK itself. All of these were however unaffected, indicating a SIK1-specific regulatory pathway.

The reduction of E-cadherin expression may be due to repression of the synthesis or an increased degradation rate. p120CAS, another structural protein, binds to the juxtamembrane domain of E-cadherin and regulates its turnover (Ireton, Davis et al. 2002; Davis, Ireton et al. 2003). By immunofluorescence we showed that p120CAS was reduced and did not localize properly in our SIK1 deficient cells, however, pCAS120 mRNA was upregulated, possibly due to a compensatory activation of the pCAS120 gene. β -catenin that normally binds to the extracellular domain of E-cadherin, was not altered neither in expression nor localization.

The expression and regulation of E-cadherin have been extensively studied, and besides promoter hypermethylation (Graff, Herman et al. 1995), microRNA regulation (Ma, Young et al. 2010) and stabilization/internalization turnover, degradation (Yang, Zong et al. 2006), transcriptional repressors have been ascribed important roles. The transcription factors (TF) Snail1, Snail2 (Slug), Zeb 1, Zeb 2 (Sip) and Twist have been reported to repress E-cadherin by binding to the E-box sequences within the promoter region (Batlle, Sancho et al. 2000; Bolos, Peinado et al. 2003; Peinado, Olmeda et al. 2007). In the SIK1-shRNA cells, the mRNA level of all above mentioned TFs, except Snail1, increased significantly. Most prominent was the increase in Snail2 and Twist, which will be discussed further.

To further investigate the functional relevance of SIK1 in the regulation of the E-cadherin promoter, we used a Luciferase reporter assay. SIK1-shRNA and sc-shRNA cells were transiently transfected with a vector containing the E-cadherin promoter

driving the production of luciferase. The E-cadherin promoter activity was significantly higher in cells having normal levels of SIK1 (sc-shRNA) compared with the cells with reduced SIK1 levels. We further transiently transfected both cell types with SIK1 W.T. or a SIK1 T322A mutant lacking the calcium calmodulin phosphorylation site (as reported in Article I). In the sc-shRNA cells, overexpressing the SIK1 W.T. increased the E-cadherin promoter activity, whereas no increase was detected in the cells overexpressing the T322A mutant. This is in line with the fact that E-cadherin is calcium-dependent, and suggests that SIK1 may be the signaling molecule mediating the calcium effect. In the SIK1-shRNA cells, overexpressing the SIK1 W.T. did not increase the E-cadherin promoter activity, excluding the possibility of SIK1 controlling the E-cadherin promoter directly.

4.3.2 *In vivo* confirmation studying *sik1*^{-/-} mice lungs

Also in mice with an ablation of the *sik1* gene, the expression of E-cadherin mRNA and protein was significantly lower in the lung tissue when compared with lung tissue from the *sik1*^{+/+} mice, although not as dramatically reduced as seen *in vitro*. Although an increased SIK2 mRNA expression was observed in the *sik1*^{-/-}-animals, the protein expression was not significantly increased, ruling out a compensatory effect of SIK2. In accordance with the *in vitro* findings, Snail2 mRNA expression was increased in the *sik1*^{-/-} animals compared with the *sik1*^{+/+} animals.

4.3.3 The role of LKB1

LKB1 activates SIK1 (Lizcano, Goransson et al. 2004; Hashimoto, Satoh et al. 2008), and loss of LKB1 is a signal for reduced E-cadherin expression and EMT (Roy, Kohno et al. 2010). Therefore, we sought to determine if SIK1 mediates the signals from LKB1 to E-cadherin. In MLE-12 cells, transient overexpression of SIK1 W.T. resulted in an increased E-cadherin mRNA expression, and in parallel, decreased Snail2 mRNA levels. This effect was blocked by overexpressing a SIK1 mutant, T182A, where the LKB1 activation site is interrupted by site directed mutagenesis.

We further expressed LKB1 together with STRAD and MO25, two proteins needed for full LKB1 activity (Baas, Boudeau et al. 2003; Boudeau, Baas et al. 2003; Zeqiraj, Filippi et al. 2009), in both SIK1-shRNA and sc-shRNA cells. This increased the E-cadherin mRNA expression MLE-12 cells with normal levels of SIK1, whereas this effect was absent in SIK-shRNA cells, further supporting our hypothesis.

4.3.4 Cytoskeletal and TJ organization

By immunofluorescence microscopy we could conclude that the reduction of E-cadherin was not due to changes in the cytoskeletal structures and organization or other intercellular junction proteins, as actin, vinculin and ZO-1 were all expressed and localized normally.

MARK2, another kinase involved in polarity and microtubule regulation (Lizcano, Goransson et al. 2004), was upregulated at both mRNA and protein level in SIK1-shRNA cells when compared with the scramble control cell line. This indicates that SIK1 controls the expression of MARK2, and thereby contribute to the establishment

of cell polarity in a LKB1-independent manner. The organization of the microtubules was not affected by the upregulation of MARK2 in response to decreased SIK1 levels.

4.3.5 Increased SIK1 leads to increased levels of E-cadherin

We further studied the correlation between SIK1 and E-cadherin in cells modified to have higher SIK1 activity. Previous work in our group has shown that cells expressing a mutated form of the cytoskeleton protein α -adducin, previously reported to be associated with high blood pressure and increased sodium reabsorption in both humans and rats, have higher SIK1 activity when compared with the normotensive (N.T.) α -adducin variant (Stenstrom, Takemori et al. 2009). A substitution of Phenylalanine to Tyrosine at position 316 in the rat protein corresponds to G460W/S586C SNPs in the human ADD1 gene (Efendiev, Krmar et al. 2004), however, the mechanism by which the mutated form of α -adducin increases SIK1 activity is unknown. We used epithelial cell lines originating from human proximal tubules, HK-2 (Ryan MJ 1994), modified by stable transfections with either α -adducin W.T. (N.T.) or the α -adducin bearing the hypertensive mutation, F316Y (H.T.).

By TaqMan mRNA expression analysis and Western blot we could conclude that both SIK1 and E-cadherin mRNA and protein expression truly increased in the H.T. cells compared with the N.T., without changing the phenotype. The TFs Snail2, Zeb 1 and Zeb 2 decreased significantly in the H.T. cells.

To investigate if the increase in E-cadherin was SIK1 dependent, we reduced SIK1 with siRNA in the H.T. cells. After 48h, decreased mRNA expression levels of both SIK1 and E-cadherin were observed whereas Snail2 increased. After 72h, the effect was clearly detectable also at protein level.

4.3.6 SIK1 regulates Snail2 expression via CREB

SIK1 has been shown to repress the TF CREB (cAMP response element binding protein) both directly and via phosphorylation of Transducer of Regulated CREB activity 2, TORC2 (Takemori, Kajimura et al. 2007; Takemori and Okamoto 2008). Reduced levels of SIK1 would therefore lead to increased expression of CREB target genes. By using a CRE-luciferase reporter vector we compared the promoter activity in cells with different levels of SIK1. In MLE-12 with reduced levels of SIK1 (SIK1-shRNA), the CRE activity was significantly higher than in the scramble control. On the contrary, in HK-2 cells with more SIK1 (HK-2 H.T.) the CRE activity was lower than the normotensive cells (HK-2 N.T.). *In silico* analysis revealed that several CREB- and C/EBP-binding sites were present in the Snail2 promoter region, which caught our interest. CREB1 was shown by siRNA experiments to regulate Snail2 mRNA expression, without affecting the SIK1 mRNA levels.

4.3.7 SIK1 regulates the stability of intercellular junctions

TER was used to investigate the functional significance of reduced SIK1, and thereby reduced E-cadherin levels, in MLE-12 cells. We observed a decreased resistance over the epithelial layer, indicating a leaky, less tight epithelium, likely to be caused by the lack of E-cadherin and abnormal p120CAS catenin distribution. When subjecting the

cells to Ca^{2+} -free medium, the TER decreases and when restoring the Ca^{2+} (referred to as the Calcium-switch technique) the TER increased again in cells having SIK1. The recovery of TER was absent in cells with reduced SIK1 and E-cadherin levels. When reducing the levels of SIK1 by siRNA in other cell lines known to form tight epithelial layers (MDCK and T84), the TER reduced significantly, supporting the role of SIK1.

ATP is a known regulator of intercellular junction stability and polarity, and the Na^+, K^+ -ATPase is the major consumer of ATP in the cell. Therefore, we investigated both ATP levels as well as Na^+, K^+ -ATPase activity in SIK1-shRNA MLE-12 cells compared with the scramble control. The Na^+, K^+ -ATPase activity was lower in the cells with less SIK1, and the ATP levels were higher, accordingly. Also the activity of the Na^+/H^+ -exchanger decreased, whereas sodium-independent transporter activity ($\text{Cl}^-/\text{HCO}_3^-$) remained unchanged. The basal membrane potential was lower and the cells did neither depolarize in response to KCl nor increase in membrane potential in response to pinacidil in the cells with reduced levels of SIK1 (MLE-12 SIK1-shRNA).

In summary, our findings support a role of SIK1 in the regulation of E-cadherin expression, and thereby the integrity, maintenance and function of polarized epithelia. This is important for vectorial transport of sodium as well as for organogenesis and tumor metastasis formation.

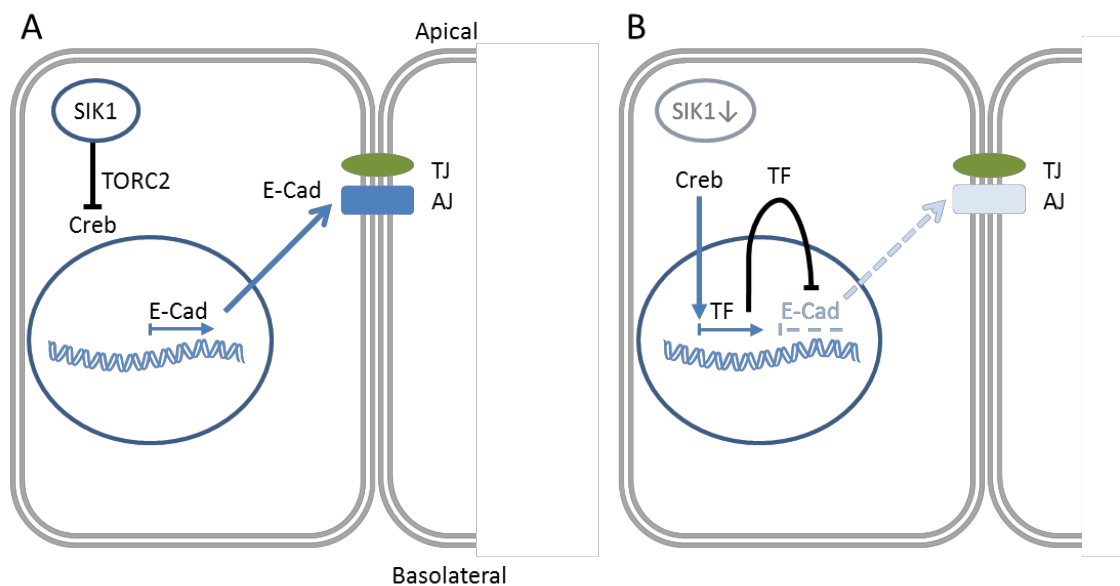


Figure 6. Proposed mechanism for SIK1 controlling E-cadherin expression. A) When expressed at normal levels, SIK1 inhibits CREB-dependent transcriptional regulation via TORC2. **B)** When SIK1 levels are decreased, CREB translocate into the cell nucleus and initiate transcription of transcription factors (TF), which in turn represses E-cadherin expression. The integrity and function of the epithelial layer is then impaired.

5 DISCUSSION

In the first study, we discovered that the acute, short term regulation of sodium homeostasis by the Na^+, K^+ -ATPase in response to increased $[\text{Na}^+]_i$ is regulated by SIK1. The rapid activation is coupled to a series of phosphorylation and dephosphorylation events leading to increased activity of the Na^+, K^+ -ATPase. It has been thought that increased substrate availability by itself, i.e. that sodium *per se*, could be a triggering signal for increased Na^+, K^+ -ATPase activity, but this hypothesis do not take into consideration neither the distribution of the Na^+ and the Na^+, K^+ -ATPase in space nor the time for ion diffusion and Na^+, K^+ -ATPase activation. Our findings disclose a signaling network able to activate Na^+, K^+ -ATPase already in the plasma membrane, which may constitute a limited pool of Na^+, K^+ -ATPases that could be in close association with NCX. The $[\text{Ca}^{2+}]_i$ associated properties of the disclosed mechanism is highly similar to a sodium handling mechanism in plants where SOS2, a SNF kinase (Liu, Ishitani et al. 2000), regulates a NHE (SOS1) via a calcium-dependent kinase, SOS3 (Qiu, Guo et al. 2002). The high similarity suggests a conserved pathway for sodium handling throughout evolution (Bertorello and Zhu 2009).

In renal epithelial cells, the regulation of vectorial sodium transport is crucial for control of the blood pressure. Too much sodium reabsorption, as in salt-sensitive hypertensive patients caused by dysregulation of the Na^+, K^+ -ATPase, increases the plasma volume and could potentially lead to hypertension with the complications that arises thereof. High blood pressure is *per se* worsening kidney damage and fibrosis, with the consequences of decreased kidney function and subsequent end stage renal failure (He, Jenner et al. 2010). High SIK1 activity might be causing increased Na^+, K^+ -ATPase activity and lead to enhanced sodium reabsorption, thereby being detrimental for blood pressure control. In cells with a mutant form of α -adducin associated with high blood pressure and increased Na^+, K^+ -ATPase activity, inhibition of SIK1 decreases the active sodium transport (Stenstrom, Takemori et al. 2009). Very recent findings from our group suggest that there exists a genetic variant of SIK1 associated with lower blood pressure, as well as left ventricular mass (Popov, Silveira et al. 2011).

The second study revealed that all SIK isoforms are expressed in the alveolar epithelial cells. SIK1 and 3 have been shown to be ubiquitously expressed (Feldman, Vician et al. 2000; Berggreen, Henriksson et al. 2012) whereas SIK2 is mainly detected in the liver, brain and in adipocytes (Dentin, Liu et al. 2007; Sasaki, Takemori et al. 2011; Henriksson, Jones et al. 2012).

In the lung, an increase in Na^+, K^+ -ATPase activity facilitates lung edema clearance. Stimulation of SIK1 activity in the damage lung would increase the vectorial transport of sodium and thereby enhance water reabsorption over the remaining epithelial layer. β -adrenergic agonists have been the target for many attempts of increasing AFR, increasing the reabsorption in both animals (Litvan, Briva et al. 2006) as well as patients with ALI (Atabai, Ware et al. 2002; Perkins, McAuley et al. 2006). Our results indicate that SIK1 is needed for the upregulation of Na^+, K^+ -ATPase activity in response to Iso via translocation of active Na^+, K^+ -ATPases to the plasma membrane.

Recently after our findings were published, a follow-up study (BALTI-2) showed low tolerance for the β -adrenergic agonist Salbutamol (Gao Smith, Perkins et al. 2012). The mortality increased following intravenous administration of Salbutamol in ventilated patients within 72h after ARDS. The extravascular fluid volume decreased, but due to poor tolerance for salbutamol, the authors cannot recommend routine use of Salbutamol treatment of ARDS patients, at least not the dose given. Central administration of Salbutamol was poorly tolerated by patients with ALI, and aerosolized administration to the damaged, flooded alveolar epithelium is associated with obvious drawbacks regarding uptake and efficiency. The β -adrenergic receptors may be downregulated due to the injury, further complicating the difficult task of drug delivery (Gao Smith, Perkins et al. 2012). If the renal SIK1 is activated by β -adrenergic agonists, this could lead to higher sodium absorption and increased blood pressure, which is detrimental in patients with lung edema (Gandhi, Powers et al. 2001). The hormonal regulation of Na^+, K^+ -ATPase is however known to be tissue specific (Bertorello and Sznajder 2005).

Whereas Article I and II account for the short term, non-genomic aspect of this thesis, Article III elucidates the gene-regulating, long term angle.

In the third study the long term effects of malfunctional sodium regulation were examined by disruption of SIK1 expression using shRNA. As hypothesized, Na^+, K^+ -ATPase activity was downregulated, and the decrease in TER indicates that vectorial sodium transport was impaired. This further support the role of SIK1 as an important sodium regulator. The most striking finding though, was the correlation between SIK1 and E-cadherin expression, a hallmark protein of cell polarity. Reduction of SIK1 leads to downregulation of E-cadherin expression in the lung cell model, and increased SIK1 levels coherently induced E-cadherin expression in a cell line originating from human lung (HK-2). The experiments performed in the human kidney cells support the possibility for the proposed mechanism to be conserved both in different epithelial tissues as well as in different species.

The role of SIK1 as a gene transcription regulator has been investigated, both via CREB inactivation via TORC1 and 2 (Kato, Takemori et al. 2006; Takemori, Kajimura et al. 2007; Li, Zhang et al. 2009), via activation of myocyte enhancer factor-2, MEF2, in response to increases in intracellular sodium (Popov, Venetsanou et al. 2012) and HDAC modulation (Berdeaux, Goebel et al. 2007). Our results in Article III suggest a new role for the SIK1 – CREB mediated gene regulation, highly connected to sodium transport.

It is somewhat surprising that the expression and localization of β -catenin at the cell membrane seems to be unchanged during E-cadherin downregulation, and neither degraded nor accumulated in the cytosol or nucleus. Other researchers have shown that β -catenin that is not bound to E-cadherin is either phosphorylated by GSK3 β and degraded by the proteasome, or protected from degradation by the Wnt signaling pathway. Wnt inhibits GSK3 β and β -catenin can accumulate in the cytosol and translocate into the nucleus, acting as a transcriptional activator leading to expression of genes associated with cancer progression and metastasis, nicely reviewed by (Valenta, Hausmann et al. 2012). Although interesting, we did not explore the expression and localization of β -catenin further as it was not in the scope of the present study.

Earlier studies suggest an important role for SIK1 for the development of the heart, both due to the specific expression pattern of *sik1* in the monolayer of the future myocardial cells during mouse embryogenesis (Ruiz, Conlon et al. 1994) and in the *sik1^{flp/flp}* knock-out mouse embryonic stem cell line used in the study by Romito *et al* (Romito, Lonardo et al. 2010). SIK1 protein may control cardiomyogenesis via cell cycle regulation via p57^{kip2} and by phosphorylation of class II HDACs leading to MEF2-dependent gene transcription (Berdeaux, Goebel et al. 2007; van der Linden, Nolan et al. 2007). The *sik1^{-/-}* strain of mice generated by Lexicon Pharmaceuticals used in this study were confirmed to be SIK1 negative. The mice developed normally, without reproduction, life span or cardiac developmental dysfunction, and grow old without obvious signs of increased cancer development, which may be due to a compensatory role of other SIK isoforms or any unknown mediator.

The role of SIK1 for regulating proteins important for the epithelial phenotype is important in both kidney and lung. In the kidney, a tight epithelial layer and regulated vectorial sodium transport is crucial for proper urine filtration and for controlling the blood pressure. If exposed to damage, high blood pressure etc. EMT and subsequent kidney fibrosis may occur, and SIK1 may be needed for proper control to avoid these events. This is somewhat contradictory as SIK1 activity can lead to high blood pressure via sodium reabsorption (Article I) but also be needed for keeping the epithelial phenotype and functions (Article III).

The integrity and impermeable features of the lung epithelium is necessary for keeping the alveolar cavity free from excess fluid leaking from the capillaries. Loosening of the junctional structures in response to decreased SIK1 levels, as indicated by the TER experiments, would increase the risk of edema formation. Also the impaired active sodium transport by lower Na⁺,K⁺-ATPase activity could decrease the AFR.

Loss of E-cadherin is a hallmark of EMT, which is needed for the cells to detach, migrate and invade surrounding tissue. Also, it is highly associated with cancer, metastasis and poor outcome (Roy, Kohno et al. 2010; Harada, Miyake et al. 2012). A myriad of different oncogenic pathways are described. Our finding that reduced E-cadherin expression as a consequence of decreased SIK1, support the suggested role of LKB1 and SIK1 in anoikis and metastasis (Cheng, Liu et al. 2009; Roy, Kohno et al. 2010).

6 SUMMARY AND CONCLUSION

The overall aim of this thesis was to investigate mechanisms of sodium regulation within transporting epithelia.

- We identified a sodium-sensing network with SIK1 at its core, able to detect small increases in intracellular sodium, and being responsible for up-regulation of Na⁺,K⁺-ATPase activity in renal epithelial cells. (Article I)
- All SIK isoforms are also present in alveolar epithelial cells type 1 and type 2 in mouse and rat, and in cell lines originating from human and mouse. SIK1 is also important for the responses in Na⁺,K⁺-ATPase activity in response to Iso. (Article II)
- In epithelial cells, SIK1 regulates the expression of E-cadherin and is needed for proper intercellular junction stability. SIK1 deficiency also disrupts the sodium handling characteristics of the cells. (Article III)

SIK1 is needed for regulation of sodium homeostasis; short term via regulation of the Na⁺,K⁺-ATPase catalytic activity, and long term by changing the gene expression level of proteins important for the establishment of a polarized epithelium, able to perform vectorial sodium transport.

7 FUTURE PERSPECTIVES

There's a growing body of evidence that SIK1 is an important kinase in many aspects, ranging from sodium transport and gene regulation involved in cell polarity as studied in the work of this thesis, as well as being important for related situations:

- regulation of blood pressure control (Stenstrom, Takemori et al. 2009; Popov, Silveira et al. 2011),
- cardiomyogenesis (Romito, Lonardo et al. 2010) and cardiac hypertrophy (Popov, Venetsanou et al. 2012),
- as a link in the LKB1-p53 tumor suppressing pathway and important for detaching cancer cells to undergo anoikis instead of forming metastases (Cheng, Liu et al. 2009)
- as a gene transcription regulator via CREB regulation (Kato, Takemori et al. 2006)
- in TGF β responses (Kowanetz, Lonn et al. 2008; Lonn, Vanlandewijck et al. 2012)

The now available *sik1*^{-/-} mice strain opens up for numerous possibilities for investigating the role of SIK1 *in vivo*. By introducing lung damage and edema formation in the knockout animals, the role of SIK1 in lung edema clearance can be elucidated. Studying the response to different agonists may open up for deeper understanding of the mechanisms and possible targets for drug development can be discovered.

Feeding the SIK1 deficient mice with a high salt diet could reveal a role for SIK1 in salt-sensitive hypertension and hypertension induced cardiac hypertrophy.

Further studies in samples from cancer patients could shed light on the relevance of co-regulation of SIK1 and E-cadherin for metastasis formation and patient outcome. If SIK1 deficiency leads to loss of E-cadherin and thereby higher risk of metastasis, investigating the underlying cause of SIK1 downregulation may be of highest importance. As the SIK1 activator LKB1 is a major player in non-small cell lung cancer (NSCLC) and NSCLC metastasis, by being among the most commonly mutated genes, after p53 and Ras (Sanchez-Cespedes, Parrella et al. 2002; Carretero, Medina et al. 2004; Ding, Getz et al. 2008), SIK1 downregulation may be very common as well. Marcus and Zhou (Marcus and Zhou 2010) discuss the different pathways overseen by LKB1 that are interrupted in metastasis formation, covering the fields of epithelial cell polarity, cell polarity during motility, cell detachment and anoikis. SIK1 is important linking LKB1 to p53 induced anoikis (Cheng, Liu et al. 2009), and the lack of SIK1 could not only be detrimental due to metastasis formation but also in the EMT process leading to cell detachment. Our findings in Article III adds further complexity to the picture, indicating that LKB1 – SIK1 – E-cadherin pathway is important for the characteristics of epithelial cell polarity via transcriptional repression.

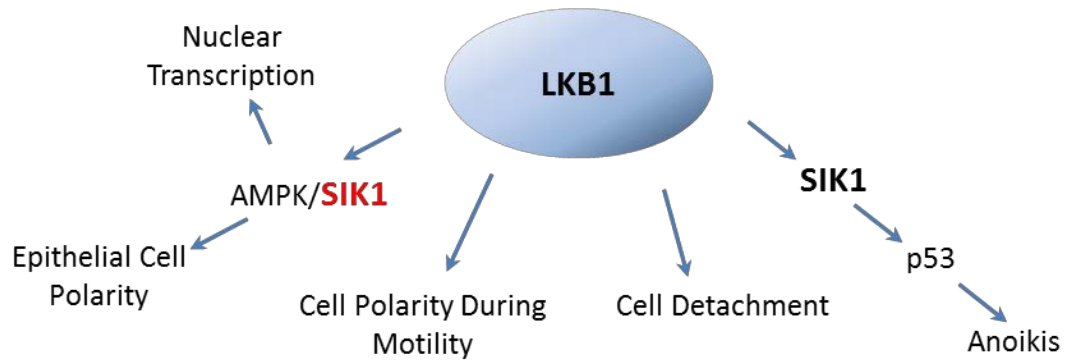


Figure 7. The relationship between LKB1, SIK1 and polarity associated effects. Modified from (Marcus and Zhou 2010).

The other SIK isoforms, SIK2 and 3, are not as intensely studied, but may as well be important factors in disease development. SIK2 has been ascribed a role in gluconeogenesis in liver cells (Dentin, Liu et al. 2007; Wang, Li et al. 2012) and lipid metabolism in adipocytes (Henriksson, Jones et al. 2012). There are reports elucidating the role of SIK3 in fat metabolism in mice (Berggreen, Henriksson et al. 2012; Uebi, Itoh et al. 2012) and in flies (Wang, Moya et al. 2011), showing that SIK3 is responsible for fat storage. Overexpression of SIK3 may also contribute to cancer cell growth by inhibition of p21 and p27 in ovarian cancers. (Charoenfuprasert, Yang et al. 2011). In addition to their individual roles, crosstalk and redundant functions may exist among the closely related SIK isoforms.

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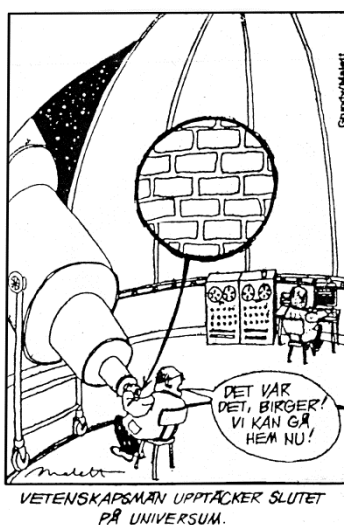
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*Scientists find the end of the universe.
“That’s it, Birger! We can go home now!”**

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