Regulatory Mechanisms of Epithelial Sodium Channel

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Synopsis

Epithelial sodium channels (ENaC) are of immense importance, controlling Na^+ transport across epithelia and thus playing a critical role in all aspects of fluid clearance as well as numerous other functions. Although extensive studies have been carried out to invistegate the regulation mechanism of ENaC, many questions still remain unclear. Therefore, we employed various techniques including electrophysiology and molecular biology approaches to investigate the mechanisms underlying the regulation of ENaCs by lipid metabolites, oxygen and mechanical stress.

We have identified profound regulation mechanisms of ENaC in distal renal epithelial cells and vascular endothelial cells by lipid metabolites, heme and mechanical forces. Our results revealed a novel O_2 sensitive regulation pathway of ENaC channels, in which hemeoxygenase acts as the O_2 sensor and the substrate and product of which either inhibits or stimulates ENaC activity. This finding may eventually lead to novel clinic strategies in dealing with diseases e.g. renal failure, kidney reperfusion injury, hypertension, pulmonary edema and pre-clampsia. In addition, we, at the first time, have revealed that ENaC is functionally expressed in a variety of endothelial cells and is able to serve as mechano-transducing sensors in the vascular endothelial cells which play important roles in vascular physiology and pathology progresses.

Dedication

I dedicate this work to my family, without their support this work may not have been possible.

Acknowledgement

I would like to thank my supervisor, Dr Yuchun Gu for his guidance and advices. I also would like to thank my colleagues, namely Fei Meng, Wilson To and Juyou Wu.

Abbreviations

$[Ca^{2+}]_i$: intracellular calcium concentration

$[Na^{+}]_i$: intracellular sodium concentration

4-AP:4-aminopyridine

A-a gradient: Alveolar to arterial gradient

AA: arachidonic acid

ACh: acetylcholine

AEA: anandamide

ATP: Adenosine-5'-triphosphate

ADP: Adenosine-5'-diphosphate

AMP: Adenosine-5'-monophosphate

BK_{Ca}: large conductance calcium activated potassium channel

cADPR: cyclic ADP-ribose

cAMP: Cyclic adenosine monophosphate

CB: carotid body

CCD: cortical collecting duct

CCE: capacitative Ca2+entry

CCT: cortical collecting tubule

cGMP: Cyclic guanosine monophosphate

CI: Carboxyheptyl imidazole

CICR: calcium induced calcium release

CO: carbon monoxide

COX: cyclooxygenase

cPGES: cytosolic PGES

cPLA2: cytosolic PLA2

CYP450: Cytochrome P-450 monooxygenases

DA: dopamine

DAG: diacylglycerol

DGK: diacylglycerol kinase

DMSO: Dimethyl sulfoxide

DP: prostaglandin D2 receptor

EC: endothelial cells

EDHF: Endothelium-Derived Hyperpolarizing Factor

EET: epoxyeicosatrienoic acids

EGTA: ethylene glycol tetraacetic acid

Em: membrane potential

eNOS: endothelial NOS

ENaC: epithelial sodium channel

EP: prostaglandin E2 receptor

EPA: eicosapentaenoic acid

ER: endoplasmic reticulum

EST: expressed sequence tag

ET-1: endothelin-1

ETC: electron transport chain

ETYA: eicosatetraynoic acid

FADH2: flavin adenine dinucleotide

FIH-1: factor inhibiting HIF-1

FP: prostaglandin F2 receptor

GDP: guanosine diphosphate

GFR: glomerular filtration rate

GMP: guanosine monophosphate

GTP: guanosine triphosphate

HIF-1: hypoxia-inducible factor-1

HMEC: human micro-vascular endothelial cells

HO: heme oxygenases

HUVEC: human umbilical vein endothelial cells

iNOS: inducible NOS

IP: prostacyclin receptor

IP3: 1,4,5,-trisphosphate

IP3R:IP3 receptor

IPA: isolated pulmonary artery

iPLA2: calcium independent PLA2

KATP: ATP activated potassium channel

KIR: inward rectifier potassium channel

Kv: voltage-gated potassium channels

LOX: lipoxygenase

MAP: kinase: mitogen-activated protein kinases

NADPH: nicotinamide adenine dinucleotide phosphate

NCX: calcium sodium exchanger

nNOS: neuronal NOS

NO: nitric oxide

NOS: NO synthase

NSCC: none selective cation channel

PGES: PGE2 synthase

PGH2: prostaglandin endoperoxide H2

PGHS: prostaglandin H2 synthases

PGI2: prostacyclin

Pl_3K: Pl_3 kinase

PKA: cAMP-dependent protein kinase

PKC: protein kinase C

PLA2: phospholipase A2

PLC: phospholipase C

P_{O2}: pressure of oxygen

PPS: physiological salt solution

ROC: receptor operated calcium channel

ROS: reactive O_2 species

siRNA: small interfering RNA

SNP: sodium nitroprusside

SOC: store-operated calcium channel

SOD: superoxide dismutase

SR: sarcoplasmic reticulum

TEA: Tetraethylammonium

TP: thromboxane receptor

TRPC: classical or canonical TRP cation channels

TXA2: thromboxane A2

VGCC: voltage-gated calcium channel

VSMC: vascular smooth muscle cell

Chapter 1

General Introduction

1.1 Epithelial sodium channel

The epithelial Na^+ channel (ENaC) is localized at the apical or outward-facing membranes of many salt-reabsorbing epitheliums which facilitate Na^+ movement across this membrane as a first step in the process of transepithelial Na^+ transport. The channels can be defined functionally as components of the membrane that transports Na^+ into the cell by electro-diffusion without coupling movements of other solutes and without the direct input of metabolic energy (Garty and Palmer 1997).

ENaCs are located in the apical membrane of polarized epithelial cells where they mediate Na^+ transport across tight epithelium. In contrast to other Na^+ selective channels involved in the generation of electrical signals in excitable cells, the basic function of ENaC in polarized epithelial cells is to allow vectorial trans-cellular transport of Na^+. This transepithelial Na^+ transport through a cell basically involves two steps; the passive entry of Na^+ into the cell on the apical membrane driven by the large electrochemical gradient for Na^+ existing across the membrane; and the active Na^+ transport across the basolateral membrane is accomplished by the Na^+-K^+- ATPase (Rick, Dorge et al. 1981; Huf and Mikulecky 1983; DeLong and Civan 1984; Palmer and Frindt 1986; Palmer and Frindt 1986). ENaCs are a diverse group of ion channels displaying complex gating kinetics, intricate regulatory patterns and biophysical and pharmacological properties that are dependent upon their biochemical state and physical surroundings.

1.2 ENaC/DEG channel super family

1.2.1 Members of super family

The ENaC/degenerin gene family represents a new class of ion channels which include seven different gene branches. The most relevant ENaC/DEG sequences available to date can presently be distinguished as (Driscoll and Chalfie 1991; Canessa, Schild et al. 1994; Adams, Anderson et al. 1998; Darboux, Lingueglia et al. 1998; Alvarez de la Rosa, Canessa et al. 2000; Hong, Mano et al. 2000; Venter, Adams et al. 2001): a), the ENaC α-, β-, γ-, and δ-subunits; b), the degenerins from Caenorhabditis elegans; c), acid-sensing ion channels (ASICs); d), the Drosophila proteins RPK/dGNaC1 and PPK/dmdNaC1; e), the peptide-gated Na^+ channel FaNaC of mollusks; and f), FLR-1 in C. elegans that is clearly distinct from the degenerins. g), the mammalian BLINaC (brain- liver- intestine amiloride-sensitive Na^+ channel) and hINaC (human intestine Na^+ channel) genes encode Na^+ channels that are clearly distinct from ENaC.

The amino acid sequence identity between the different ENaC/DEG subfamilies is ≈15–20%, whereas the identity within subfamilies is ≈30% for the different ENaC subunits; ≈30% for degenerins; 45–60% between the four ASIC genes ASIC1, ASIC2, ASIC3, and ASIC4; 38% for the two characterized Drosophila members; and ≈65% for the three FaNaC members. Acids-sensing ion channels (ASICs) and (BLINaC)/(hINaC) are the three ENaC/DEG channel groups found in vertebrates; the four ENaC/DEG channel

groups of invertebrates are: the degenerins from Caenorhabditis elegans; the Drosophila channels RPK/dGNaC1 and PPK/ dmdNaC1; FMRFamide-gated sodium channel (FaNaC), which is expressed in mollusks; FLR-1, which is the only characterized member of a group of C. elegans ENaC/DEG family members that are different from the degenerins. Degenerins and ENaC were the first two genes identified in this super family and were found to have substantial sequence homology. The name degenerin (DEG) comes from the cellular phenotype induced by mutations of the deg-1 gene and other related genes that result in selective degeneration of sensory neurons involved in touch sensitivity. ENaC has long been characterized by its Na^+ selectivity and amiloride sensitive and known to play a crucial role in Na^+ absorption in the distal part of the kidney tubule and to be the target of aldosterone action. Additional members of this ion channel family were subsequently identified by sequence homology and characterized by functional expression. The acid-sensing ion channels (ASICs) were originally called mammalian degenerins (MDEG) or brain Na^+ channels (BNaC, BNC) because they were found to be expressed mainly in the central and peripheral nervous system. After the discovery of their activation by extracellular protons these channels were named acid-sensing ion channels (ASICs). At about the same time the FMRF-amide-gated ion channel (Fa- NaC) was cloned from the mollusk Helix aspersa. This channel forms its own subfamily within the ENaC/DEG family of ion channels.

The known members of the ENaC/DEG gene family are wildly distributed in different tissues including transporting epithelia as well as neuronal excitable tissues. They

exhibit a high degree of functional heterogeneity including Na^+ reabsorption, taste reception, touch sensation, and mechano-transduction. Depending on their function in the cell, these channels are either constitutively active like ENaC or activated by mechanical stimuli as postulated for C. elegans degenerins, or by ligands such as peptides or protons in the case of FaNaC and ASICs.

1.2.2 Degenerins

Degenerin genes MEC-4, MEC-10, UNC-8 UNC-105 and DEG-1 are the core elements of the mechanosensory complex of Caenorhabditis elegans. Elegant genetic analysis has identified these genes (Chelur, Ernstrom et al. 2002; Goodman, Ernstrom et al. 2002; Goodman and Schwarz 2003; Bianchi, Gerstbrein et al. 2004; O'Hagan, Chalfie et al. 2005). The mutation of these genes specifically disrupts gentle body touch sensation and swelling and subsequent death of the cells. These genes are called degenerins because their mutations cause cell degenerative phenotype (Driscoll and Chalfie 1991; Waldmann, Champigny et al. 1996; Mano and Driscoll 1999; Goodman, Ernstrom et al. 2002; O'Hagan, Chalfie et al. 2005; Cueva, Mulholland et al. 2007; Zhang, Bianchi et al. 2008).

Degenerin subunits MEC-4 and MEC-10 form the core of MEC channel complex, which is believed to be the machano-transducer ion channel in C. elegans touch receptor neurons (Driscoll and Chalfie 1991; Hong, Mano et al. 2000). The MEC channel has

long been supported by genetic and molecular data to respond to mechanical stimuli and was demonstrated to be gated by mechanical forces recently. A transient rise in intracellular Ca^{2+} concentration induced by MEC under mechano-stimuli has been observed. Furthermore, mutation on a certain point of the mec-4 causes channel hyperactivity, which is believe to permit excessive Na^{+} influx and possible Ca^{2+} entry into the cell which finally cause cell death (Mano and Driscoll 1999; Bianchi, Gerstbrein et al. 2004).

UNC-8 is expressed in interneurons, motoneurons, and some sensory neurons in Caenorhabditis elegans and is believed to form channel complex along with other proteins including DEL-1 (degenerinlike) in parts of the neurons. Similar to that of the MEC-4, mutation on UNC-8 causes hyperactive channels and results in swelling and death of motoneurons (Mano and Driscoll 1999). The hyperactivation-causing mutation is in the extracellular domain of UNC-8 (G387E) just after the first cysteine-rich domain. UNC-8 loss-of-function mutation experiments suggested that UNC-8 may function as a stretch receptor to mediate proprioception in motoneurons (Tavernarakis, Shreffler et al. 1997) (Rajaram, Spangler et al. 1999; Tavernarakis, Everett et al. 2001; Sedensky, Siefker et al. 2004; Bianchi 2007).

UNC-105 is expressed in the muscle cells of the body wall, and semidominant unc-105 alleles induce hypercontraction of body wall muscles. One of these mutations causes hyperactivity of the channel as assayed in Xenopus oocytes. Hyperactivity of the mutant

channel is thought to cause excess ion influx with consequent hypercontraction. These data suggest that UNC-105 may be a stretch-sensitive channel gated by changes in molecular interactions occurred during locomotion.

1.2.3 Super family Structures

The size of the ENaC/DEG proteins ranges from ≈530 to ≈740 amino acids. The pore of ENaC has been the best characterized among the members of the ENaC/DEG channel family, and it is likely that the basic structural features of the channel pore are conserved within this family(Renard, Lingueglia et al. 1994). Regions of conserved amino acid sequences in ENaC/DEG family members are likely to represent structural elements important for channel function (Kellenberger, Gautschi et al. 1999) (Canessa, Schild et al. 1994; Rossier, Canessa et al. 1994).

The conserved domains are located essentially in the transmembrane segments and their proximity, and in the extracellular loop. A few sequences are completely conserved among the ENaC/DEG family members; they include a HG motif located in the NH2-terminal cytoplasmic domain in close proximity to the M1 segment, a FPxxTxC sequence following M1 (post-M1) and completely conserved residues in the M2 region(Canessa, Schild et al. 1994; Duc, Farman et al. 1994; Rossier, Canessa et al. 1994). The extracellular loop contains cysteine-rich domains (CRD) II and III(Firsov, Robert-Nicoud et al. 1999). The conserved Cysteine residues might be involved in the

formation of disulfide bonds to maintain the tertiary structure of the large extracellular loop. Candidate cysteine residues in the extracellular loop of α-ENaC involved in disulfide bridges have been identified by tandem Cysteine mutations and functional analysis(Garcia-Anoveros, Ma et al. 1995). Other domains are conserved only within particular ENaC/DEG subfamilies. Two domains in the extracellular loop are conserved exclusively within the degenerin subfamily, the cysteine-rich domain CRDI and the extracellular regulatory domain ERD (Lingueglia, Renard et al. 1994; Renard, Lingueglia et al. 1994). ENaC subunits contain conserved proline-rich motifs in the cytoplasmic COOH terminus that are unique to the ENaC subfamily. These motifs have the consensus sequence PPPXYXXL and are involved in protein-protein interactions (Canessa, Schild et al. 1994; Duc, Farman et al. 1994; Garcia-Anoveros, Samad et al. 2001).

1.3 ENaC Primary Structure and Membrane Topology

The ENaC channel consists of three non-identical, but homologous subunits, α-, β-, and γ-ENaC. Each subunit can be defined in terms of four distinct domains: the cytoplasmic N-terminus, the large extracellular loop, the two short hydrophobic segments, and the cytoplasmic C-terminus (Canessa, Merillat et al. 1994; Canessa, Schild et al. 1994; Chassande, Renard et al. 1994; Renard, Lingueglia et al. 1994; Rossier, Canessa et al. 1994).

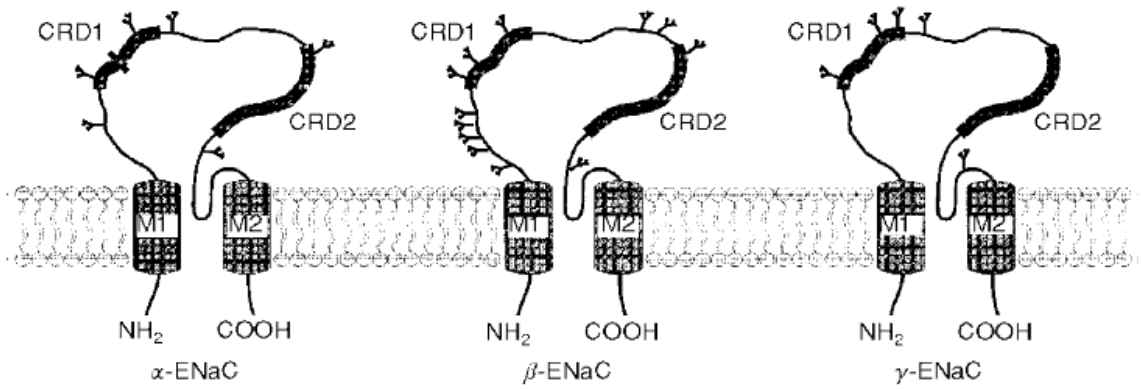

Figure 1.1 ENaC subunits are composed of cytoplasmic N-terminus, the large extracellular loop, the two short hydrophobic segments, and the cytoplasmic C-terminus.

1.3.1 The N-terminal

1.3.1.1 The N-terminal is important in channel gating, assembly, delivery and expression

There is considerable sequence homology in the cytoplasmic N-terminus of α-, β-, and γ-ENaC. The N-terminus has been suggested to participate in many key functions including subunit assembly, gating, and endocytic retrieval and degradation (Adams, Snyder et al. 1997; Grunder, Firsov et al. 1997; Horisberger 1998; Prince and Welsh 1998). However, there are still controversies regarding the role of the N-terminus in

channel assembly, delivery and expression (Lingueglia, Voilley et al. 1993; Rossier, Canessa et al. 1994; Urzua, Sessler et al. 1994; Fyfe and Canessa 1998; Fyfe, Quinn et al. 1998; Thomas, Auerbach et al. 1998). There are several amino acids that are highly conserved among ENaC subunits and species. These include lysine residues, which play roles in ubiquitination, endocytosis, and channel degradation. Deletion of the entire cytoplasmic N-terminus of α-, β-, and γ-ENaC completely eliminated Na^+ current and the co-expression of wild type α-, β-, and γ-ENaC with N-terminal mutants of each rENaC subunit dramatically reduced Na^+ current (Chalfant, Denton et al. 1999; Chalfant, Denton et al. 1999). These observations suggest that the N-terminus is essential for functional channel expression in oocytes and subunit assembly and delivery to the plasma membrane as seen in other multimeric ion channels such as the acetylcholine receptor and voltage gated K^+ channels (Li, Jan et al. 1992; Verrall and Hall 1992; Shen and Pfaffinger 1995; Smith, Mackler et al. 1998) (Beguin, Hasler et al. 1998; Fyfe, Quinn et al. 1998; Snyder, Cheng et al. 1998).

1.3.1.2 N-terminus in channel ubiquitination

The N-terminus is important in channel ubiquitination. The cytoplasmic, N-terminus of α- and β-rENaC play an important role in determining the half life of the channel and thereby the number of channels in the plasma membrane. ENaC has a half life of ≈1 h and is ubiquitinated at key lysine residues in the N-terminus of the α- and β- subunit. Point substitutions of the lysine residues in γ-rENaC decreased channel ubiquitination

and degradation and increased the number of channels in the membrane and Na^+ current(Hendron, Patel et al. 2002).

A sequence residue KGDK (amino acids 47-50) in α-rENaC has been recently demonstrated as an endocytic motif which regulates the number of channels in the plasma membrane. The deletion of this amino acid sequence in α-rENaC has increased Na^+ current ≈4 fold by increasing the number of channels in the plasma membrane without effect on the single channel conductance or on Po. Moreover, the increase in the number of channels in the membrane was due to a decrease in the endocytic retrieval of channels from the plasma membrane.

1.3.2 The extracellular loop

Nearly 70% of the constituent amino acids of each subunit of the Deg/ENaC superfamily lie in the extracellular compartment, which constantly exposed in the extracellular solution. There are a number of potential N-linked glycosylation sites on the extracellular loop domain of each subunit. The function of these glycosylation sites remains uncertain; the results from previous experiments suggested that glycosylation is not essential for efficient channel assembly and translocation to the plasma membrane and for its macroscopic conduction properties, at least in the case of the α-subunit (Canessa, Merillat et al. 1994; Canessa, Schild et al. 1994; Renard, Lingueglia et al. 1994; Snyder, McDonald et al. 1994). Coexpression of unglycosylated α-ENaC with

wild-type β-, and γ-ENaC did not exhibit differences in the magnitude of Na^+ current and ion selectivity. The two cysteine-rich domains (CRD1 and CRD2) spanning nearly 50% of the extracellular loop, and they are highly conserved among all ENaC subunits. Each CRD domain contains 16 cysteines and all these cysteines are conserved among every ENaC subunit. Most of them are conserved among other branch members of ENaC/DEG super family (Take-Uchi, Kawakami et al. 1998; Firsov, Robert-Nicoud et al. 1999). Point mutation analysis revealed that these CRD domains are related to the surface expression of ENaCs mainly by affecting channel delivery without effects on channel assembly and degradation (Green and Wanamaker 1997; Firsov, Robert-Nicoud et al. 1999; Green 1999).

1.3.3 Transmembrane domain

Topological analyses predicted that DEG/ENaC family contains two hydrophobic segments (Canessa, Merillat et al. 1994; Canessa, Schild et al. 1994; Rossier, Canessa et al. 1994). Both hydrophobic segments span the cell membrane (Coppola, Waldmann et al. 1994; Lingueglia, Renard et al. 1994; Voilley, Lingueglia et al. 1994). The first hydrophobic segments (H1M1) lie between the N-terminus and extracellular loop and is composed of the transmembrane domain preceding the short hydrophobic post-membrane-spanning region (H1). The second hydrophobic segments (M2) lie between the extracellular domain and the C-terminus and is composed of a short hydrophobic region (H2) preceding the transmembrane domain (M2) (Canessa, Merillat

et al. 1994). There is also evidence suggesting that the H2 domain is inserted into the membrane. Both H1M1 and H2M2 contain around 50 amino acids. The M1 domain is much less conserved among family members than the M2 region is. Mutational analysis demonstrated that both hydrophobic segments of α-rENaC affect the channel unitary conductance, channel dwell time and amiloride affinity, while a M2 mutation also reversed the cation selectivity (Langloh, Berdiev et al. 2000; Ji, Parker et al. 2001).

1.3.4 Carboxyl terminal tails

The hydrophobic carboxy-terminal lies in the cytoplasm and is important in channel regulation. The Carboxyl-terminus is the least conserved among family members, which may explain why ENaC channels expressed on different tissue or species respond differently to similar agonists or antagonists (Matalon, Benos et al. 1996; Zhu, Yue et al. 1996; McNicholas and Canessa 1997). The carboxyl terminus closely regulates channel gating and internalization. It also is the phosphorylation target of protein kinase. The carboxyl terminus of each subunit conserves a proline-rich (PY) motif in spite of the fact that the C-terminus domain is the least conserved among subunits and subfamilies. The importance of this conserved sequence is underlined by studies of a human genetic hypertensive disease, Liddle's syndrome (Hansson, Nelson-Williams et al. 1995; Hansson, Schild et al. 1995). In this disease, either mutations truncating part of the amino acids or point mutations within the C-terminus result in a gain of channel function such that sodium is re-absorbed with great avidity.

1.4 Distributions and physiology role

The epithelial sodium channels are wildly distributed in different tissues in including: kidney, lung, gastrointestinal tract, and sweat glands. The regulatory responses of ENaC channels to similar agonists or antagonists vary from tissue to tissue and species to species. Same regulator may result in different effects on this channel. cAMP stimulates amiloride-sensitive sodium transport in airway and renal epithelia, but has no effect on ENaC channels in colon. Different proportions of subunits resulted in different channel composition and the presence or absence of intermediate effector or accessory proteins in different proteins. Malfunctions of ENaC results in deleterious consequences for the cell and tissue.

1.4.1 Kidney

In the kidney, ENaC is mostly expressed in the distal nephron where it is under strictly hormone control and constitutes the regulated, rate-limiting entry step in Na^+ reabsorption (Schlatter and Schafer 1987; Farman 1988; Garty and Palmer 1997; Liang, Butterworth et al. 2008). Na^+ reabsorption can be measured in in vitro microperfused tubules in the distal nephron, as a large amiloride-sensitive Na^+ flux in sodium-deprived or aldosterone-treated animals. The first descriptions of the amiloridesensitive Na^+ current at the single-channel level using the patch-clamp technique were obtained from principal cells of micro dissected cortical collecting ducts (CCDs) and from a cell line

derived from the distal nephron of amphibians that responds to aldosterone. Additionally, the most recent studies suggest that this hormone regulated, sodium channel mediated Na^+ reabsorption of kidney nephrons extends at least into the outer medullary collecting duct. The presence of the amiloride-sensitive Na^+ current correlates with the expression of ENaC both at the mRNA and protein levels in the distal convoluted tubule, the connecting tubule, the CCD, and the outer medullary collecting duct. There is a clear axial heterogeneity of ENaC expression along the distal nephron; expression of ENaC is higher in superficial cortical regions of the distal nephron such as the connecting tubule or the CCD than in the deeper medullary regions such as the inner medullary collecting duct. Western blot showed that all three ENaC subunits are expressed in cortex, outer medulla and the inner medulla of the kidney, and the Na^+ current that detected by whole cell patch clamp confirmed ENaC's functionality in this section.

1.4.1.1 ENaCs expression and function on the cortical collecting duct

The cortical collecting duct (CCD) is the most important expression site of the ENaC in kidney. Although the reabsorption of sodium largely takes place in the proximal tubule and Loop of Henle, the distal tubule and connecting duct are where this reabsorption is closely regulated according to the needs of the body. The amilorde sensitive sodium channel plays an important role in both acid-base balance and water balance (Reif, Troutman et al. 1986; Frindt, Sackin et al. 1990).

In vitro microperfusion on cortical collecting duct demonstrated that the Na^+ was transported in this section by an amiloride-sensitive mechanism (Barbry and Lazdunski 1996) (O'Neil and Hayhurst 1985), which was first described in the amphibian skin. like the Na^+ transport mechanism of the amphibian skin (Koefoed-Johnsen and Ussing 1958; Macrobbie and Ussing 1961; Rick, Dorge et al. 1980), the Na^+ move into the epithelial cells through the sodium channels that only localized on the apical (outer) side of the cells, and is driven out by the Na^+-K^+ ATPase that is localized only on the basolateral (inner side) membrane (Koefoed-Johnsen and Ussing 1958; Dorge, Gehring et al. 1974; Rick, Dorge et al. 1981; Huf and Mikulecky 1983; DeLong and Civan 1984; Van Driessche and Zeiske 1985; Reif, Troutman et al. 1986; Frindt, Sackin et al. 1990; Hillyard, Viborg et al. 2007). Patch clamp experiments, which enabled the most direct observation of Na^+ activity on the epithelial cells, confirmed that both cortical collecting tubule and amphibian skin transport Na^+ in this amilorde-sensitive pathway (Palmer and Frindt 1986; Palmer and Frindt 1986; Hunter, Horisberger et al. 1987; Lazrak, Samanta et al. 2000). The abundance of expression and high Na^+ activity along the CCD makes the regulation of the channels in this section very important in the overall Na^+ balance (Kaissling 1985; Hamilton and Eaton 1986; Beck, Dorge et al. 1989; Wald, Scherzer et al. 1989; Zeidel 1993; Kamel, Quaggin et al. 1994; Malnic, Fernandez et al. 1994; Watanabe, Matsushita et al. 1999).

1.4.1.2 ENaCs expression and function on the medullary duct

The outer medullary collecting duct contributes to the renal Na^+ reabsorption as well. The immunoblots experiments showed that all three ENaC subunits are expressed in both inner and outer medullary collecting tubule of the kidney. The channel activities have been recorded by whole cell patch clamp measurements (Vehaskari, Hempe et al. 1998; MacDonald, MacKenzie et al. 2000; Kim, Wang et al. 2005). Whole cell recording on outer medullary collecting duct indicated that the transepithelial Na^+ current of the outer medullary collecting duct cell can reach to as high as that of the CCD cells cell. However the Na^+ current was undetectable on the primary cells obtained from control rats (Frindt, Ergonul et al. 2007)

This amiloride-sensitive Na^+ reabsorption was also found in the inner medullary collecting duct (Sonnenberg, Honrath et al. 1987), but in a much lower exchange rate in compare to that of the CCD or outer medullary collecting duct (Diezi, Michoud et al. 1973).

1.4.1.3 ENaCs expression and function on the proximal tubule

Although almost two-thirds of the Na^+ is reabsorbed in the proximal tubule, the amiloride-sensitive sodium channel is not a major contributor to Na^+ retention in this section. The Na^+ entry into the epithelial cells in proximal tubule is mainly mediated by

active transport and facilitated diffusion. The uptake of Na^+ is coupled with the uptake of organic solutes and ions such as H^+. Furthermore, the sodium exchange in this part of the nephron tubule appears to be spontaneous rather than regulated by the needs of the sodium homeostasis (Edwards, Baer et al. 1973; Stein, Osgood et al. 1973; Chou, Porush et al. 1977; Ullrich, Fromter et al. 1977; Bagrov, Vasil'eva et al. 1979; Molitoris, Geerdes et al. 1991; Greger, Lohrmann et al. 1992; Larsen and Mobjerg 2006; Efendiev, Das-Panja et al. 2007; Girardi, Fukuda et al. 2008; Stevens, Saad et al. 2008; Kastner, Pohl et al. 2009; Segawa, Aranami et al. 2009; Verrey, Singer et al. 2009; Weinstein and Sontag 2009).

However, Na^+ selective, amiloride sensitive channels were detected by patch clamp technique in the luminal membrane of rabbit straight proximal tubule segments (Gogelein and Greger 1986; Duc, Farman et al. 1994). Their regulation mechanism and physiological functions remain to be determined. Functional epithelial sodium channels were detected on the epithelium of the urinary bladder of toad (Crabbe 1961) and rabbit(Lewis and Diamond 1976; Lewis, Eaton et al. 1976). The channels are tightly regulated by hormones (Bentley 1968; Spooner and Edelman 1976; Duc, Farman et al. 1994).

1.4.2 Lung

Epithelial sodium channels in lung, unlike those in other parts of the body such as kidney, colon, and sweat and salivary ducts, do not participate in maintaining body sodium homeostasis and fluids balance. It is the mechanism by which the epithelium reabsorbs and secretes fluids in order to maintain an appropriate level of hydration (Durand 1988; Liu, Johnson et al. 2003). Fluid absorption is largely mediated by Na^+ absorption, and Na^+ enters the alveolar cells mainly through apical Na^+ selective channels. Patch clamp experiment results demonstrated the presence of ENaCs on the apical membranes of fetal distal lung epithelial cells (FDLE) and alveolar type II cells (ATII)(Matalon, Kirk et al. 1992; Yue, Shoemaker et al. 1994) (Hamill, Marty et al. 1981), where they are regulated by various agents and second messengers. However, different types of Na^+ channel are present in the apical membrane of alveolar cells, whose conductance ranges from 4-25pS.

Na^+ transport has been well studied in excised amphibian lung. The simple architecture of the amphibian lung makes it possible to directly study the ion movements in lung. An amiloride sensitive Na^+ current was observed when the excised bullfrog lung was mounted in an Ussing chamber (Gatzy 1976). The excised lung exhibited a transmural electrical potential difference with the pleural surface positive and a high resistance. Later Xenopus lung epithelium experiments revealed that Na^+ transport across the lung epithelium occurs in the same way as in the kidney and other tight epithelia (Fischer,

Van Driessche et al. 1989).

The sodium channel has been also studied in the trachea. The results suggest that it is amiloride sensitive (Patton, Jenkins et al. 1982) and can be regulated by hormones. Additionally, this amiloride-sensitive sodium transport in the upper airway also mediates chloride secretion.

The ability of fluid secretion and absorption of airway epithelium is regulated by the movements of chloride and sodium ions. Thus a balance between these two systems is crucial in maintaining a proper level of hydration of the fluid layer lining the inner surface of the airway epithelium (Bijman and Quinton 1984; Frizzell, Halm et al. 1986; Quinton 1986). According to a previous studies on the trachea epithelium of dog, human and rabbit, the Na^+ transport mediated by epithelial sodium channels are closely coupled with the Cl^- activities (Al-Bazzaz and Al-Awqati 1979; Welsh and Widdicombe 1980; Liu, Luo et al. 2007). However, this active sodium transport regulates the Cl^- secretion but the Cl^- secretion did not appear to have any influence on the Na^+ transport (Al-Bazzaz and Al-Awqati 1979) Thus the appropriate function of epithelial sodium channel is important in maintaining the thickness and viscosity of the fluid layer lining the cells; disruptions of its functions could cause serious consequences as seen in Cystic Fibrosis(Reddy and Quinton 1989; Hartmann and Verkman 1990; Welsh 1990).

1.4.3 Mechanical sensory cells

Epithelial sodium channel subfamily members have long been implicated in the mechano-sensation and transduction processes in mechanical sensory cells. The epithelial sodium channel has been linked to mechanical sensation by genetic studies in Caenorhabditis elegans that revealed the homology between the rENaC channels and the genes involved in neurodegeneration and mechanical-stimulus transduction in C.elegans (Corey and Garcia-Anoveros 1996; Chelur, Ernstrom et al. 2002; Bianchi 2007). This similarity brought the speculation that the members of the same channel family might be involved in mechanosensory functions in vertebrates (Chalfie, Driscoll et al. 1993; Hamill and McBride 1996; Adams, Anderson et al. 1998; Chelur, Ernstrom et al. 2002). C.elegans degenerins are implicated in touch sensation and proprioception. Based on the genetic analysis of C.elegans mutants with impaired touch and stretch sensation, a model of the touch-transducing complex was proposed in which the degenerins MEC-4 and MEC-10 constitute the channel core of the mechanosensory transduction complex (Huang and Chalfie 1994; Gu, Caldwell et al. 1996). MEC-4 and MEC-10 subunits are coexpressed in touch receptor neurons (Tavernarakis and Driscoll 1997; Duggan, Garcia-Anoveros et al. 2000). The similarity between the subfamily members of epithelial sodium channel and the channels involved in mechanotransducer channel on C. elegans brought the speculation that the members of the same channel family might be involved in mechanosensory functions in vertebrates(Chalfie, Driscoll et al. 1993; Hamill and McBride 1996; Adams, Anderson et al. 1998; Price, Lewin et al.

2000; Chelur, Ernstrom et al. 2002)

The Epithelial sodium channel contributes to arterial baroreceptor mechano-transduction (Drummond, Price et al. 1998; Bianchi 2007). The gamma subunit of ENaC localizes to the site of mechano-transduction in baroreceptor nerve terminals innervating the aortic arch and carotid sinus. The b baroreceptor activity and control of blood pressure were abolished by an amiloride analog that inhibits ENaC channels (Drummond, Price et al. 1998). Further study in the topic revealed that ENaC transcripts and proteins are expressed in mechanosensory neurons and at the putative sites of mechano-transduction in baroreceptor sensory-nerve terminals. The potent ENaC channel blockers amiloride and benzamil disrupted mechanical transduction in arterial baroreceptor neurons(Drummond, Welsh et al. 2001), which indicated that the sodium channels had a functional role in transducing mechanical stimuli into neuronal responses. In addition to its mechanosensitivity in sensory neurons, ENaCs are also participated in the vessel responses to pressure. RT-PCR, immunoblotting and immunolocalization experiments confirmed that ENaCs are expressed in isolated blood vessels and vascular smooth muscle cells. The inhibition of ENaC with amiloride and benzamil which blocked myogenic constriction in isolated rat cerebral arteries proved the ENaC's functionality (Drummond, Gebremedhin et al. 2004).

The response of epithelial sodium channels to mechanical forces such as stretch and shear force granted its role in vascular epithelial cells sensing and responding to

changes in blood flow status. This topic will be discussed further in my thesis.

1.5 Regulation of ENaC

1.5.1 Aldosterone Regulations of the epithelial sodium channel

The mineralocorticoid aldosterone is the primary hormone regulating the Na^+ transport across the distal renal and gastrointestinal epithelium and responsible for maintaining electrolyte homeostasis and blood pressure in all terrestrial vertebrates. It is considered the most potent modulator stimulating Na^+ reabsorption (Palmer LG 1986; Kemendy, Kleyman et al. 1992; Eaton, Becchetti et al. 1995; Rozansky 2006). In addition, aldosterone-induced Na^+ influx alters the electrochemical gradient in these epithelial cells which in turn regulates the activities of other ions such as K^+ and H^+ secretion (Paillard 1977; de Wardener 1978; Rozansky 2006). Aldosterone's effects on Na^+ transport have been intensively studied on a variety of native and cultured epithelia from amphibian, avian, and mammalian sources. These studies involved short circuit measurement, current fluctuation analysis, microelectrode impalements, patch clamp recording and many molecular biology experimental techniques. The fundamental features of the aldosterone-induced increase in Na^+ transport elucidated by all the previous studies can be summarized as(Lipton and Edelman 1971; Spooner and Edelman 1975; Geering, Girardet et al. 1982; Geering, Girardet et al. 1982; Palmer, Li et al. 1982; Garty 1986; Rozansky 2006).

a), the aldosterone induced increase in transepithelial Na^+ transport is mainly mediated by apical epithelial sodium channel and partially by basolateral Na^+-K^+-ATPase, which facilitates the extrusion of Na^+ from the cell.

b), aldosterone's effects can be divided into three phases according to different cellular events: 1 The latent phase, during which protein synthesis is initiated without significant change in Na^+ transport; 2 The early phase, starts from 1-3 hours, during which an increase in Na^+ reabsorption but no significant increase in ENaC mRNA are detected; 3 The late phase, initiates from 6-24 hours and can persist up to several days, during which newly synthesized ENaC subunits and Na^+-K^+-ATPase are observed.

c), in all the time domains of the response to aldosterone, Na^+ transport increase is mediated by steroid receptors and is dependent on transcription and translation. This has been proved by the observation that the aldosterone-induced apical Na^+ actions were fully abolished by the inhibitors of transcription and translation.

d), the aldosterone stimulates Na^+ absorption by increasing the expression of epithelial sodium channel on the apical cell membranes and may have additional effects on the channel kinetic characteristics (open probability). Furthermore, aldosterone enhances the expression level and activity of the basolateral Na^+-K^+-ATPase.

Long term aldosterone's action is characterized by the observation that the increase in apical Na^+ transport begins before the significant elevation in ENaC subunit mRNA level are detected. Although ENaC mRNA remains stable during this period, the increase in Na^+ transport was fully blocked by applying inhibitors of translation and transcription,

which indicated that aldosterone stimulates protein synthesis of other ENaC modulator rather than the channel itself (Lipton and Edelman 1971; Rozansky 2006).

1.5.2 Regulation by SGK1

1.5.2.1 SGK

Serum glucocorticoid induced kinase (SGK), is an aldosterone induced protein in the distal nephron and colon epithelial cells. SGK1 was originally identified through its glucocorticoid regulated mRNA in mammary epithelial cells and termed SGK to reflect its regulation at the transcriptional level by serum and glucocorticoids (Alliston, Maiyar et al. 1997; Waldegger, Barth et al. 1997). It was subsequently cloned and its expression level was found to be regulated by a variety of hormonal and non-hormonal stimuli, including serum, follicle stimulating hormone (FSH), osmotic shock and mineralocorticoids (Chen, Bhargava et al. 1999; Shigaev, Asher et al. 2000). It has the characteristic motifs of a serine/threonine kinase and kinase activity has been demonstrated in vitro (Kobayashi, Kitano et al. 1999; Kobayashi and Cohen 1999; Park, Leong et al. 1999; Brunet, Park et al. 2001). SGK1 is found in most vertebrate tissues, although at variable levels, and its expression pattern within tissues shows considerable heterogeneity with respect to both basal and stimulated levels of expression.

In kidney CD, basal levels of SGK1 are low and are strongly induced by

mineralocorticoids, whereas in proximal tubule, expression is undetectable (by in situ hybridization) in the absence or presence of mineralocorticoids or glucocorticoids. Moreover, although SGK was initially discovered as a mRNA induced by glucocorticoids, as in this case in distal nephron and colon epithelial cells, where both mineralocorticoid receptor and glucocorticoid receptor are expressed, and both receptors mediate a similar, if not identical physiological effects in gene transcription, SGK can be induced by mineralocorticoid aldosterone and glucocorticoids such as dexamethasone (Shigaev, Asher et al. 2000; Wagner, Ott et al. 2001; Friedrich, Feng et al. 2003). Experiments in Xenopus cortical collecting duct cell line, rabbit cortical collecting duct cell and rat cortical collecting duct cell demonstrated that aldosterone increased the SGK mRNA level within 0.5 hour and protein abundance rapidly, with maximal induction at approximate 6h. And both SGK mRNA and protein level started to drop within 24h, in some cases, the mRNA and protein level decreased to near baseline by 24h. This time course and dose response of SGK to aldosterone expression is consistent with that of the early increase in Na^+ transport(Loffing, Zecevic et al. 2001; Faletti, Perrotti et al. 2002; Asher, Sinha et al. 2003; Rauh, Dinudom et al. 2006).

1.5.2.2 SGK stimulates the ENaC-mediated apical Na^+ transport

The early phase of the stimulatory effect of aldosterone on sodium reabsorption in renal epithelia is thought to involve the increase of apical sodium channel activity by SGK. Since aldosterone's action clearly requires changes in gene transcription, these

observations suggested the hypothesis that a key mineralocorticoid-regulated gene (or genes) encoded a regulatory protein that increases the plasma membrane localization and/or activity of already synthesized ENaC subunits (Palmer, Li et al. 1982; Naray-Fejes-Toth and Fejes-Toth 2000; Rozansky 2006). Nevertheless, regulation of α-subunit biosynthesis might be important for the late effects of aldosterone. However, in most cases, the ENaC mediated early effect of aldosterone accounts for more than 60% of the total increase in Na^+ current and, importantly, occurs without changes in ENaC gene transcription or mRNA levels (Kemendy, Kleyman et al. 1992; Kleyman, Coupaye-Gerard et al. 1992; Eaton, Becchetti et al. 1995; Ling, Zuckerman et al. 1997). Thus, it has been suggested that mineralocorticoids stimulate electrogenic Na^+ transport in tight epithelia by altering the transcription of the SGK gene rather than stimulating the expression of channel gene. In other words, the earliest mineralocorticoid effect is to increase the activity of the epithelial sodium channel (ENaC), ENaC mRNA and protein levels do not change. Instead, physiologic observations suggest that a mineralocorticoid target gene(s) encodes an ENaC regulator(s) (Chen, Bhargava et al. 1999; Naray-Fejes-Toth, Canessa et al. 1999; Al-Baldawi, Stockand et al. 2000; Naray-Fejes-Toth and Fejes-Toth 2000). To begin to identify and characterize mineralocorticoid-regulated target genes, suppression-subtractive hybridization was utilized to generate a cDNA library from A6 cells, a stable cell line of Xenopus laevis of distal nephron origin. SGK was identified from this screen. Sequence comparison revealed that frog, rat, and human SGK are 92% identical and 96% similar at the amino acid level. SGK mRNA was confirmed by Northern blot to be strongly and rapidly

corticosteroid stimulated in A6 cells (Becchetti, Kemendy et al. 2000). In situ hybridization revealed that SGK was strongly stimulated by aldosterone in rat collecting duct but not proximal tubule cells (Naray-Fejes-Toth, Canessa et al. 1999). Low levels of SGK were present in rat glomeruli, but SGK was unregulated in this structure. Finally, SGK stimulated ENaC activity approximately sevenfold when coexpressed in Xenopus laevis oocytes (Chen, Bhargava et al. 1999; Naray-Fejes-Toth and Fejes-Toth 2000). These data suggest that SGK is an important mediator of aldosterone effects on Na^+ transport in tight epithelia. In addition, in view of the existence of SGK homologues in invertebrates, it is interesting to speculate that SGK is an ancient kinase that was adapted to the control of epithelial Na^+ transport by early vertebrates as they made the transition from a marine to a freshwater environment. To test that whether increased SGK mediates the early response to aldosterone, SGK was co-expressed with ENaC in Xenopus oocytes, and this co-expression increased the Na^+ current 7-fold in comparison to control, and this is mainly a result of an increase in the number of ENaC channels at the cell surface (Chen, Bhargava et al. 1999; Naray-Fejes-Toth and Fejes-Toth 2000). The co-expression of SGK and renal outer medullary potassium channel (ROMK), a principal potassium channel found in collecting duct apical membrane, under the same conditions produced no effect. This further confirmed the view that changes in K^+ transport depend on the alterations in the electrochemical gradient established by Na^+ (Shigaev, Asher et al. 2000; Loffing, Zecevic et al. 2001; Faletti, Perrotti et al. 2002; Rauh, Dinudom et al. 2006).

1.5.2.3 Possible SGK regulation pathway

SGK1 stimulation of ENaC-mediated Na^+ transport has been demonstrated to involve its kinase activity. However, phosphorylation of ENaC subunits by SGK1 has not been demonstrated. Moreover, SGK1 and probably SGK2 and SGK3 increase ENaC localization to the plasma membrane but an effect on channel open probability has not been ruled out. Hence, it seems likely that the target proteins of the SGKs are involved in ENaC translocation to the plasma membrane, its removal, or both. In conclusion, the most efficient way to achieve the rapid and potent effects that SGK1 on ENaC is to alter both insertion and removal of channels. The possible regulation pathways of SGK over ENaC are described as follow;

1.5.2.3.1 SGK stimulate ENaC activity by phosphorylation

The fact that SGK has sequence similarity to serine-threonine kinase and a kinase-dead mutant of SGK did not stimulated ENaC activity suggested that SGK might increase ENaC surface expression by protein phosphorylation (Rauh, Dinudom et al. 2006). However, the target substrate of the SGK phosphorylation is unknown. It is possible that SGK phosphorylates ENaC directly. Consistent with such a direct mechanism, aldosterone has been reported to phosphorylate the C terminus of the β- and-ENaC, while the physiological consequences of this phosphorylation remain undetermined (Chen, Bhargava et al. 1999). However, an indirect mechanism has been proposed based on the

observations which demonstrated that ENaC is not phosphorylated by SGK. In this indirect mechanism, SGK may phosphorylate one or more proteins involved in regulating the expression of ENaC at the cell surface (Alvarez de la Rosa, Zhang et al. 1999; Wang, Barbry et al. 2001; Faletti, Perrotti et al. 2002; Snyder, Olson et al. 2004; Zhou and Snyder 2005).

1.5.2.3.2 SGK increases Na^+ transport by facilitating the ENaC insertion

The means by which SGK increases the ENaC surface expression is also unknown. One possible explanation is that SGK helps channel trafficking to the cell surface. This statement is because SGK is thought to be a member of the PKB/Akt family of serine/threonine kinases, a kinase family involved in the insulin-induced exocytosis of a glucose transporter, and parallels between the glucose transporter (GLUT4) mediated glucose transport and ENaC-mediated Na^+ transport. This similarity perhaps reflects the common themes that underlie the hormonal control of protein trafficking and led to the speculation that SGK might regulate ENaC trafficking through a similar mechanism (Naray-Fejes-Toth and Fejes-Toth 2000; Pearce, Verrey et al. 2000; Kanelis, Rotin et al. 2001; Asher, Sinha et al. 2003; Friedrich, Feng et al. 2003; Dieter, Palmada et al. 2004; Snyder, Olson et al. 2004; Zhou and Snyder 2005; Zhou, Patel et al. 2007).

1.5.2.3.3 SGK increase Na^+ transport by reducing the ENaC removal

Several studies reported the interactions between SGK and Nedd4, ubiquitin-ligase that

reduce ENaC surface expression by initiating channel internalization. Nedd4 is considered a modulator that suppresses ENaC cell surface expression and activity (Chen, Bhargava et al. 1999; Shigaev, Asher et al. 2000; Chigaev, Lu et al. 2001; Kanelis, Rotin et al. 2001; Snyder, Olson et al. 2002; Pearce 2003; Dieter, Palmada et al. 2004; Snyder, Olson et al. 2004; Li, Koshy et al. 2009). This suppression effect is vitally important in mediating Na^+ homeostasis and the malfunction of this interaction can cause serious pathological conditions such as Liddle's syndrome (Schild 1996; Schild, Lu et al. 1996; Tamura, Schild et al. 1996; Staub, Gautschi et al. 1997; Kellenberger, Gautschi et al. 1998; Dahlmann, Pradervand et al. 2003; Pradervand, Vandewalle et al. 2003; Zhou, Patel et al. 2007; Rotin 2008).

1.5.2.3.3.1 PY motif and channel internalization

ENaC was found to be composed of three non-identical but homologous subunits, each containing two transmembrane domains, a large extracellular loop, and short intracellular NH_2 and COOH termini. Other domains involve an NH_2-terminal gating domain, an extracellular cysteine-rich region important for channel expression at the cell surface, and a pre-M2 shown to contain the ion-selectivity filter and the amiloride binding site. All three ENaC subunits have conserved proline-rich regions conforming to the consensus of PY motifs in their COOH-terminal end, namely xPPxY, where P is proline, Y is tyrosine, and x is any amino acid. Such motifs have been shown to act as ligands for WW domains. These motifs have gained considerable interest with the mapping of Liddle's syndrome to mutations in the genes encoding either the β- or the

γ-subunit (Kellenberger, Gautschi et al. 1998). It has been demonstrated that the SGK-induced phosphorylation of Nedd4 inhibits the interaction with ENaC subunits (Chen and Sudol 1995). This syndrome is a rare hereditary disorder in which the kidneys excrete K^+ but retain too much Na^+ and water, leading to high blood pressure (Hansson, Nelson-Williams et al. 1995). It was described as an autosomal dominant syndrome associated with early onset of severe hypertension, variable degrees of low serum K^+ level and elevated blood pH, and suppressed plasma renin activity and aldosterone levels. In all cases of Liddle's syndrome mapped until now, mutations are located at the COOH terminus of either β- or the γ- ENaC and cause either the deletion or the modification of the corresponding PY motif (Canessa, Horisberger et al. 1995; Hansson, Nelson-Williams et al. 1995; Puoti, May et al. 1995; Schild, Canessa et al. 1995; Dinudom, Harvey et al. 1998; Debonneville, Flores et al. 2001). To date, a total of 12 mutations have been identified in the COOH-terminal domains of β- and γ-subunits. Previous studies have shown that the expression of Liddle's channels yield higher channel activity compared with wild-type channels (Dinudom, Harvey et al. 1998; Debonneville, Flores et al. 2001). This increase was attributed to elevated channel numbers at the cell surface, as determined by transient expression in Madin-Darby canine kidney (MDCK) cells. There are also other reports provided evidence that both cell channel number and open probability were increased by expressing extracellularly FLAG-tagged ENaC constructs in X. laevis oocytes. Two mechanisms have been proposed to be perturbed and to be responsible for the observed increase in channel density: endocytosis or ubiquitination. The first proposal involves a defective

tyrosine-based endocytosis signal (YXXθ, where Y is tyrosine, X is any amino acid, and θ is hydrophobic amino acid), which overlaps with the above-mentioned PY motif. It was shown that mutation of the leucine after the PY motif in β-ENaC (PPPNYDSL; the tyrosine motif is underlined) caused increased amiloride-sensitive Na^+ currents (McDonald, Price et al. 1995; Schild, Canessa et al. 1995; Snyder, Price et al. 1995). Moreover, this leucine is also involved in interaction with the WW domain of Nedd4, as recently demonstrated by resolving the structure of a complex between the third Nedd4 WW domain and a β--ENaC PY peptide (McDonald, Price et al. 1995; Schild, Canessa et al. 1995; Dinudom, Harvey et al. 1998; Debonneville, Flores et al. 2001). Hence, mutation of this leucine may also affect the interaction with Nedd4. Further support for the endocytosis model came from experiments in X. laevis oocytes co-overexpressing ENaC with dominant-negative dynamin, a protein known to inhibit clathrin-mediated endocytosis (Puoti, May et al. 1995). This caused an increase in ENaC activity, suggesting that ENaC is internalized via such a mechanism.

1.5.2.3.3.2 The ENaC cell surface expression and activity are regulated by ubiquitination

The notion that ENaC may be regulated by ubiquitination first came from a two-hybrid screen using the COOH terminus of β-ENaC (including the PY motif) as bait. In this screen, Nedd4 was identified as a binding partner for the PY motifs in ENaC (Staub, Dho et al. 1996). Nedd4 was originally identified in a subtractive screen between mouse embryonic and adult brain (Kumar, Tomooka et al. 1992). It is part of a protein family,

which comprises 12–13 human proteins, the Nedd4/Nedd4-like family of ubiquitin-protein ligases (Harvey, Dinudom et al. 1999; Harvey and Kumar 1999; Rotin, Staub et al. 2000; Staub, Abriel et al. 2000). These proteins are composed of one to four WW domains (serving protein-protein interaction), a domain homologous to the E6-AP-COOH terminal domain (HECT), the catalytic portion of these proteins, and most contain an NH2-terminal C2 (Ca^{2+}-dependent lipid binding) domain (Andre and Springael 1994; Chen and Sudol 1995; Hofmann and Bucher 1995; Sudol, Chen et al. 1995). Two closely related Nedd4 isoforms (or paralogues) exist: Nedd4–1 (also named Nedd4, KIAA0093, or RPF1) and Nedd4–2 (also known as KIAA0439, LdI-1, Nedd4La, Nedd18, or Nedd4-L). Nedd4–1 is composed of one C2 domain, a HECT domain, and three to four WW domains (Huibregtse, Scheffner et al. 1995; Scheffner, Nuber et al. 1995). The rat and mouse species contain three WW domains, whereas in humans there are four WW domains (Debonneville, Flores et al. 2001; Kamynina, Debonneville et al. 2001; Kamynina, Debonneville et al. 2001). The difference in the number of WW domains may be due to alternative splicing, as there is evidence for multiple transcripts in human Nedd4–1. Nedd4–2 contains four WW domains and a HECT domain (Debonneville, Flores et al. 2001; Kamynina, Debonneville et al. 2001; Kamynina, Debonneville et al. 2001; Kamynina, Tauxe et al. 2001). Only human and X. laevis Nedd4–2 comprise a C2 domain, whereas such a domain appears to be lacking in mouse Nedd4–2 (Chen and Sudol 1995; Huibregtse, Scheffner et al. 1995; Kamynina, Debonneville et al. 2001). However, there is evidence for alternative splicing of this isoform as well, and there may be isoforms that contain, and others that do not contain,

a C2 domain.

The finding that ENaC interacts with Nedd4 suggested that ubiquitination plays a role in the regulation of ENaC. A model has been proposed in which Nedd4 interacts with the channel via its WW domains to the ENaC PY motifs and ubiquitinates one or several ENaC subunits (Staub, Dho et al. 1996). Such ubiquitination would cause a decrease in ENaC at the cell surface, likely by enhanced endocytosis. This proposed model also accounts for the hyper-active channel seen in Liddle's syndrome, where one of the PY motifs (and hence a binding site for Nedd4) is missing, proper binding (and, consequently, ubiquitination) of Nedd4 would be impaired, leading to increased Na^+ channel density (Staub, Gautschi et al. 1997).

The regulation of ENaC by ubiquitination has been well demostrated by studies of Liddle's syndrome (Schild, Canessa et al. 1995; Snyder, Price et al. 1995). Previouse invistigations have shown that the expression of Liddle's channels yield higher channel activity compared with wild-type channels. This increase was attributed to an elevated channel numbers at the cell surface, as determined by transient expression in Madin-Darby canine kidney (MDCK) cells (Staub, Gautschi et al. 1997). There are also other reports suggesting that both increase in channel number and open probability were increased by expressing extracellularly FLAG-tagged ENaC constructs in X. laevis oocytes (Damke, Baba et al. 1994; Kellenberger, Gautschi et al. 1999; Kellenberger, Hoffmann-Pochon et al. 1999). Two mechanisms have been proposed to be perturbed

and to be responsible for the observed increase in channel density: endocytosis or ubiquitination. The first proposal involves a defective tyrosine-based endocytosis signal (YXXθ, where Y is tyrosine, X is any amino acid, and θ is hydrophobic amino acid), which overlaps with the above-mentioned PY motif. It was shown that mutation of the leucine after the PY motif in β-ENaC (PPPNYDSL; the tyrosine motif is underlined) caused increased amiloride-sensitive Na^+ currents (Awayda, Ismailov et al. 1996; Bubien, Ismailov et al. 1996; Ismailov, Berdiev et al. 1996; Ismailov, Shlyonsky et al. 1999; Ji, Fuller et al. 1999). Moreover, this leucine also interacts with the WW domain of Nedd4, as recently demonstrated by resolving the structure of a complex between the third Nedd4 WW domain and a β--ENaC PY peptide (Kanelis, Rotin et al. 2001; Rotin, Kanelis et al. 2001). Hence, mutation of this leucine may also affect its interaction with Nedd4. Further support for the endocytosis model came from experiments in X. laevis oocytes co-overexpressing ENaC with dominant-negative dynamin, a protein known to inhibit clathrin-mediated endocytosis (Damke, Baba et al. 1994; Kellenberger, Gautschi et al. 1999; Kellenberger, Hoffmann-Pochon et al. 1999). This caused an increase in ENaC activity, suggesting that ENaC is internalized via such a mechanism.

1.5.2.3.3.2 SGK increase the channel activity by deactivating the ubiquitination protein, Nedd4

SGK1 phosphorylates Nedd4 and interferes with Nedd4-ENaC interaction. The observation that both Sgk1 and Nedd4–2 are able to control ENaC density at the cell

surface suggested that the two proteins may act on the same regulatory pathway. The recent identification of the consensus phosphorylation motif for Sgk1 [RXRXX(S/T)] (Kobayashi and Cohen 1999; Kobayashi, Deak et al. 1999; Polo, Sigismund et al. 2002) and the presence of a conserved PY motif (hence a putative binding site for the WW domains of Nedd4–2) on Sgk1 prompted the research for Sgk1 phosphorylation sites on Nedd4–2. Two motifs were subsequently identified (Debonneville, Flores et al. 2001), and a third, present only on certain spliced forms of human Nedd4–2, was described (McDonald, Western et al. 2002; Snyder, Olson et al. 2002). Nedd4-2 but not Nedd4-1 which does not contain such consensus sites in X. laevis oocytes was phosphorylated by Sgk1 and that this phosphorylation is impaired when the Sgk1 PY motif is mutated. In addition,, the mutation of the Nedd4–2 phosphorylation sites impairs the ability of Sgk1 to increase ENaC activity at the cell surface and provided evidence that Sgk1-dependent phosphorylation of Nedd4–2 reduces its ability to interact with ENaC (Debonneville, Flores et al. 2001). Subsequent studies demonstrated that in vitro translated Sgk 1 can interact with Nedd4–2 in a PY motif-dependent manner (Snyder, Cheng et al. 1998). However, there is some controversy over the involvement of the ENaC PY motifs in Sgk1-dependent regulation of ENaC.

Hence, on the basis these data, the model for ENaC activity regulation, involving Nedd4–2, Sgk1, aldosterone, was proposed. ENaC cell surface expression is routinely regulated by the balance between the insertion of more channels from vesicular stocks and their retrieval via Nedd4–2- mediated ubiquitination. Aldosterone, on binding to the mineralocorticoid and glucocorticoid receptors and translocation into the nucleus,

induces the expression of Sgk1 (and a number of other genes). Sgk1 will bind via its PY motif to a WW domain of Nedd4–2 and initiate the phosphorylation. Such phosphorylation weakens the interaction between ENaC and Nedd4–2 and reduces ENaC ubiquitination, resulting in the accumulation of ENaC at the cell surface and increased Na^+ reabsorption (Schild, Lu et al. 1996; Kanelis, Rotin et al. 2001; Lu, Pribanic et al. 2007).

1.5.3.1 AA regulation over ENaCs

Arachidonic acid (AA) is a polyunsaturated fatty acid that is present in the phospholipids of membranes of the body's cells. The chemical structure of arachidonic acid is a carboxylic acid with a 20-carbon chain and four cis double bonds. AA is involved in cellular signaling, mediating inflammation and helping to maintain the mammalian cell membrane correct fluidity at physiological temperatures. Arachidonic acid is found in the sn-2 position of membrane phospholipids, where it can potentially be liberated by the deacylating action of different lipases (Bonventre 1999). Arachidonic acid and its metabolites have been implicated in the regulation of a number of important physiologic processes in the kidney, including water and Na^+ reabsorption and K^+ secretion. Phospholipase A2 (PLA2) is the principal enzyme responsible for arachidonic acid production in most mammalian cells (Balsinde, Balboa et al. 1999; Kudo and Murakami 2002). After its liberation, AA is metabolized into a wide range of products by

three types of oxygenase: cyclooxygenases (COX) (Bonvalet, Pradelles et al. 1987; Smith 1992; Kuwamoto, Inoue et al. 1997; Jakobsson, Thoren et al. 1999), lipoxygenase (LOX)(Werz and Steinhilber 2006; Mahipal, Subhashini et al. 2007) (Samuelsson, Dahlen et al. 1987; Schweiger, Furstenberger et al. 2007) and cytochrome P450(Capdevila, Chacos et al. 1981; Schwartzman, Ferreri et al. 1985; Nelson, Koymans et al. 1996). Both the product of AA and AA itself have well-substantiated bioactivities. Its metabolites perform a diverse range of physiological and pathological roles in the cardiovascular system (Lee, Lu et al. 1999; Node, Huo et al. 1999; Roman 2002) and kidney (Breyer, Jacobson et al. 1996; Breyer 1998; Breyer, Zhang et al. 1998; Breyer and Breyer 2000; Breyer and Breyer 2000; Zhang, Guan et al. 2000). Among the wide range of AA metabolites, the prostaglandin via cyclooxygenases (COX) path way has been well studied for its role in water and Na^+ homeostasis in the kidney; the Hydroxyeicosatetraenoic acids (HETEs) formed through hydroxylase pathway are involved in cell proliferation and migration of vascular smooth muscle cells, suggesting contribution to atherosclerosis and may cause vasoconstriction, increased myogenic mechanism sensitivity in renal and cerebral arteries; the epoxyeicosatrienoic acids (EETs) formed by CYP epoxygenase path way are believed to participate in modulating ion transport, gene expression, producing vasorelaxation factors and anti-inflammatory effects in cardiovascular system and kidney (Harrison and Cai 2003; Pratt, Medhora et al. 2004; Imig 2005; Fleming 2007; Gauthier, Yang et al. 2007; Saltin 2007; Spector and Norris 2007; Kaspera and Totah 2009; Spector 2009).

1.5.3.2 AA metabolites regulate ENaCs

1.5.3.2.1 Prostaglandins regulate ENaC

1.5.3.2.1.1 Prostaglandins

Prostaglandins comprise a diverse family of autocoids derived from cyclooxygenases-mediated metabolism of AA to prostaglandin H_2, generating five primary bioactive prostanoids: PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and thromboxane A_2 (Breyer, Bagdassarian et al. 2001; Helliwell, Adams et al. 2004). Each of these prostanoids interacts with a unique G protein-coupled receptor (GPCR) (Breyer, Davis et al. 1996). The importance of these autacoids to systemic blood pressure and volume control is highlighted by the deleterious side effects of cyclooxygenase inhibitors nonsteroidal anti-inflammatory drugs (NSAIDs), which may induce hypertension, Na^+ retention, and oedema, suggesting an antihypertensive role for endogenous prostaglandins (Radi and Ostroski 2007). Among the prostanoids that have been well described, PGE_2 is the major prostaglandin produced along the collecting duct and potently regulates the Na^+ and water homeostasis in kidney (Machida, Ueda et al. 1988; Teraoka, Takahashi et al. 1989; Taub, Wang et al. 1992; Breyer and Breyer 2000; Hebert, Carmosino et al. 2005). The maintenance of normal renal blood flow and function during physiological stress is especially dependent on endogenous prostaglandin synthesis. Prostaglandins effectively buffer the vasoconstrictor effects in kidney to preserving normal renal blood flow,

glomerular filtration rate and salt excretion. PGE_2 not only dilates the glomerular microcirculation, supplying the renal medulla but also modulates salt and water transport in the distal tubule.

1.5.3.2.1.2 PGs regulation through PG receptors

PGs have multiple effects on epithelial solute and water transport in kidney by interaction with the prostaglandin receptors on the cell surface along the nephron; it may either stimulate or inhibit solute and water transport. Three distinct effects of basolateral PGs on transport have been described(Pitts, Dominguez et al. 1984; Boumendil-Podevin and Podevin 1985; Jabs, Zeidel et al. 1989; Ardaillou, Nivez et al. 1990; Lear, Silva et al. 1990; Hebert, Jacobson et al. 1991; Shimizu, Nakamura et al. 1993; Kokko, Matsumoto et al. 1994; Kaji, Chase et al. 1996; Guan, Zhang et al. 1998; Natochin, Bogolepova et al. 2000; Wegmann and Nusing 2003; Nusing, Treude et al. 2005): stimulation of basal water absorption; inhibition of vasopressin-stimulated water absorption; and inhibition of Na+ absorption. Numerous studies have demonstrated that PGE_2 directly inhibits solute absorption in in vitro microperfused thick ascending limbs, as well as water and solute absorption in the collecting duct. These findings provide a cellular basis for the well-described natriuresis and diuresis after acute renal PGE2 infusions in intact animals. Tubule microperfusion studies also demonstrate a more complex picture, because PGE2 can either increase or decrease water absorption. When added to vasopressin-prestimulated collecting ducts, PGE2 potently inhibits

water absorption, consistent with the aforementioned in vivo diuretic effects of PGE2 infusion. However, observations of PGE_2 increases osmotic water and Na^+ absorption have been reported as well; infusion of PGE_2 in glomerular renal filtrate increases Na^+ transport and PGE_2 augment Na^+ transport in frog skin and in MDCK cells (Boumendil-Podevin and Podevin 1985; Jabs, Zeidel et al. 1989; Lear, Silva et al. 1990; Kaji, Chase et al. 1996). The controversial effects are generally considered the results of the diverse signaling pathways initiated by distinct EP receptors.

In contrast to classic circulating hormones such as insulin and vasopressin, prostanoids act locally on the tissues in which they are synthesized or on the tissues adjacent to those in which they are synthesized. Prostaglandin synthesis occurs in all cells and tissues, the kidney is a particularly rich source where PGE_2 is the major renal metabolite and urinary PGE_2 concentrations are typically higher than that of the circulating PGE_2. Thus the kidney is an important biologic target of PGE_2 due to its autocrine and paracrine action. Nevertheless, PGE2 has multiple effects and under certain circumstances, P opposing functional effects on a given target tissue, such as seen in its vasodilator and vasoconstrictor effects on different vascular beds. The underlying explanation for such controversial PGE_2 effects are accounted for by multiple receptor subtypes for individual prostaglandins; there are at least three types of EP prostaglandin receptors expressed in the kidney. Each receptor has a distinct distribution and appears to modulate distinct renal actions.

However, another possible explanation is that PGE_2 as well as other prostanoids which are membrane permeable, may directly cause cellular event without interaction with their receptors; for instance, PGs may directly bind to ion channels to alter their activities which in turn initiate different cellular responses. In regard of PGs regulating water and Na^+ reabsorption, epithelial sodium channel may be one of their direct targets. The investigation of effects of PGs on ENaC channel could help to understand different signaling pathway of prostanoids action.

1.5.3.2.2 EET may directly regulate ENaC channels

1.5.3.2.2.1 EETs

Epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids are formed by CYP pathways. Early studies indicated that the EETs produce important biological effects, particularly in the vascular and renal systems. EETs are synthesized in the endothelium and are believed to function as an endothelium-dependent hyperpolarizing factor (EDHF) under certain circumstances. The EDHF effects mediated by EETs are considered the result of their abilities to modulate ion channels. EETs are demonstrated to directly regulate ion transport by interactions with ion channels. The involvement of EETs in ion homeostasis was best demonstrated by their ability to release Ca^{2+} from aortic smooth muscle microsomes. EETs were found to transiently increase intracellular Ca^{2+} concentration in this type of cells by stimulating Ca^{2+} influx through L-type Ca^{2+} channels.

11,12-EET particularly increase Ca^{2+} in pig ventricular myocytes. EETs also mediate Ca^{2+} release from intracellular stores in glomerular mesangial cells. Ca^{2+} mobilization from both intracellular store or influx from extracellular fluid has been shown repeatedly to occur concurrently with EET-stimulated biological responses, indicating that an increase in the cytoplasmic Ca^{2+} concentration may be a central component of the cellular response to EETs. The intracellular Ca^{2+} elevation mediated by EETs is believed to be the basis for their action as an endothelial-derived hyperpolarization factor. EETs mediate smooth muscle cell hyperpolarization and vasorelaxation by increasing the open probability of large conductance Ca^{2+} dependent K^+ channel (BK_{Ca}). This EET-induced BK_{Ca} activation is thought to be triggered by an EET-mediated increase in intracellular Ca^{2+}. The further investigations of interactions between EETs and ion channels revealed that EETs may have the potential to mediate the activation of BK_{Ca} by directly interactions with the channel itself. Patch clamp results demonstrated that EETs were able to activate the BK_{Ca} channels in cell-free patch clamps, where the Ca^{2+} concentration is held constant. Therefore, the activation of BK_{Ca} under such circumstance is not dependent on intracellular Ca^{2+} or other messengers and cellular regulators. However, this elevation could also be caused by the interactions between EETs and the channel associated proteins or the cell membrane lipid domains that surrounds the channels.

1.5.3.2.2.2 EETs and renal Na^+ transport

EETs have been shown to inhibit the amiloride-sensitive ion transport in renal epithelial cells and modulate Na^+ transport in kidney by regulating the function of membrane-bound transport proteins (Escalante, Staudinger et al. 1995; Sakairi, Jacobson et al. 1995; Yu, Xu et al. 2000; Wei, Lin et al. 2004; Wei, Sun et al. 2006). 14,15-EET stimulate Na^+/H^+ exchanger activity in glomerular messangial cells by approximately 40%-60% (Staudinger, Escalante et al. 1994; Escalante, Staudinger et al. 1995) whereas 5,6-EET inhibits Na^+ reabsorption in renal CCD cells. EET inhibits the open probability of Na^+ channels in rat ventricular myocardium cells by 70%. However, in neither case is there enough evidence presented to provide a mechanism by which EETs affect Na^+ transport. Nevertheless, the observation that the stimulation effects of EETs on Na^+ transport in rabbit CCD cells pretreated with cyclooxygenase inhibitors were completely abolished has led to the speculation that EETs modulate Na^+ transport by interacting with other factors such as the prostaglandin synthesis pathway (Sakairi, Jacobson et al. 1995). The understanding of the effects of EETs on Na^+ movement and Na^+ channel has not been well established.

The previous studies on EETs regulation over K^+, Cl^- and Ca^{2+} channels lead to the hypothesis that the overall effects of EETs on Na^+ movement in the renal and vascular system are partially contributed by the direct interactions between EETs and ENaCs. In addition, ENaCs are expressed in vascular endothelium, where EETs are synthesized

(Revtyak, Hughes et al. 1988; Carroll, Balazy et al. 1993; Fang, VanRollins et al. 1995; Rosolowsky and Campbell 1996) and exert their EDHF actions (Campbell, Gebremedhin et al. 1996; Quilley, Fulton et al. 1997; Hoebel, Steyrer et al. 1998; Edwards, Thollon et al. 2000; Campbell, Falck et al. 2001; Snyder, Krishna et al. 2002; Archer, Gragasin et al. 2003; Dhanasekaran, Al-Saghir et al. 2006). The cellular targets of the EDHF effects of EETs have been previously summarized as Ca^{2+} channels and K^{+} channels; the activation of these channels lead to hyperpolarization of smooth muscle cells (Baron, Frieden et al. 1997; Hayabuchi, Nakaya et al. 1998; Benoit, Renaudon et al. 2001; Ungvari and Koller 2001; Fleming, Rueben et al. 2007). Nevertheless, the ENaC channel is likely to be another cellular target of EETs in vascular endothelium. Thus, the understanding of the effects of EETs on ENaC channels will contribute to explain EET action in both the renal and vascular system. Furthermore, previous studies indicated that deletion of soluble epoxide hydrolase (sEH), the enzyme that converts EETs to dihydroxyeicosatrienoic acids, decreased blood pressure in male mice; sEH inhibitors decreased blood pressure in hypertensive rats (Yu, Xu et al. 2000; Imig, Zhao et al. 2002; Keseru, Barbosa-Sicard et al. 2008; Hercule, Schunck et al. 2009). These results suggested a role of EETs in the blood pressure control. And it is reasonable to speculate that the overall effects of EETs on blood pressure is contributed by not only their known EDHF function, but also their regulation over Na^{+} movement both in kidney and vascular endothelium.

1.5.4 Vasopressin regulation of ENaC

1.5.4.1 Vasopressin is an important regulator of ENaC

Anti-diuretic hormone (ADH), also known as Vasopressin, is an important regulator of Na^+ reabsorption, which causes rapid Na^+ influx increase in Na^+ reabsorbing epithelia. It acts by stimulating the apical sodium channels and this action is one important constituent of Na^+ homeostasis. Vasopressin and its analogues can induce a 2- to 5-fold increase in Na^+ channel activity within 5-20 minutes. Its effect on Na^+ transport is similar to that of the aldosterone but happens more rapidly, as the most immediate cellular response to vasopressin does not involve transcriptional effects on ENaC channel or channel regulatory proteins. The vasopressin effects can also be divided into different stages based on the different cellular events; the rapid effect and long-term effect. Previous studies indicated that the acute vasopressin-induced Na^+ transport increase mainly results from accelerated Na^+ channel insertion whereas the long-term effects mainly depend on increased ENaC channel synthesis, both of which lead to an increased ENaC surface expression. The rapid vasopressin effect, which occurs within 5-20 minutes, does not involve protein transcription and translation, whereas long treatment of vasopressin has been reported to markedly increase the expression of β- and γ-ENaC and other regulatory protein and mRNA. In addition, there are also reports indicating that vasopressin may enhance ENaC open probability by direct phosphorylation of channel proteins.

1.5.4.2 The acute vasopressin effects is mediated by cAMP

Vasopressin binds to basolateral V2 receptor, which activates adenylate cyclase, resulting in an increased cellular cAMP, and which in turn accelerates ENaC trafficking from intracellular pool to the cell surface, and probably also enhances the channel open probability. V2 receptors are found expressed in both the collecting duct and connecting tubule of rat, mouse and human.

Numerous previous studies have clearly demonstrated that vasopressin effects on Na^+ can be reproduced by membrane permeable cAMP analogues, inhibitors of phosphodiesterase and other activators of adenylate cyclase such as forskolin. All these observations proved that vasopressin effects are mediated by cAMP. Although uncertaintys remain, the mechanism by which cAMP increases ENaC mediated Na^+ current can be summarized as: cAMP increase the ENaC surface expression by facilitating channel trafficking/insertion to cell surface which is responsible for the rapid vasopressin effects. In addition cAMP may also enhance ENaC channel open probability, which is observed in purified channels such as in lipid bilayers.

1.5.4.3 The rapid vasopressin effects, cAMP increases ENaC channel trafficking

The rapid vasopressin induced Na^+ transport increase results from an increased ENaC surface expression contributed by a cAMP mediated increase in channel trafficking/insertion rate.

Functional studies on Xenopus laevis kidney A6 cells demonstrated that the acute activation of Na^+ channels by vasopressin depends on intact microtubules and Golgi

apparatus. This observation suggests that the vasopressin-induced activation of ENaC is caused by enhanced trafficking of channels from an intra-cellular pool to the apical plasma membrane. On a coexpression of α-, β-, and γ-ENaC in Fischer rat thyroid epithelial cell, cAMP-mediated stimulation of ENaC has been investigated by channel modification, protein labeling and electrophysiology measurements; a covalent modification of cysteines was introduced into ENaC, which can almost completely and irreversibly block the ENaCs that are already inserted in the cell surface without effects on the intracellular channels. The rate of the increase in transepithelial current of cAMP-treated cells and control cells were measured by a short circuit current technique. As all pre-existing ENaC channels were blocked by the modification, the increased current was contributed by the newly inserted unmodified ENaC channels at the cell surface. The cAMP treated cells clearly displayed a higher rate of current increase compared to that of the control cells, which indicates a higher rate of channel insertion induced by cAMP. The protein labeling method also confirmed the same conclusion. More direct evidence was provided by a combination of antibody detection of ENaC and short circuit current measurements on MDCK cells expressed with ENaC; the increase in transepithelial current is proportional to the increase in surface density of ENaC induced cAMP. Cell surface biotinylation of endogenous ENaC on mpkCCD cells further confirmed the model in which the acute ENaC stimulation by cAMP results from an accelerated ENaC insertion from intracellular pool. However, despite all in vitro evidence, evidence that vasopressin increase the ENaC surface abundance in vivo is still lacking.

1.5.4.4 Long-term vasopressin effects

The long-term vasopressin effect is distinguished by its transcriptional effects on ENaC and ENaC regulatory proteins. Long-treatment with vasopressin or its analogue markedly increased the expression of β-, and γ-ENaC in a rat cortical colleting duct cell line and in rat kidneys. This effect is thought to account for the increase of Na^+ transport occurring in these cells since the enhanced trafficking of channel molecules to the cell surface does not appear to be related to the long-term vasopressin stimulation; in DDAVP (a synthetic vasopressin analogue) treated connecting tubule and collecting duct cells, immunohistochemistry revealed that the induction of β-, and γ-ENaC led to an intracellular accumulation of the channel subunits without detectable change in the cell surface abundance of ENaC subunits. However, the role of enhanced channel trafficking in long-term vasopressin effects remains uncertain.

1.5.4.5 Overview of the vasopressin effects

The vasopressin effect is similar to that of the aldosterone, but occurs much more rapidly, which indicates that it may compose the most immediate step in the renal regulation over Na^+ reabsorption.

1.5.5 Regulation of ENaC by insulin

The stimulating effect of insulin on ENaC-mediated renal Na^+ reabsorption exhibits a

high similarity to that of the aldosterone. In cultured renal epithelial cells, both aldosterone and insulin have been shown to increase the phosphorylation of the COOH terminal ends of all the ENaC subunits. The aldosterone induced protein SGK is thought to be the integrator of the aldosterone and insulin stimulation effect. In this hypothesized mechanism, aldosterone regulates the SGK abundance; insulin regulates the SGK activity.

As described in the section on aldosterone's action, one of the transcriptional effects of aldosterone is the increase of SGK expression. Subsequently, SGK decreases the removal of ENaC channel from cell surface by phosphorylating the Nedd4, which acts as a channel ubiquitin ligase. Importantly, SGK must be phosphorylated at two residues to have its kinase activity. This phosphorylation occurs through the PI3K pathway; inhibitors of the PI3K pathway prevented SGK from phosphrylating a peptide substrate thus abolishing the aldosterone-induced increase in Na current in A6 cells. In distal nephron epithelial cells, PI3K pathway can be activated by insulin receptors. Thus it has been proposed that SGK might integrate signals from aldosterone and insulin to regulate epithelial Na^+ absorption.

1.5.6 Regulation of ENaC by proteases

1.5.6.1 Proteases are involved in the activation of ENaC

Proteases are involved in the processing and activation of ENaC. Current evidence indicates that proteases contribute to ENaC regulation by cleaving specific sites in the channel subunits both along their biosynthetic pathway and at the cell surface. The regulation of ENaC by proteolytic processing is an important and complex mechanism.

1.5.6.2 Extracellular channel activating-proteases

Membrane-anchored channel-activating proteases (CAPs) with extracellular proteolytic activity were first identified using the Xenopus laevis oocytes expression system and were shown to activate ENaC when coexpressed with the channel. Electrophysiological studies in the Xenopus laevis oocytes expression system clearly demonstrated a large and rapid stimulatory effect of extracellularly applied trypsin and chymotrypsin on ENaC activity. Various membrane-bound or secreted proteases such as CAPs are likely to exist in ENaC-expressing epithelia and are thought to act on the extracellular domain of the channel after its surface insertion or act on the channel regulatory proteins.

There are many candidates of endogenous ENaC-activating protease in ENaC regulation in mammals. One attractive candidate, prostasin, the mammalian homologue of Xenopus CAP1, is expressed in the kidney and cultured collecting duct cells and its expression appears to be regulated by aldosterone. However, the prostasin is practically inactive due to the urine pH and may act indirectly by altering the activity of

downstream proteases. Another candidate is tissue kallikrein which recently has been reported to be involved in ENaC processing in the kidney. Tissue kallikrein is synthesized in large amounts in the connecting tubule and released into the urine, and act on ENAC either directly on the extracellular channel domain or indirectly by activating other proteases. Also, tissue kallikrein synthesis and release in connecting tubule is subject to aldosterone regulation. However, tissue kallikrein failed to activate ENaC heterologously expressed in Xenopus laevis oocytes and renal ENaC cleavage is preserved in a kallikrein-deficient mice. Extracellularly applied trypsin demonstrated a markedly stimulating effect on ENaC probability as well. In addition, trypsin also has been reported to indirectly stimulate ENaC channel by activating G-proteins. These findings all suggest that ENaC regulation by proteases is complex.

1.5.6.3 Intracellular channel-activating proteases

Candidates for intracellular ENaC activating proteases include furin and other Golgi-associated convertases, the proteolytic cleavage by which is thought to be important in the maturation of ENaC in the biosynthetic pathway. Western bolt analysis of a range of ENaC expressing cells and tissues has revealed the presence of distinct ENaC cleavage products in particular of the α- and γ- subunit. However, the presence of uncleaved ENaC subunits in the plasma membrane suggests that this cleavage is not a prerequisite for channel synthesis and trafficking.

1.5.6.4 Overview of ENaC regulation by proteases

Although the precise molecular mechanism of proteolytic channel activation remains unclear, the overall result of proteases action can by summarized as, proteolytic cleavage may cause a conformational change of the channel favoring its open-state.

1.6 ENaCs is suggested to be mechano-sensitive

1.6.1 Machano-sensitivity of ENaC implied by the homology between DEG subunits and ENaC subunits

Specific members of the degenerin/epithelial Na^+ channel (DEG/ENaC) family of ion channels are activated by mechanical stimuli. DEG subunits MEC-4 and MEC-10 form the core of MEC channel complex, which is believed to be the machano-transducer ion channel in C. elegans touch receptor neurons (Barr and Sternberg 1999; Barr, DeModena et al. 2001; Yoder, Hou et al. 2002; Nauli, Alenghat et al. 2003). Genetic and molecular data support a model of the mechano transducer in C. elegans in which DEG subunits MEC-4 and MEC-10 compose the core of the channel complex. The most direct evidence that the MEC channel is gated by mechanical forces has come from studies employing intracellular Ca^{2+} concentration measurements (Suzuki, Kerr et al. 2003). This set of experiments clearly demonstrated that the MEC channel is specifically required for responses to gentle touch and not for general function of touch neurons. A transient

rise in intracellular Ca^{2+} concentration is observed in touch neurons of C. elegans that are mechanically stimulated, whereas this Ca^{2+} rise is absent in Mec-4 and Mec-2 mutants, suggesting that the MEC channel is indeed needed for cellular responses to gentle touch. Although DEG and ENaC channels participate in strikingly diverse biological processes and are activated in response to markedly distinct stimuli the overall secondary structure of channel subunits encoded by this gene superfamily is highly conserved. DEG and ENaC subunits range from about 500 to 1,000 amino acids in length and share several distinguishing blocks of sequence similarity (Canessa, Schild et al. 1994; Adams, Snyder et al. 1997; Firsov, Gautschi et al. 1998; Eskandari, Snyder et al. 1999). Their subunit topology is invariable: all members of the DEG and ENaC subfamily have two membrane-spanning domains with Cysteine-rich domains (CRDs, the most conserved is designated CRD3) situated between the transmembrane segments. N- and C-termini project into the intracellular cytoplasm, whereas most of the protein, including the CRDs, is extracellular. Based on the homology of the members of the DEG/ENaC family, it has been suggested that ENaC is also a mechano-sensitive channel.

1.6.2 Machano-sensitivity of ENaC implied by reconstitution in bilayers and expression system

1.6.2.1 Reconstituted ENaC in bilayers

Initial reports regarding the role of ENaC in mechanosensation is from reconstitution of

the ENaCs in planar lipid bilayers. ENaC subunits (α-, β-, and γ-ENaCs) have been cloned from bovine renal epithelial channel and reconstituted to form the core conductive unit of Na^+-selective, amiloride-sensitive channels in planar lipid bilayers. The re-built channel displayed voltage-independence and amiloride-sensitivity consistent with the biophysical characterizations of ENaCs and exhibited mechano-sensitivity in which the single-channel open probability was maximally activated with a hydrostatic pressure gradient (Awayda, Ismailov et al. 1995; Ismailov, Berdiev et al. 1997; Jovov, Tousson et al. 1999; Berdiev and Benos 2006). With the use of a rabbit nuclease-treated in vitro translation system, mRNA coding for α-ENaC was translated and the polypeptide products were reconstituted into liposomes. On incorporation into planar lipid bilayers, in vitro-translated α-ENaC protein 1) displayed voltage-independent Na^+ channel activity with a single-channel conductance of 40 pS, 2) exhibited mechano-sensitivity in that the single-channel open probability was maximally activated with a hydrostatic pressure gradient of 0.26 mmHg across the bilayers, 3) was blocked by low concentrations of amiloride. This observation of stretch activation of alpha-ENaC was confirmed in dual electrode recordings of heterologously expressed α-ENaC whole cell currents in Xenopus oocytes swelled by the injection of 15nl of a 100mM KCl solution. Another set of experiments of reconstituted ENaC channel in lipid bilayers also displayed mechano-sensitivity. Hydrostatic pressure gradient across bilayers increased channel open probability by five-fold without effects on channel unitary conductance (Ismailov, Berdiev et al. 1997).

1.6.2.2 ENaC in cell expression system

ENaC subunit (α-rENaC) has been cloned and transfected into LM cells, a null cell for stretch-activated channels. The ENaCs expressed in LM cells exhibited stretch-activation (Kizer, Guo et al. 1997). A cDNA clone was obtained of the entire coding region of rat α-rENaC (α-rENaC). The α-rENaC cDNA was cloned into an expression plasmid and transfected into LM (TK2) cells, a null cell for SA-CAT activity. The channel is only activated when negative pressure is applied during cell-attached patches, cell swelling, or patch excision. The open probability (Po) of the reconstituted channels was increased in a graded manner with increased negative pressure on the pipette. Po was increased by a maximum of 0.39 at 280 mmHg. After observing stretch-activated ion channels in a cell attached patch, the patch was excised to the inside-out configuration and subjected to asymmetrical solutions so current–voltage (I–V) plots could be constructed to determine the ion selectivity of the channels. Expressed α-rENaC channels had a conductance of approximate 24.2pS when activated in symmetrical NaCl Ringer's solutions. These results provided evidence that ENaC is mechano-sensitive and implied the potential role of ENaC channels in mechano-signal transduction (Kizer, Guo et al. 1997).

ENaC has also clearly demonstrated its mechano-activation in Xenopus Oocytes (Awayda, Ismailov et al. 1995). Xenopus laevis oocytes injected with α-, β-, and γ-mENaCs cRNA were examined by using a two-electrode voltage clamp and was

measured by whole-cell as well as single-channel recordings. The application of physiologically relevant ranges of laminar shear stress produced a significant increase in channel open probability without effects on channel conductance and channel density on the membrane surface. Flow activated whole cell Na^+ currents in oocytes expressing α-, β-, and γ--mENaC. In the absence of perfusion, the whole cell Na^+ current was 10.0μA. Initiating bath flow from 0 (no flow) to the range of 4–6 ml/min led to a increase in amiloride-sensitive Na^+ current. Amiloride-sensitive Na^+ currents were not observed in water-injected oocytes either in the absence or presence of flow. However, it was difficult to establish a dose-response relationship (flow rate vs. flow-induced current) with the oocytes expression system due to the fact that the maximal increase in Na^+ current in response to flow was quite variable. Flow-mediated increases in Na^+ current were observed within 1 min after initiation of oocytes perfusion and reached a plateau within 3–5 min. Flow activation of the sodium current was reversible, as current fell when the flow was stopped. Flow activation of Na^+ current was not associated with a significant change in the resting membrane potential of the oocyte(Satlin, Sheng et al. 2001). Cells are potentially exposed to a variety of mechanical forces including indentations, circumferential stretch, high frequency vibrations, osmotic pressure gradients, hydrostatic pressure, and fluid shear stress.

1.6.3 Mechano-sensitivity of ENaC implied by Native ENaC in distal nephron

Similar to vascular endothelial cells, epithelial cells in the distal nephron are also

exposed to a variety of mechanical forces including indentations, circumferential stretch, osmotic pressure gradients, hydrostatic pressure and fluid shear stress (Hamill and Martinac 2001). In the kidney, frictional forces of the ultra-filtrate flowing through tubules regulate the vectorial transport of glucose (Garvin 1990), chloride (Garvin 1990) magnesium (Wong, Berry et al. 1995), K^+ (Giebisch 1998) (Kunau, Webb et al. 1974; Woda, Bragin et al. 2001), Na^+ (Grantham, Kurg et al. 1970; Stoner, Burg et al. 1974; Engbretson and Stoner 1987; Satlin, Sheng et al. 2001), the organization of the cytoskeleton, the synthesis of matrix proteases, and the activity of specific transcription factors(Essig and Friedlander 2003).

ENaCs expressed in distal nephron segments are subjected to physiological variations in tubular flow rate. Increasing the rate of perfusion of isolated rabbit CCDs within a physiologically relevant range increased net Na reabsorption via an amiloride-sensitive pathway, suggesting that ENaCs within the CCD are activated in response to increases in tubular flow rates (Engbretson and Stoner 1987; Satlin, Sheng et al. 2001; Woda, Leite et al. 2002; Woda, Miyawaki et al. 2003).

The Na^+ transport in rabbit cortical collecting duct were examined to verify the effects of flow on Na^+ channels flow (Satlin, Sheng et al. 2001; Morimoto, Liu et al. 2006). Single cortical collecting duct was perfused by increasing flow rates and the rates of net Na^+ absorption were measured. The rate of net Na^+ absorption increased significantly as flow rate was increased. 0.1mM amiloride added to the luminal perfusate significantly

inhibited net Na^+ absorption compared with transport rates measured at comparable flow rates in the absence of the inhibitor. In addition, net Na^+ absorption in the CCD reflects the sum of opposing processes of Na^+ absorption and secretion. To determine whether a flow-induced reduction in bath-to-lumen Na^+ backflow into the lumen could account for the flow stimulation of net Na^+ absorption, net Na^+ transport was measured in a group of CCDs perfused with a slightly hypotonic 0-Na^+ perfusate containing amiloride and bathed in Burg's solution. These selected conditions were used to inhibit lumen-to-bath and maximize bath-to-lumen transepithelial Na^+ transport. However, an increase in flow rate under this condition led to no significant change (Morimoto, Liu et al. 2006).

1.7 The potential role of endothelial ENaCs

1.7.1 Endothelial cells, shear force and mechano-sensitive channels

Endothelial cells are constantly exposed to a variety of mechanical forces such as pressure created by the hydrostatic forces of blood within the blood vessel, which is applied perpendicularly against the endothelial cells; circumferential stretch or tension, created as a result of defined intercellular connections between the endothelial cells that exert longitudinal forces on the cell during vasomotion; and shear stress, the

dragging frictional force produced by blood flow, which is applied tangential to the apical membrane of endothelial cells. Shear stress has been demonstrated to play important roles in physiological processes such as cell growth, differentiation, remodeling, metabolism, morphology, and gene expression. The blood pressure has also been implicated to cause both endothelium-derived vasoconstriction and vasodilatation. Numerous studies suggest that endothelial cells can detect these mechanical forces produced by blood flow and consequently modulate the tone of vascular smooth muscle cells, which in turn controls blood pressure and blood flow by adjusting the caliber of arteries and arterioles.

Endothelial mechano-sensitive ion channels represent one of the earliest responses in mechanical signal transduction. Mechano-sensitive ion channels function as transducers that respond to mechanical stimuli by changing the permeability of the membrane to specific ions. Mechano-sensitive ion channels in endothelial cells represent the most immediate cellular response to fluid flow and these responses happen in seconds and minutes (Olesen, Clapham et al. 1988; Barakat, Leaver et al. 1999; Nakao, Ono et al. 1999; Marchenko and Sage 2000). In other words, the virtually immediate endothelial response to shear stress is the activation of flow-sensitive ion channels. Therefore, mechano-sensitive ion channels in endothelial cells are hypothesized to play a role in shear stress sensing and transduction. Several types of ion channels have been suggested to mediate the initial shear force signaling events, including stretch-activated cation channels, P2X4, TRPM7, TRPV, TRPC, K^+ channels, Ca^{2+}

activated Cl^- channels and volume-regulated anion channels (VRACs) (Olesen, Clapham et al. 1988; Olesen, Davies et al. 1988; Rossitti, Frangos et al. 1995; Nilius, Viana et al. 1997; Traub and Berk 1998; Nakao, Ono et al. 1999; Marchenko and Sage 2000; Nilius and Droogmans 2001). The activations of shear force sensitive ion channels eventually lead to alterations of membrane potential with or without $[Ca^{2+}]_i$ elevation(Folgering, Sharif-Naeini et al. 2008).

1.7.2 ENaC is a potential candidate of mechano-sensitive ion channels

ENaC are expressed in ECs.

ENaC mRNA and proteins have been recently identified in vascular endothelial cells including the human vascular endothelial cell line (ECV) (Golestaneh, Klein et al. 2001), human umbilical vein endothelial cells (Kusche-Vihrog, Sobczak et al. 2008) and bovine aortic endothelial cell line (BAEC). PCR analysis followed by sequence alignment demonstrated that both the mineralocorticoid receptor (MCR) and the epithelial sodium channel (ENaC) genes are expressed in the human vascular endothelial cell line (ECV). The growth and multiplication of the ECV in culture were influenced by both aldosterone and the MCR-specific antagonist ZK 91587. The PCR analysis of the total RNA from exponential HBMEC cultures was used to screen for the possible expression of ENaC at the level of mRNA. ENaC also has been described in the human bone marrow endothelial cells (HBMEC) (Chen, Valamanesh et al. 2004). The PCR analysis of the total

RNA from exponential HBMEC cultures revealed that ENaC mRNA and DNA are expressed in this type of cells. In addition, ENaC has been recently identified in human umbilical vein endothelial cells (Kusche-Vihrog, Sobczak et al. 2008). However, their function and regulation in vascular endothelial cells are poorly understood.

Recent reports suggest that ENaC are required for myogenic vasoconstriction.

Myogenic vasoconstriction is an intrinsic property of most resistance vessels characterized by a decrease in luminal diameter in response to an increase in intraluminal pressure. The response is important in establishing basal vascular tone and maintaining blood flow autoregulation. And ENaCs are required for myogenic vasoconstriction in mouse interlobar arteries (Jernigan and Drummond 2005), thus ENaC proteins may be components of mechano-sensitive complexes in the vascular system including both endothelial cells and smooth muscle cells. Selective DEG/ENaC inhibition by low doses of amiloride and benzamil abolishes pressure-induced vessel constriction and increases cytosolic Ca^{2+} and Na^{+} without diminishing agonist-induced responses in isolated mouse interlobar arteries. Under control conditions, mouse interlobar arteries constricted in response to an increase in intraluminal pressure in a pressure-dependent manner, whereas both low dose amiloride and benzamil inhibited the development of myogenic tone by preventing active vaso-constriction. Resistance vessels crossing many vascular beds respond to increases in blood pressure by vaso-constriction, a mechanism important for maintaining blood flow autoregulation.

This response is initiated by conversion of a mechanical stimulus into a cellular event such as membrane potential depolarization and is independent of neural, metabolic, and hormonal vasoactive factors. This observation that ENaC are required for myogenic vasoconstriction in mouse interlobar arteries has further permited the speculation that ENaC may function as mechano-sensitive ion channels in the vascular system, which initiates pressure induced contraction by depolarizing the cell membrane potential, which in turn activates voltage-gated Ca^{2+} channels.

1.8 Other important cellular pahways

1.8.1 Na^+ /Ca^{2+} exchanger (NCX)

The NCX family belongs to the super family of membrane proteins, which exchanges three Na^+ ions for one Ca^{2+} ion or four Na^+ ions for one Ca^{2+} ion depending on intracellular Na^+ concentrations ($[Na^+]_i$) and intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (DiPolo, 1979; Hilgemann et al., 1992). In resting excitable cells, when $[Ca^{2+}]_i$ rise and the cells require the return of $[Ca^{2+}]_i$ to resting levels (Salvaterra et al., 1989), this exchange transport mechanism couples the uphill extrusion of Ca^{2+} to the influx of Na^+ ions into the cells down their electrochemical gradient. This mode of operation, defined as forward mode (Wang et al., 2000), which keeps the 10^4-fold difference in Ca^{2+} concentrations across the cell membrane. In contrast, in other physiological circumstances, when $[Na^+]_i$ rise or membrane depolarization occurs, thus reducing the

trans-membrane Na^+ electrochemical gradient, the NCX mediates the extrusion of $[Na^+]_i$ and the influx of Ca^{2+} ions. This mode of operation is defined as reverse mode (Wang et al., 2000). Furthermore, submicromolar concentrations (0.1–0.3 μM) of intracellular Ca^{2+} could activate the antiporter (Robertson et al., 2000b). This regulatory function of low micromolar Ca^{2+} is more evident when the NCX is working in the reverse mode.

1.8.2 Ca^{2+}-activated K^+ channel

K^+ channels can be activated by several factors. One such class of K^+ channels is Ca^{2+}-activated K^+ channels, whose gating is mediated by the rise of intracellular concentration of free Ca^{2+} ions. Three broad categories of Ca^{2+}-activated K^+ channels have been identified and classified according to their conductance and are called large-conductance, 100–300 pS (BK_{Ca}) (Marty 1981), intermediate-conductance, 25–100 pS (IK_{Ca}) (Ishii, Silvia et al. 1997; Logsdon, Kang et al. 1997), and small-conductance, 2–25 pS (SK_{Ca}) Ca^{2+}-activated K^+ channels (Blatz and Magleby 1986). Among them, BK_{Ca} channels were first studied in smooth muscle cells where they are the key players in setting the contractile tone.

BK_{Ca} channels from vascular smooth muscle are multimeric complexes, which consist of 2 distinct subunits: pore-forming subunit α and regulatory subunit β, which are arranged in a 1:1 stoichiometry (Knaus, Folander et al. 1994; Knaus, McManus et al. 1994; Toro, Wallner et al. 1998). Each channel exists as a tetramer, composed of 4

α-subunits either alone or in association with β-subunit pairs, which could change physiological properties of the channel (Garcia-Calvo, Knaus et al. 1994; Shen, Lagrutta et al. 1994; McManus, Helms et al. 1995); for example, α –subunit splice variants and association with β1 subunit give rise to diverse electrophysiological phenotypes of the channel.

BK channel activity is highly regulated by its molecular composition. The α-subunit consists of 7 trans-membrane spanning domains (S0–S6) at the N-terminus, the P-loop (or the pore-forming loop) between the S5 and S6 domains, the four hydrophobic segments (S7–S10) at the large intracellular carboxyl (C) terminus. Acidic residues in the S2 domain (Seoh, Sigg et al. 1996) and S3 domain (Papazian, Shao et al. 1995) along with basic residues (Arg) at every third position in the S4 domain (Papazian, Timpe et al. 1991) confer voltage sensitivity to the channel (act as voltage sensor). Ca^{2+} regulatory domain(s) are complex and not simple linear structures. Although the hypothesis has been formed from the results of gene mutation, for example, when the regulator of conductance for the K^+ domain (RCK) (Jiang, Lee et al. 2002; Xia, Zeng et al. 2002) mutated together with mutation of the Ca^{2+} bowl, which located just before S10, practically abolish BK_{Ca} physiological regulation by Ca^{2+}; recent evidence suggests that neither the Ca^{2+} bowl nor the RCK domain is necessary for Ca^{2+} activation of BK_{Ca} channels (Piskorowski and Aldrich 2002). At this point, I can only state that BK_{Ca} channels probably have several Ca^{2+}-binding sites with complex 3D structure that regulate channel activity. Nonetheless, they are activated both by elevated cytosolic

Ca^{2+} levels and by membrane depolarization. Quite surprisingly, these channels can open even in the absence of Ca^{2+} or voltage activation (voltage less than -80mV) (Horrigan and Aldrich 2002) and it is thought that the Ca^{2+} and membrane potential dependence of the channels are independent of each other, both of which can enhance the channels' open probability (Pallotta 1985).

1.8.3 Heme oxygenase and oxygen metabolic pathway

1.8.3.1 Heme oxygenase

Heme 0xygenase (HO) activity serves an essential metabolic function as the rate-limiting step in heme degradation (Tenhunen, Marver et al. 1969; Tenhunen, Marver et al. 1969; Tenhunen, Marver et al. 1970; Tenhunen, Ross et al. 1970). The diverse physiological roles of HO have been invistigated in basic research fields including molecular cell biology, genetics and pharmacology and also in clinical sciences such as pulmonology (Lee, Alam et al. 1996; Mumby, Upton et al. 2004; Hoetzel, Leitz et al. 2006; Hoetzel, Dolinay et al. 2008), cardiology(Vulapalli, Chen et al. 2002; Liu, Pachori et al. 2006), exercise physiology (Sato, Balla et al. 2001; Thompson, Basu-Modak et al. 2005), and transplant immunology (Sato, Balla et al. 2001; Akamatsu, Haga et al. 2004). There are three isoforms of HO: HO-1, HO-2 and HO3, among which the HO-2 is constantly produced and HO-1 is highly inducible as a stress response protein and critical mediator of cellular homeostasis (Applegate, Luscher et al. 1991; Vulapalli, Chen et al. 2002).

HO-1 and HO-2 catalyze heme degradation by cleaving heme-b at the α-methene bridge carbon, to generate carbon monoxide (CO), biliverdin, and ferrous iron (Tenhunen, Marver et al. 1969; Tenhunen, Marver et al. 1969). The reaction requires molecular oxygen as well as reducing equivalents from NADPH cytochrome p450 reductase. The expression of HO-1 occurs at low levels in most tissues under physiologic conditions except in the spleen, the site of erythrocyte hemoglobin turnover. HO-2 is constitutively expressed under basal conditions in most tissues.

1.8.3.2 The physiology role of HO-1

HO-1 has been suggested to play a very important role in homeostasis and tissue protection. The central role of HO-1 in vascular and tissue homeostasis is exemplified by HO-1 deficiency in both human (Kawashima, Oda et al. 2002) and experiment animals. Analysis of HO-1 gene deleted mice indicated that HO-1 is an important molecule in systemic responses to stress and iron homeostasis (Poss and Tonegawa 1997; Poss and Tonegawa 1997; Zakhary, Poss et al. 1997). The protective role of HO-1 in injury and disease is suggested by the phenotypic studies of HO-1 deletion mice since endothelial cells derived from HO-1 deficient mice are more susceptible to cytotoxicity induced by pro-oxidant stimuli, such as heme or hydrogen peroxide, and produce more intracellular reactive oxygen species (ROS) when challenged with such stimuli(Poss and Tonegawa 1997). Furthermore, the HO-1 deficiency mice also displayed increased mortality during pulmonary(Fujita, Toda et al. 2001) and renal ischemia/reperfusion (I/R) injury,

suggesting the protection role of HO-1 against oxidative stress (Zhang, Bedard et al. 2002; Shen, Ke et al. 2003; Di Filippo, Marfella et al. 2005; Tsuchihashi, Zhai et al. 2005; Feitoza, Goncalves et al. 2007).

1.8.3.3 CO

CO, the byproduct of heme degradation, has been demonstrated to have many important physiological roles including anti-apoptosis, anti-inflammation and anti-proliferation. When applied at low concentrations, CO can influence a number of signaling pathways in cultured cells, including those regulated by soluble guanylate cyclase and Mitogen-activated protein kinases (MAPK). At a cellular level, CO can stimulate guanylate cyclase to increase the production of cGMP, which has been demonstrated in vascular smooth muscle cells (Morita, Perrella et al. 1995). CO treatment can modulate the activation of MAPK pathways that are critical for cellular signal transduction in response to stress and inflammation. The MAPK signaling pathway has been implicated in the anti-inflammatory, anti-apoptosis and anti-proliferation.

Accumulating experimental evidences suggest that CO confers tissue protection in preclinical animal models of injury and disease. These models include acute lung injury, vascular injury, pulmonary hypertension, liver injury, organ I/R injury, organ transplantation, and numerous others. In specialized applications, CO inhibited the

tissue injury during mechanical ventilation in mice, by reducing inflammation and preventing alveolo-capillary barrier dysfunction (Dolinay, Szilasi et al. 2004; Hoetzel, Dolinay et al. 2008; Hoetzel, Schmidt et al. 2009). Low concentration CO can reverse established pulmonary hypertension in rats, induced by chronic hypoxia or monocrotaline administration. All these previous results suggest CO as potential therapies for human disease.

1.9 Hypothesis and aims

1.9.1 Hypotheses

We hypothesized that AA and its metabolites, namely EETs and PGs, in addition to their regulation of Na^+ reabsorption through cellular receptors, may direct affect channel activity.

We also aimed to identify the possible oxygen regulation pathway. In this study, we propose that HO mediated heme degeneration reaction, in which the HO-1 acts as oxygen sensor may serve as one of the oxygen regulation mechanism.

We hypothesized that ENaCs are mechano-sensitive and endothelial-expressed ENaCs may serve as mechano-transducing ion channels.

1.9.2 Specific aims

To verify the proposed hypotheses, the flowing specific objects were investigated

1.9.2.1 Determine the direct effects of AA on ENaC activity

The results of this investigation were demonstrated and discussed in chapter 3 with more detailed discussions in chapter 7.

1.9.2.2 Determine the direct effects of prostaglandins on ENaC activity

The results of this investigation were demonstrated and discussed in chapter 3 with more detailed discussions in chapter 7.

1.9.2.3 Determine the direct effects of EETs on ENaC activity

The results of this investigation were demonstrated and discussed in chapter 3 with more detailed discussions in chapter 7.

1.9.2.4 Verify the possible oxygen sensitive regulation pathway of ENaCs that underlines the impact of hypoxia on renal Na+ transport

The results of this investigation were demonstrated and discussed in chapter 4 and chapter 6 with more detailed discussions in chapter 7.

1.9.2.5 Determine whether ENaCs are able to sense machano-stimulation

The results of this investigation were demonstrated and discussed in chapter 6 with more detailed discussions in chapter 7.

1.9.2.6 Determine the ENaC expression in vascular ECs

The results of this investigation were demonstrated and discussed in chapter 5 and chapter 6 with more detailed discussions in chapter 7.

1.9.2.7 Determine whether ENaCs expressed in vascular ECs are functional

The results of this investigation were demonstrated and discussed in chapter 5 and chapter 6 with more detailed discussions in chapter 7.

1.9.2.8 Investigate the characterizations of endothelial expressed ENaC and potential regulation mechanisms

The results of this investigation were demonstrated and discussed in chapter 5 and chapter 6 with more detailed discussions in chapter 7.

1.9.2.9 Determine the effects of proposed intracellular inhibitory molecules on ENaC in ECs in vitro

The results of this investigation were demonstrated and discussed in chapter 4, chapter 5 and chapter 6 with more detailed discussions in chapter 7.

1.9.2.10 verify the hypothesis that removing or neutralizing the intracellular inhibitors restores ENaC activity

The results of this investigation were demonstrated and discussed chapter 6 with more detailed discussions in chapter 7.

1.9.2.11 Verify the hypothesis that after channel activity is restored, ENaC is able to mediate cellular response to mechano-stimulation such as shear force

The results of this investigation were demonstrated and discussed in chapter 6 with more detailed discussions in chapter 7.

1.9.2.12 Identify the possible endogenous factors that are able to remove or neutralize the intracellular inhibitory factors

The results of this investigation were demonstrated and discussed in chapter 6 and chapter 7 with more detailed discussions in chapter 7.

Chapter 2

General Materials and Methods

2.1. Cell culture

2.1.1 M1 cells

Mouse kidney cortical collecting duct cells (M1) were purchased from the European Collection of Cell Cultures. Cells were grown in a medium containing DMEM: Ham's F12 medium (1;1) (Sigma), 2 mM glutamine (Gibco), 5 µM dexamethasone (Sigma) and 5% FBS (Sigma) in a 5% CO_2 and 37ºC incubator. M1 cells were maintained as follows: the Culture medium was changed to freshly supplemented Medium every 4-5 days until the cells were approximately 80% confluent. Subcultures were performed when the cells reached 80% confluence. 1.5 µM aldosterone (Sigma) was added into the culture medium 24 hours before the experiments to stimulate cells. When cells reached 70% confluence, cells were seeded in a low density approximately 40%-60% to either the coverslip or the culture inserts (BD).

2.1.2 HMEC cells

Human dermal microvascular endothelial cells (HMEC) and primary cultured human dermal microvascular endothelial cells (pHMEC) were kindly provided by Prof A Ahmed (University of Birmingham, UK). pHMECs were used within five passages. The culture medium contains both basal culture midia and supplement.

Subculture: Subcultures were performed when the cells reached 80% confluence.

Remove all of the old culture medium from the flask. Add 3ml of PBS solution and incubate for 2 minutes. After remove the PBS, add 2-3ml trypsin/EDTA solution to the flask and make sure the entire bottom surface is covered. Trypsin incubation should not last longer than 3 minutes. When 90% of the cells are rounded up, rap the flask very gently to dislodge cells from the surface of the flask. Add culture medium to the flask at a ratio of 2:1 (culture medium/trypsin) to neutralize the trypsin. Harvest the mixture and centrifuge at 200g for 5 minutes to pellet the cells. Remove the supernatant from the tube and then re-suspend cell. The cell pellet is diluted and seeded on coverslips or culture flasks. The density of seeding on coverslips varies depend on experimental need but for culture purpose, 2500-5000 cell per cm^2 is desired.

Maintainece: the Culture medium was changed to freshly supplemented Medium every 48 to 72 hours until the HMEC cells were approximately 80% confluent. The fresh culture medium is required to be warmed to 37^oC before replacing the old medium.

2.1.3 HUVEC

Primary cultured HUVEC cells, culture media and stimulatory supplement were purchased from Lonza and Promocell.

Subculture: The cells were seeded at a density of 2500 to 5000 cells/cm^2. The mixture of culture media was added into culture flask at a rate of 1mL/$5cm^2$. Minimum of 30 minutes equilibrium time of the culture media in 5% CO_2 humidified incubator was

required.

Subculture was immediately performed when the cell confluence reached to 80%. For each 25cm^2 of cells to be subcultured, 5ml of PBS buffer solution, 3ml of EDTA, and 2ml of EDTA/trypsin solution were applied. After incubation with PBS and EDTA solution, cells were covered by 2ml of trypsin/EDTA solution in the 25cm^2 flask for not longer 2 minutes. When 90% of the cells were rounded up, a gentle shock is required to apply to the bottom of the flask to release the majority of cells from the culture surface. A seconde shock was applied after 30 seconds If only a few cells were detached. I neutralize the trypsin by culture media at a ratio of 2:1 (culture media/trypsin) and then transfer the mixture to a sterile 15ml centrifuge tube. The mixture was centrifuged at 220g for 5 minutes to pellet the cells. The cell pellet was then diluted and seeded on coverslips or culture flasks. The density of seeding on coverslips varies depend on experimental need.

Maintenance: I change the growth medium the day after seeding and every 48 hours thereafter. The fresh culture medium is required to be warmed to 37^{o}C before replacing the old medium.

2.1.4 HEK cell culture

Human human embryonic kidney epithelial cells (HEK 293) cells were cultured in the

DMEM medium containing 10% FCS under standard condition of 5% CO2 and 37^{o}C.

Subculture: HEK Cells were split every 2–4 days as they reach 70–80% confluence. As a general rule, HEK 293 cells should not be confluent nor should they be seeded too sparsely. Therefore the cells were seeded approximately at a density of 15% confluence when splitting. Experiments were not performed until cells reached 40% confluence. The cells were split according to the following protocol; culturing medium was removed and cells were washed once with pre-warmed sterile PBS (containing no Ca^{2+} or Mg^{2+}). Cells were detached by EGTA-trypsin incubation for 2 minutes. Afterwards, 5-10 ml of growth medium was added into the mixture of trypsin-EDTA and cells to stop the trypsinization. The cell pellet was collected after 5minutes centrifuge at the speed of 1000rpm. The cells were then resuspended gently but thoroughly and appropriate amount cell suspension was aliquot into culture flasks and plates.

2.2. Patch clamp protocal

2.2.1 Single channel recording

The single-channel patch clamp is one type of the voltage-clamp technique. This technique permits the direct measurement of the dynamic activity of individual membrane-bound channel proteins. In this recording configuration, a blunt pipette is sealed onto a patch of membrane and the membrane at the tip of the pipette is preserved rather than ruptured as seen in whole cell recording configuration. The

current recorded is only the current that flows through the membrane at the tip of the pipette. Since this membrane area is very small, there is likely just one or a few ion channels are located in the patched membrane. Therefor, individual ion-channel currents can be recorded. Previously, the only way to estimate kinetics or conductance of these channels was the technique of “noise” or “fluctuation” analysis. Noise analysis yields an estimate of the mean lifetimes of the channels and the mean amplitudes, but no information about actual opening and closing transitions nor about the shape of the conductance changes.

In single-channel recording, a good seal is vitally important to avoid voltage error and guarantee the accuracy of the results of the experiments. A high seal resistance is a prerequisite of low-noise recordings. The recording pipette must be engaged with the cell membrane properly to ensure that the interior of the pipette is isolated from the extracellular solution. The quality of the seal is judged by the resistance, i.e. if the resistance of the seal is infinite, in most cases a few giga ohms, the seal is then considered good and no current can leak across it. The reason why the success of single channel recording is highly dependent on seal quality is because; firstly, depending on the size of the seal resistance, a fraction of the current passing through the membrane patch will leak out through the seal and will not be measured i.e. the lower the seal resistance the larger the fraction of undetected current. Secondly, thermal movement of the charges in the conducting pathways of the seal will constitutes a major source of noise in the recording unless the seal resistance is very

high. High-resistance seals, often called “gigaseals,” require that very clean pipettes be used and that they only be used once. If this is the case, gigaseals will routinely form after the application of gentle suction. When good gigaseals are formed, the noise due to the leakage current is virtually eliminated and other sources of noise remain the dominant limitations in the resolution of the recording technique. A competent amplifier is able to minimize such noises.

2.2.1.1 Equipment

2.2.1.1.1 Amplifier

The patch amplifier employed in the project is Axon 1D from Axon Instruments. Appropriate choice of patch amplifier is the prerequisite of a success patch-clamp recording since after a good gigaseal is formed the noise resulted from the leakage current is virtually eliminated whereas other noise sources become the main body of the noise in the recording. These noise sources include the noise of the electronics, the pipette glass, the input capacitance and the feedback resistor. We use this type of amplifier because it is able to minimize the noise of the electronics and the feedback element in the headstage.

2.2.1.1.2 Micromanipulator

The micromanipulator MP285 from Sutter instruments was utilized to control the movements of the headstage (recording pipette).

2.2.1.1.3 Patch pipette

Patch pipettes were fabricated from selected glass tubing. We use glass tubing with walls of 0.3 mm thick because thick wall generates lower electrical noise and increases bluntness at the tip, which preventing penetration into the cell during seal formation. The outside diameter of the glass tubing is 1.5 mm and the inside diameter is 1.2 mm.

The physical characters of patching pipette varied in different cell types. For example, the optimum pipette is range from 1.5µm to 2.0µm in M1 cell experiments, whereas this size is less than 1.5µm in HUVEC experiments. Currents were recorded at 20 kHz sample acquisition and filtered by a 5 kHz low-pass filter.

2.2.1.2 Data analysis

2.2.1.2.1 Filtering

A filter is a circuit that removes selected frequencies from the signal. Filtering is most often performed to remove unwanted signals and noise from the data. The filter method is particularly important in measuring currents from single-ion channels. The most common form of filtering in single channel recroding is a low-pass filter, which limits the bandwidth of the data by eliminating signals and noise above the corner frequency of the filter. We use digital low-pass filters that are implemented in the recording software (clampfit from Axon instruments) which consist of a series of mathematical calculations that process digitized data. All recordings were filtered by the

Gaussian Low-pass filter in clampfit 9.0 at the parameters of 15 coefficients (200Hz cut-off).

2.2.1.2.2 Single channel current detection

Open probability analysis: The channel open probabilities were analyzed by the single-channel search function of pclampfit 9.0. A 50% threshold crossmethod was utilized to determine valid channel openings. When multiple channel events were observed in a patch, the total number of functional channels (N) in the patch was determined by observing the number of peaks detected on all-point amplitude histograms. NPo, the product of the number of channels and the open probability, or the open probability (Po) itself, was used to measure the channel activity within a patch.

2.2.2 Whole-cell recordings

The whole-cell patch clamp is voltage-clamp technique. This technique permits the measurement of the activity of all the membrane-bound channel proteins on the cell. Similar to the single-channel protocol, a blunt pipette with low-resistance is sealed by suction onto the surface of the membrane to achieve the giga-seal. In contrast to the single channel recording, the patch of membrane enclosed within the tip of the pipette is ruptured by applying a gentle negative pressure or a short voltage pulse known as zap. The electrolyte solution in the pipette then forms an electrical continuity with the

interior of the cell. It is equivalent to an extremely low resistance intracellular micropipette. The voltage at the top of the pipette is controlled by a voltage-clamp circuit.

2.2.2.1 Equipments

2.2.2.1.1 Amplifier

The patch amplifier employed for the whole cell recordings is as described in 2.2.1.1.1

2.2.2.1.2 Micromanipulator

The micromanipulator is as described in 2.2.1.1.2

2.2.2.1.3 Patch pipette

The properties of pipettes used for whole-cell recordings are similar to that of the single-channel recording. However, reducing the noise of the pipette is much more crucial in single-channel recording than in whole-cell recording since the dominant noise source in whole-cell recording is the pipette resistance that is in series with the capacitance of the entire cell. Hence, the noise contribution of the pipette is not as important. In order to provide sufficient bandwidth for whole-cell current recording and to limit command voltage errors, the resistance of a pipette used in wholecell recording is slightly bigger than that used in single-channel recordings. In both single-channel and whole-cell recording, capacitance currents following voltage steps must be sufficiently

small and simple in time course to allow compensation by circuitry in the patch-clamp amplifier.

Physical character of patching pipette: the size of the pipette for whole cell recording is generally smaller than that used in single channel recordings; usually range from 1µm to 1.5µm. This size is chosen because it optimizes the seal performance in whole cell configuration. Large tips may potentiate the negative suction effects which make it easier to break the membrane clamped in the pipette, but this advantage is compensated by the high leakage current after whole cell formation. For example, In HUVEC whole cell recordings, due to the cell biophysical characterization, small tip pipettes maintain good quality recordings, although it was difficult to break the cell membrane. Currents were recorded at 20kHz sample acquisition and filtered by a 5kHz low-pass filter.

2.2.2.2 Data analysis:

Filtering: all recordings were filtered by clampfit 9.0 (purchased from Axon instruments) under the parameters; The Gaussian Low-pass filter, 15 coefficients (6000Hz cut-off). This filter was selected because it can effectively remove high frequency noise from single channel recordings without interfering with whole cell currents. Current-Voltage relationship analysis: whole cell currents were analyzed by clampfit 9.0.

2.2.2.3 Perforated-patch recording method

Perforated-patch recording techniques measure whole-cell currents and transmembrane voltages much less invasively than standard patch-clamp or microelectrodes do. Unlike the conventional whole cell patch configuration where the entire cell membrane within the tip of recording pipette is broken, the perforated-patch only drills micro-holes in the cell membrane and these micro-holes are impermeable to molecules as large as or larger than glucose. Because perforated-patch does not permeate pipette solution into cells, the intracellular solution can be preserved and the mechanisms important to cell signalling and channel regulation remain operative.

After giga omh seal was achieved, amphotericin B was utilized to gain electrical access to the cell's interior. These polyene antibiotics form pores in cholesterol- or ergosterol-containing cell membranes, which allows the measurements of electrophysiology activity of whole-cell without changing the intracellular environments. Because of their limited water solubility, amphotericin stock solutions of 15 mg/mL are prepared in DMSO. These stock solutions lose activity upon prolonged storage and freezing and the perforating activity of this compound begins to decrease about 3 hours after preparing. The stock solution, therefore, is best to prepare them freshly before use and use within 2 hour. My approach is to aliquot the antibiotic powder into centrifuge tubes and to store them indefinitely in the freezer without loss of activity. At the time of use, the appropriate amount of powder was solubilized by fresh DMSO. Solubilization is

enhanced by sonication or vortex mixing. Stock solution can be added directly to the pipette filling solution to achieve a desirable final concentration. It is not clear if higher concentrations offer any advantage. Vortex mixing for a few seconds or sonication for a minute is recommended for maximum solubilization of the antibiotic in the pipette filling solution. When the antibiotic is in excess, some of it settles out of solution and adheres quite well to the wall of a plastic tube or syringe. Therefore, the saturated solution can be used without adding much particulate matter to the pipette interior. The filling solution can be filtered after adding the antibiotics, but care must be taken since some filters might inactivate these compounds. The final solution is yellow in color and slightly turbid. After filtration with either a 0.22 or 0.45 micron filter, the final solution is perfectly clear. With nystatin, it is possible to make up larger volumes of the stock solution and adjust the pH to neutrality before use. In my experience, amphotericin B seems to interfere with seal formation. Although it is possible to seal some cells when either compound is present in the pipette filling solution, the success rate is lower than when an antibiotic-free filling solution is used with the same cells. In the first set of experiments of Perforated-patch (more than 500 attempts); the gig-seal formation is achieved normally as seen in antibiotic-free backfill solution condition, but immediately after seal formation, the resistance started to drop until it reached to less than 200 mega-ohm, which result in an unacceptable leakage current in whole cell recording. Therefore, it is best to fill the tip of the pipette with an antibiotic-free solution and then to backfill with the antibiotic-containing solution. The tip-filling process can be accomplished by simply dipping the tip into the desired solution.

However, the distance of antibiotic-free solution from the tip is critical in determining the time length of whole cell formation; the time required for the diffusion of the antibiotic from the backfilling solution to the tip determine how long it requires for the antibiotic to partition the cell membrane. For example, if the pipette is filled up to 1.0 mm from the tip with an antibiotic-free solution, more than an hour will be required for the antibiotic to reach a tip-concentration comparable to that in the backfilling solution. Ideally, the tip should be filled to a distance that allows the antibiotic to reach the tip within a few seconds following a gig-ohm seal formation. Since the time required for seal formation depends on the cells and the setup, tip filling distances should be optimized by each investigator. My approach is to overfill the tip with amphotericin B and then force the excess solution out by applying positive pressure to the back of the pipette while observing it under a dissecting microscope in the first few attempts, after I acquired more experience without microscope observation. When the antibiotic-free solution is approximately 0.5mm from pipette tip, the time required for whole-cell formation after gig-ohm seal is less than 90 seconds. However, the quality of the seal of whole-cell is not stable until the partition effects of amphotericin B stops, thus sometimes a waiting period of 10 minutes is necessary. I monitor the whole-cell forming process by cell membrane capacitance and access resistance, and start the formal current recordings only after these two indicators remain stable.

2.3 Western blot

1. Cultured cells were homogenized with CelLytic M lysis/extraction reagent (Sigma: c2978) containing Protease Inhibitor cocktail (Sigma: P8340).

2. The standard concentration curve was drawed using readouts of a serial diluted standard concentration protein mixed with Bradford reagent via a spectrophotometer (read outs against concentrations). Concentrations of same proteins were determined by their Bradford Assay readout using standard curve.

3. Equal amounts of total protein were loaded into the SDS-PAGE gel wells, the amount of protein was calculated as following: the volume that needed for each sample = [the lowest protein concentration of all samples x volume of the well (30µl)]/ concentration of the sample that is going to be loaded. β-actin was used as the inner marker to calibrate the protein quantity. Samples were prepared with 5x loading buffer (for 10ml buffer: 1.25ml of Tris-HCl pH6.8; 2 ml of 20% SDS; 2 ml of glycerol; 0.02g of bromophenol blue; 0.469g of DL-Dithiothreitol, add water up to 10 ml), heat at 94°C for 5 min.

4. Separated protein samples on 10% SDS-PAGE gels (for 15ml separating gel: 5ml of 30% acrylamide/bisacrylamide; 3.75ml of 1.5M Tris-HCl solution pH 8.8; 0.075ml of 20% SDS; 0.2ml of 10% Ammonium persulfate; 6ml of distilled water; 10µl of N,N,N′,N′

-Tetramethylethylenediamine; For 6.5 ml stacking gel: 640µl of 30% acrylamide/bisacrylamide; 780µl of 1M Tris-HCl pH6.8; 36µl of 20% SDS; 62µl of 10% Ammonium persulfate; 5ml of distilled water; 4µl of N,N,N′,N′-Tetramethylethylenediamine) under voltage 120 V for 90min and then transferred to PVDF membranes under voltage 100 V for 1h.

5. The membranes were rinsed with Tris-Buffered Saline Tween-20 (TBS-T) and then blocked in TBS-T containing 5% milk for 1 hour (at room temperature). The membrane was incubated with the first antibody, overnight at 4°C, followed by washes in TBS-T and incubation with the secondary antibody for 1 hour at room temperature.

6. Membranes were washed with TBS-T and blots were detected by Pico chemiluminescent substrate (Pierce: 34080). A negative control was performed without incubation of either primary antibody or secondary antibody.

2.4 Isolation of caveolae compartment from HUVEC cells

HUVEC were grown to confluence in 75ml culture flasks as described in 2.1.3, and used for fractionation. The homogenates in MBS (25 mM 2-(N-morpholino)-ethanesulfonic acid (MES), pH 6.5, 0.15 M NaCl) containing 1% Triton X-100 were adjusted to 40% sucrose by the addition of 2 ml of 80% sucrose prepared in MBS and placed at the bottom of an ultracentrifuge tube. A 5–30% discontinuous sucrose gradient was formed

above (4 ml of 5% sucrose/4 ml of 30% sucrose, both in MBS lacking detergent) and centrifuged at 39,000 rpm for 18 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA, USA). A light-scattering band at the 5–30% sucrose interface was collected or fractionated into 12 subfractions. A 100 µl aliquot of each fraction was used to measure alkaline phosphatase activity. After direct addition of substrate solution (p-nitro-phenyl phosphate, R&D systems, Minneapolis, MN, USA), the absorbance at 405 nm was measured.

2.5 Mechano-stimulation application

2.5.1 Flow application method

To apply laminar solution flow, an Open Diamond Bath recording chamber (RC-26, purchased from Warner instruments) was utilized. The diamond shaped geometry facilitates laminar solution flow throughout the bath area evenly to reproduce the most physiological shear force. The flow speed was controlled by gravity and measured by the ratio of volume against time. The shear force was therefore calculated accordingly.

2.5.2 Membrane stretch application method

Membrane stretch was produced by applying a positive pressure or a negative pressure to the rear of the patch pipette. However, the positive pressure seems to be more

important since it simulate the effect of blood pressure. Channel activity measured as the probability of channel being open which is dependent on the degree of membrane stretch. A pressure gauge was introduced in the pressure application system to measure the exact amplitude of the pressure.

2.6 Calcium imaging

Fura-2: The growth medium was removed and cells were rinsed once in Earl's Balanced Salt Solution (EBSS). To prepare stock solution, 50 µg Fura-2 AM (F1221, Molecular Probe, Ltd.) was dissolved in 20 µl of 20% pluronic acid (0.01g in 50 µl Dimethyl sulfoxide (DMSO)). Prior to the experiment, a mixture of 1 µl stock dye with 200 µl EBSS was loaded onto the cells and incubated for at least 30-40 minutes. Prior to placing the coverslips into the recording chamber, cells were rinsed in modified thyroid medium (mM): NaCl 115, KCl 3, NaH_2PO_4 1.25, $MgCl_2$ 1.3, $NaHCO_3$ 24, $CaCl_2$ 2 and d-glucose 11, at pH 7.4 to remove residual dye. Data acquisition and analysis were performed by Wasabi programming (Hamamatsu, Ltd.). A CCD camera (C9102, Hamamatsu, Ltd.) was used to capture the fluorescent image. Images were collected in a format of 1344 x 1024 pixels. Fluorescent changes in Fura-2 were measured with double wavelength excitation at 340 and 380 nm, and emission at 510 nm. Absolute Ca^{2+} was calibrated by using Fura-2 calcium imaging calibration kit (Invitrogen F6774). Changes in Ca^{2+} concentration in the region of interest were calculated according to a ratio of 340/380. Time lapse recording initially captured the images at 2s intervals. In order to minimize

cell photo bleaching in the long experimental protocol, 3 or 5 s intervals were applied in the experiments. Most data presented in the figures were acquired at 3 s intervals. The hypoxic medium was made of modified tyrode buffer bubbled with 95% N_2 and 5% CO_2. The O_2 level in the recording chamber was measured by using an OCM-2 amplifier (Cameron Instrument Company) and a mini Clark-style O_2 electrode (Diamond General Development Corp). The EGTA solution for calcium imaging recording contained (mM) NaCl 117, KCl 4.46, $NaHCO_3$ 24, d-glucose 11 and EGTA 0.2. The high K^+ bath solution contained (mM) NaCl 66.3, KCl 45.3, $MgCl_2$ 1, $CaCl_2$ 1.8, $NaHCO_3$ 23 and d-glucose 11, at pH 7.4.

Post-hoc analysis was carried out using Wasabi programe. Individual cells or groups of cells were outlined and analyzed. Data from one coverslip were averaged and presented in the figures. The same experiments were repeated on different coverslips, the number of total cells and batches were presented using n and N, respectively. Data are presented as means ± S.E.M., and statistical differences were compared using a Student's paired t test, taking $P < 0.05$ being significant *.

2.7 Perfusion system for exchange of the gas bubbled media

The media were bubbled in para-film sealed bottles. Each bottle was connected to two TYGON tubes; a 1.6 mm ID tube carried the medium out of the bottle, and a 0.8 mm ID tube returned the medium to the bottle. The bubbled medium circulated in the system

constructing of the medium bottle and two tubes, and was driven by a peristaltic pump (Gilson minipuls 3 with four pumping heads). A tri-connector was placed very close to the electric valve and the recording chamber. This connector linked the delivery and return tubes, and another tube to the recording chamber (chamber tube). The chamber tube passed an electrical valve (Automation 8) and reached the recording chamber. The media flowed into recording chamber (RG 26, Warner) if the valve was open; otherwise they continually circulated to reduce dead space. The flow rate was 5 ml/min. After the coverslip was loaded into the recording chamber, a glass cover-slide (20 x 60 mm) was placed on top of the recording chamber to reduce gas exchange. An oxygen electrode was placed into the recording chamber to detect the O_2 level in the medium. According to the calibration, the O_2 level of the hypoxia medium in the recording chamber was around 20 mmHg.

2.8 Hypoxic protocol

Hypoxic challenges were performed in HUVEC and HEK experiments.

HUVEC and HEK cells were exposed to a hypoxic solution containing (mM): NaCl, 42; $NaHCO_3$, 24; $MgSO_4$, 1; NaH_2PO_4, 0.435; glucose, 5.56; $CaCl_2$, 1.8; KCl, 80, with 2min duration and 10min interval. ~5μM of $PGF_{2\alpha}$ (higher concentration may be required when certain chemical was used, which will be specified in the following chapters) was added to the solution to achieve a pretone equivalent to ~15% of control. When the

pretone was stable, cells were exposure to continuously gas of 95% N_2/5% CO_2 for 40 min, then wash with bath and return to normoxia condition. Oxygen level was monitored using a dissolved oxygen meter in all experiments: at hypoxia condition, ~2% and normoxia, ~19%. Reproducible results were obtained after 60-90min recovery time after exposure to hypoxia, so a standard 80min recovery was observed between control and testing experiments.

2.9 Gene modification

2.9. 1 Interference RNA (RNAi)

1. HMECs: One day before transfection, plate cells on coverslips in a 24 well pate format with 500 µl of growth medium without antibiotics, so that cells will be 90-95% confluent at the time of transfection.

2. For each coverslip, oligomer-Lipofectamine™ 2000 complexes were prepared as follows:

a. Dilute 20 pmol siRNA oligomer in 50 µl Opti-MEM® I Reduced Serum Medium without serum (final concentration of RNA when added to the cells is 33 nM) and then mix them gently.

b. Lipofectamine™ 2000 was mixed gently before use, then dilute 1 µl in 50 µl Opti-

MEM® I Reduced Serum Medium. Mix gently and incubate for 5 minutes at room temperature.

c. After the 5-minute incubation, combine the diluted oligomer with the diluted Lipofectamine™ 2000. Mix gently and incubate for 20 minutes at room temperature.

3. Add the oligomer-Lipofectamine™ 2000 complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.

4. Incubate the cells at 37°C in a CO_2 incubator. The cell will be suitable for experiments in a period of 48-96h.

The materials used are list as following: 500 µl Opti-MEM (Invitrogen); on-Targetplus siRNA (smart pool ENaC channel, catalogue no. L-006504-00 from Thermo Fisher Scientific); lipofectamine 2000 (Invitrogen catalogue no.:11668). Negative controls were employed by using anegative control siRNA (nontargeting pool, Dharmacon). Knockdown effects were examined by RT-PCR and western blot analysis after 24 and 48 hours.

2.9.2 Over-expression

1. HUVECs: One day before transfection, plate cells on coverslips in a 24 well pate

format with 500 µl of growth medium without antibiotics, so that cells will be 90-95% confluent at the time of transfection.

2. For each coverslip, prepare complexes as follows:

a. Dilute 0.8µg of plasmid in 50 µl of Opti-MEM® I Reduced Serum Medium without serum (or other medium without serum). Mix gently.

b. Mix Lipofectamine™ 2000 gently before use, then dilute 2µl in 50 µl of Opti-MEM® I Medium. Incubate for 5 minutes at room temperature.

c. After the 5 minute incubation, combine the diluted plasmid with diluted Lipofectamine™ 2000 (total volume = 100 µl). Mix gently and incubate for 20 minutes at room temperature.

3. Add the 100 µl of complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.

4. Incubate cells at 37°C in a CO_2 incubator for 48 hours prior to testing for transgene expression.

2.10 Statistical method

I used a t-test to analyze my data. My results usually consisted of two groups of data, where each group represents one cellular response to a certain stimulation or a matching control. I need to compare each stimulation group with its control, comparing the difference of the two means in relation to the variation in the data. The t-test is one type of parametric statistic that fits this purpose. As the t-test assumes normality, I needed to do a normality test to check whether the individual groups were normally distributed. I used graphical methods and Kolmogorov–Smirnov tests to check the normality of my data. The results of both tests indicate that my data is normally distributed. Thus it is safe and appropriate to use the t-test to analyze my experimental data.

2.11 Solutions and chemicals

Chemicals dissolved in water: suramin (Sigma-Aldrich), D609 (Sigma-Aldrich), AICAR (Calbiochem), sulfaphenazole (Sigma-Aldrich), carboxyheptyl PGI_2Na (Sigma-Aldrich), L-NAME (Torcis), acetylcholine (Sigma-Aldrich), sodium nitroprusside (Sigma-Aldrich);

Chemicals dissolved in ethanol: nifedipine (Sigma-Aldrich), 2APB (Calbiochem), OAG (Sigma-Aldrich), AA (Alexis), ETYA (Alexis), indomethacin (Alexis), isotetrandrine (Sigma-Aldrich), forskolin (Sigma-Aldrich), AH6908 (Biomol), NS398 (Sigma-Aldrich),

EETs (Biomol), PPOH (Cayman), DDMS (Cayman), Cabacyclin PGI_2 (Biomol), 15-HPETE (Biomol), SKF525A (Biomol), picotamide (Cayman), PGE_2 (Biomol), $PGF_{2\alpha}$(Biomol);

Chemicals dissolved in DMSO: amiloride (Sigma-Aldrich), CO donor (Sigma-Aldrich), amphotericinB (Sigma-Aldrich), U73122 (Calbiochem), KBR7943 (Calbiochem), compound C (Calbiochem), RHC20267 (Sigma-Aldrich);

All solutions were made on the day of the experiments. The 1000 times stock solution were made of chemicals that dissolved in ethanol/DMSO/water. The solvent, ethanol/DMSO at the same vehicle concentration, was tested alone on the controls and had no effect. Light or temperature sensitive chemicals like ATP and arachidonic acid (AA) were kept on ice and in the dark. Drugs were added into a syringe or bottle which led to the recording chamber as necessary, at room temperature or at 37◦C respectively. The activities of chemicals, e.g. suramin, U73122, 2APB, and nifedipine, from the same stock solutions as used in these experiments, were also examined in other cell lines, including human kidney epithelial tubules cells and PC12 cells to assess their efficacy. In these cells, these drugs all produce results as expected, either inducing or preventing $[Ca^{2+}]_i$ elevation.

Chapter 3

Effects of lipids on ENaC activity in cultured mouse cortical collecting duct cells

3.1 Introduction

Epithelial Na^+ channels (ENaC) expressed on the apical membrane of distal renal tubules and collecting ducts are responsible for hormone-regulated Na^+ reabsorption. Two basic steps compose this Na^+ reabsorption: Na^+ enters into the cells via ENaC on the apical membrane following the Na^+ gradient and it is then extruded to the interstitial fluid by basolateral Na^+-K^+-ATPase. The rate of Na^+ uptake is determined by the number of channels (channel density) and channel activity. Although the direct activation mechanism of ENaC remains unclear, protein kinase A (PKA), the small G protein K-Ras (Eaton, Malik et al. 2001; Staruschenko, Nichols et al. 2004; Staruschenko, Patel et al. 2004), serum glucocorticoid-induced kinase (SGK) (Chen, Bhargava et al. 1999; Friedrich, Feng et al. 2003) and the channel activated protease (CAP1) (Vallet, Chraibi et al. 1997) have been demonstrated to increase ENaC activity. Whereas protein kinase C, $[Na^+]_i$ (Anantharam, Tian et al. 2006), arachidonic acid (AA) (Worrell, Bao et al. 2001), the products of cytochrome P450 (CYP), e.g. 11,12 epoxyeicosatrienoic acid (EET) (Wei, Lin et al. 2004), and the reduction of PIP2 (Ma, Saxena et al. 2002; Yue, Malik et al. 2002) have been shown to decrease ENaC activities.

Arachidonic acid (AA), a precursor of eicosanoids, exists primarily in esterified form as a glycerophospholipid in the cell membrane. Under normal conditions, non-esterified, free arachidonic acid is virtually undetectable. Arachidonic acid is found in the *sn*-2 position of membrane phospholipids and can be liberated primarily by phospholipase

(PLA). Upon release, AA may initiate signalling or can be metabolized into a wide range of products via cyclooxygenases (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450). AA and its metabolites act in a diverse range of physiological and pathological roles in water and Na^+ homeostasis in the kidney (Breyer, Jacobson et al. 1996; Breyer, Zhang et al. 1998; Breyer and Breyer 2000). In addition to interactions with specific receptors (Breyer, Kennedy et al. 2000), lipids can also directly bind to ion channels to alter their activities, resulting in either inhibition (Oliver, Lien et al. 2004) or stimulation (Chyb, Raghu et al. 1999). By using trans-epithelial electric resistance (TEER) methods, a previous study has shown that the decrease of apical AA by a cPLA antagonist significantly increases trans-epithelial Na^+ current whereas the decrease of basolateral AA reduces Na^+ transport, suggesting that AA can lead to inhibition of ENaC activity (Worrell, Bao et al. 2001). In the oocyte expression system, AA, as well as its analogue ETYA, moderately decreases ENaC whole cell currents by inhibiting ENaC exocytosis and increasing ENaC endocytosis (Carattino, Hill et al. 2003). However, AA fails to reduce ENaC activity when CYP-epoxygenase is blocked, suggesting that 11,12-EET as a metabolite of AA inhibits ENaC activity (Wei, Lin et al. 2004). It still remains unclear whether ENaC activity, including conductance and open probability, is altered by lipids regardless of the channel density and other second messengers. To prevent the interference of second messengers, single channel recording was employed in this study to clarify the direct effects of the lipids on ENaC channels.

3.2 Specified protocols

3.2.1 Patch clamping recording protocols

Single channel recordings were performed as previously described (Gorelik, Gu et al. 2002). Briefly, a coverslip or insert containing grown M1 cells was transferred into a recording chamber mounted on a Nikon inverted microscope (Nikon TE 2000U). A patch pipette with a resistance of 7 MΩ was fabricated from a borosilicate glass capillary (1.5 od, 0.86 id) (Warner) on a Sutter Puller (P97). Bath solutions contained (in mM): 110 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPEs, 5 Na-HEPEs, pH 7.2. Pipette solution contained (in mM): 110 NaCl, 4.5 KCl, 0.1 EGTA, 5 HEPEs, 5 Na-HEPEs, pH 7.2. Currents were recorded with an Axon 1D amplifier and Axon clampex 9.0. The data were acquired at 20 KHz and filtered by a 1 kHz low pass filter. The channel events were analyzed by pclampfit 9.0 (single channel search in analyze function). Data were further filtered at 200 Hz before data analysis. A 50% threshold cross method was utilized to determine valid channel openings. When multiple channel events were observed in each patch, the total number of functional channels (N) in the patch was determined by observing the number of peaks detected on all point amplitude histograms. NPo, the product of the number of channels and the open probability, or the open probability (Po) itself was used to measure the channel activity within a patch. The NPo was calculated according to previous descriptions (Yue, Malik et al. 2002). Because the recording membrane patch usually contained multiple channels, in most cases the changes in the NPo (not Po)

were directly observed and compared. Due to the variance of the channel open probability, 2-5 minutes of single channel recording prior to lipid application was normally used as the control. The open probability of the channel during the application of lipids (whole duration excluding the noise) was directly compared with that of the control. This ratio was employed to determine the effects of lipids on ENaC activity. In some cases, the NPo of ENaC during the application of lipids was compared to that of ENaC in medium after the lipids were washed away. The data were utilized to confirm the observations. The data are presented as the mean ± S.E.M., and the statistical differences were compared using Student's paired *t* test, taking $P < 0.05$ as significant and represented as *.

There is an inevitable fluid turbulence during bath medium exchange, for example during valve opening and closing, and this will cause a transient noise which is composed of an initial spike and a base line sine wave. The noise component of the currents was excluded from the NPo analysis. Control experiments have been performed by switching perfusion with normal bath medium and the NPo of the currents was not significant altered.

3.2.2 Chemicals

The following chemicals were used: arachidonic acid (AA) (Alexis), linoleic acid (LA) (Alexis), ETYA (Alexis), stearic acid (Sigma), 11-12 EET (Biomol), 20 HETE (Biomol), PPOH

(Cayman), DDMS (Cayman), AH6809 (Biomol), SC51322 (Biomol), PGE2 (Biomol) and PGF2α (Biomol). Most chemicals were dissolved in ethanol and made up as 1000 to 5000 times stock. All recording solutions were made on the day of experiments. The solvent at the same dilution was tested alone in controls and had no effect on the results.

3.3 Results

3.3.1 Functional ENaC channels expressed in M1 cells

Single channel recording was utilized to characterize the ENaC channels in M1 cells. In a cell-attached recording, an inward current was detected when the pipette voltage was held at +20 mV and +60 mV. These inward currents exhibited a slope conductance of 5.1 ± 0.25 pS (n=15) between the command voltages (20 and 60 mV, hyperpolarization). The cell membrane under a patching pipette was excised from the cell to form either the inside-out or the outside-out recording configuration. In an outside-out recording, the currents with a slope conductance of 5.15 ± 0.15 pS (n=15) are shown in figure 3.1a. A bath application of 0.5 μM amiloride significantly reduced the channel open probability, shown as the currents flickered and the major open state stepped toward the closing level. A high concentration of amiloride (5 μM) then almost abolished this current (Fig. 1a). Currents with the same conductance were also observed in the inside-out recordings (n=60) (Fig. 1b). The summary of the excised membrane recordings was plotted as shown in figure 3.1c. The data above suggested that these

currents were carried out by ENaC channels. In addition, previous reports have also shown that the rundown of ENaC currents in the inside-out recording are due to the lack of PIP2 (Yue, Malik et al. 2002). In M1 cells, significant current rundown of ENaC was not normally observed within 10 minutes of the formation of the inside-out recording configuration (figure 3.2). The selected NPo values of ENaC in M1 cells (seven individual cells), which cover the range of NPo values observed in M1 cells, were presented in the lower panel of figure 3.2d.

3.3.2. Effects of AA and other fatty acids on ENaC activity from the extracellular side

Within 10 minutes, the bath application of AA at 10 μM significantly reduced the ENaC NPo to 0.08 ± 0.05% of the control (n=5), as shown in figure 3.2a ($P<0.005$), while the cell-attached recording was performed. Incubating cells with AA for a longer period did not enhance the inhibition effect as described previously (Carattino, Hill et al. 2003). ETYA, an analogue of AA but resistant to AA degenerative enzymes, was used to determine whether the inhibitory effect of AA is a result of the metabolites of AA. The NPo of the ENaC channel was slightly reduced to 85.1 ± 9.1% (n=6) of the control by ETYA (Fig. 2b) ($P<0.05$). In addition, extracellular application of LA also reduced the NPo of ENaC to 89.1 ± 8.2% (n=5) of the control (figure 3.2c) ($P<0.05$). In an outside-out recording, a bath application of AA reduced the NPo of ENaC to 77.5 ± 6.1% of the control (n=5). These results indicate that the lipid can alter the membrane's physical properties, resulting in a slight change in channel kinetics. However, the major

inhibition effect of AA on ENaC is accounted for by the metabolites of AA but not via a non-specific effect of the lipid.

3.3.3 Effects of AA and other lipids on ENaC activity from the cytoplasmic side

In most of the inside-out recordings, cells were held by a pipette voltage of either 60 mV or 100 mV. In the inside-out recordings, lipids can directly interact with the cytoplasmic membrane and the effects of lipids on ENaC should be instantaneous. Therefore, our standard protocol was to incubate the patch of the membrane with the lipids for up to 5 minutes. Some experiments with longer incubation times, exceeding 9 minutes, were also performed and no further effects on ENaC activity due to the lipids were observed. Application of 10 μM AA only induced a small reduction of the ENaC NPo, to 86.1 ± 4.2% (n=10) of the control (figure 3.3a) ($P<0.05$). Similar effects were observed by application of EYTA (n=9), LA (n=8) or stearic acid (n=6) on the cytoplasmic membrane (Fig. 3). These observations suggested that a significant inhibition effect of AA on the ENaC NPo resulted from the AA metabolites but not from AA itself.

3.3.4 Inhibition effect of 11, 12-EET on ENaC channels

11, 12 EET as a metabolite of AA via the epoxygenase pathway, exerted a significant inhibition on the ENaC NPo when it was applied directly to the cytoplasmic membrane. In an inside-out recording, the NPo of ENaC was reduced to 5.3 ± 2.1% (n=12) of the control by a bath application of 250 nM of 11, 12 EET and 7.4 ± 2.5% (n=11) of the

control by 100 nM of 11, 12 EET (figure 3.4). The other products, e.g. 20-HETE, as metabolites of AA via the CYP-dependent ω-hydroxylation pathway, slightly altered the NPo of ENaC as did ETYA (not shown). In the outside-out recordings, a bath application of 250 nM of 11, 12 EET inhibited ENaC activity to 8.3 ± 3.1% (n=5) of the control, which is similar to the results obtained from cytoplasmic application of 11,12-EET （P>0.05）. In a cell-atteched recording, a bath application of 10 μM AA reduced the NPo of ENaC to 55.1 ± 8 .9% (n=5) of the control when cells were pre-incubated with PPOH (15 μM), which is significantly different to AA effects on cells without treatment (P<0.05) (figure 3.2c).

3.3.5. Effects of PGE and PGF on ENaC activity

PGF2 and PGE, the major metabolites of AA via the cyclooxygenase (COX) pathway, are abundant in CCD cells. They are generated in the cellular plasma and diffuse out of the cell. In an inside-out recording, the application of PGE enhanced the NPo of ENaC to 135.1 ± 14.2% (n=12) (figure 3.5a) of the control (P<0.05) and addition of PGF2 to the cytoplasmic membrane enhanced the NPo of ENaC to 167.3 ± 15.1% (n=11) (figure 3.5b) of the control (P<0.05). Changes in the conductance of the ENaC channels were not observed with the application of either PGF2 or PGE. In addition, PGE2, at a physiological concentration of 2 μM (Els and Helman 1997), also enhanced the NPo of ENaC to 153.1 ± 111.3% (n=4). Furthermore, EP receptor antagonists, e.g. SC-51322 (2 μM) and AH6809 (5 μM), have been included in the pipette during some inside-out

recording (n=20), no significant effects were observed on the augmented effects of PG on the ENaC NPo.

In conclusion, cytoplasmic AA, EYTA, LA and SA slightly reduced the NPo of ENaC, whereas 11,12 EET almost abolished ENaC activity. Cytoplasmic PGE and PGF both significantly enhanced the NPo of ENaC (figure 3.6).

3.4 Discussion

The data presented demonstrated that AA inhibits ENaC activity in M1 cells. This inhibition effect is mediated by 11,12-EET, a metabolite of AA via CYP epoxygenase, but not via AA *per se*. The unsaturated fatty acids from either the extracellular or intercellular side of the cell membranes slightly reduced the ENaC channel open probability, whereas both PGF2 and PGE, the major metabolites of AA via COX, significantly enhanced the ENaC channel open probability. Since the lipids and their metabolites are constantly present in the cellular cytoplasm, their direct interaction with ENaC channels might serve as a mechanism in the regulation of ENaC activity and subsequently determine Na^+ reabsorption.

The previous studies have shown that ETYA, at high concentrations of 40 μM (Worrell, Bao et al. 2001) and 50 μM (Carattino, Hill et al. 2003), inhibits ENaC activity in *Xenopus* oocytes. Due to the similar effects of ETYA and AA, AA was considered a direct regulator

of ENaC. Controversially, ETYA has also been shown to exert no effect on ENaC activity from rat CCD cells (Wei, Lin et al. 2004), resulting in a conclusion that metabolites of AA, rather than AA itself, inhibit ENaC activity. We found there is a small inhibition of ENaC activity caused by ETYA, and this small inhibition is due to non-specific effects of fatty acids. Application of LA, ETYA and stearic acid from either the extracellular or intracellular side exerts similar slight inhibitions of ENaC. However, differences in AA's inhibitory effect on ENaC by cytoplasmic application revealed that metabolites of AA, rather than AA itself, primarily inhibit ENaC. This is consistent with previous work (11, 12-EET) (Wei, Lin et al. 2004).

In the toad bladder, an increase of Na^+ reabsorption is associated with an increased turnover of phospholipids (Goodman, Wong et al. 1975), which implicates the involvement of PLA (Yorio and Bentley 1978). Inhibition of PLA_2 by aristolochic acid significantly increases the Na^+ conductance in A6 cells, suggesting that cPLA2 is tonically active in A6 cells (Worrell, Bao et al. 2001). The cPLA2 constantly liberates free AA from the sn-2 position of the membrane lipids. The free AA is the substrate for a number of enzymes, e.g. COX, CYP epoxygenase and CYP hydroxylase, and it can degenerate to a number of metabolites, e.g. PGE2, PGF2, EET, HETE. Either free AA or its metabolites on the cytoplasmic side may act as signalling molecules to directly regulate ion channels. Previous studies have demonstrated that polyunsaturated fatty acids including AA and LA could directly mediate the activities of ion channels including TRP (Chyb, Raghu et al. 1999), BK (Denson, Wang et al. 2000), K^+ channels (Kim and Clapham

1989; Ordway, Walsh et al. 1989; Oliver, Lien et al. 2004), and this regulation is not due to alterations in the membrane fluidity by PUFA. In this study, we found that fatty acids, including saturated fatty acids, could slightly inhibit ENaC activity in a non-specific way. Unlike other channels, ENaC possesses only two transmembrane domains and cytoplasmic C- and N- terminals. Other evidence suggested that ENaC may associate with the cholesterol enriched microdomains (Hill, An et al. 2002), which are detergent insoluble. The fatty acids may interact with either a subunit of the ENaC channel (Carattino, Hill et al. 2003) or these lipids may form rafts to alter the physical proximity, resulting in changes in channel activity.

Prostaglandins comprise a diverse family including PGE2, PGF2α, PGD2, PGI2 and thromboxane A2 (TXA2) (Breyer and Breyer 2001; Breyer, Kennedy et al. 2002; Sugimoto and Narumiya 2007). These prostanoids are abundantly produced in the kidney (Farman, Pradelles et al. 1986; Bonvalet, Pradelles et al. 1987; Smith, Marnett et al. 1991; Smith 1992; Hebert, Carmosino et al. 2005), where they act locally via eight specific transmembrane G protein-coupled receptors (GPCR) designated EP 1-4 (for E-prostanoid receptor), FP, DP, IP and TP (Breyer, Kennedy et al. 2002; Nusing and Seyberth 2004; Wu and Liou 2005; Sugimoto 2006), respectively. PGE2 is the major prostaglandin produced along the collecting duct (Hebert, Breyer et al. 1995), where it potently regulates solute and water transport (Breyer, Jacobson et al. 1996; Breyer and Breyer 2000). It is well established that PGE2 exerts its effects by either stimulating or inhibiting salt and water reabsorption through its interaction with the cell surface EP

receptors in an autocrine or paracrine fashion (Narumiya 1994). The controversial effects are generally considered to be due to the diverse signalling pathways initiated by distinct EP receptors (Guan, Zhang et al. 1998). However, as we demonstrated in this report, PGs can also directly affect ENaC without EP receptors, because they lack a cellular mediator, e.g. Ca^{2+} or cAMP, to cope with the activation of GPCR in the inside-out recordings. Cytoplasmic prostaglandins directly enhance the NPo of ENaC. Due to their membrane permeability, luminal PGs might also stimulate ENaC after they enter into the cytoplasm. Many observations may be consistent with this direct activation. For example, infusion of renal filtrates contacting PGs increases Na^+ reabsorption (Hornych, Bedrossian et al. 1975; Lazzeri, Barbanti et al. 1995; Breyer and Harris 2001). PGE2 augments amiloride-sensitive Na^+ transport in frog skin (Kokko, Matsumoto et al. 1994; Matsumoto, Mo et al. 1997), MDCK (Wegmann and Nusing 2003) and A6 cells (Nielsen 1990).

Our results are in disagreement with the observations of Els and Helman, who found that PGE2 decreases the NPo of Na^+ channels by unknown mechanisms (Els and Helman 1997). Several explanations of this discrepancy are possible. Most obviously, there could be a species difference, since frog skin was investigated in the previous study whereas our study was performed on mouse kidney cells. Second, there could be time-dependent differences. In the tissue study of frog skin, changes in short circuit currents happen over minutes. In patch clamping experiments, the exposure of the inner surface of the membrane is fairly brief. Effects of PG on ENaC are

instantaneous and occur within seconds. Finally, the interference of second messengers should be seriously considered when the study is performed on a tissue level. Within minutes, application of PGE could stimulate a variety of signalling pathways via EP receptors (Breyer and Breyer 2001). Results would therefore be profound. In this study, we provided a simple and clear conclusion which excludes effects of second messengers.

Consistent with the previous studies, 11, 12-EET, but not HETE, plays a primary role in inhibiting ENaC activity. This effect is due to the direct interaction of 11, 12-EET with ENaC channels, although the interaction site remains unclear. Accumulated evidence has suggested that 11,12-EET is the major signalling molecule inhibiting ENaC activity (Wei, Lin et al. 2004; Sun, Lin et al. 2006; Wei, Sun et al. 2006). The expression of CYP epoxygenase, which mediates the product of 11,12-EET from AA, could be regulated by the diet of Na^+ uptake (Sun, Lin et al. 2006). It partly explains the observation that removal of an epoxygenase inhibitor decreases the blood pressure in rats maintained on a high Na^+ diet (Makita, Takahashi et al. 1994). However, some studies reported there is either lack of epoxygenase or deficient of epoxygenase activity in cultured cells (Michaelis, Fisslthaler et al. 2005). Our results in this study confirm that 11, 12-EET strongly inhibits ENaC by direct interaction.

There are opposite regulations in ENaC channel activity by different AA metabolites. Products of epoxygenase inhibit ENaC channels whereas products of COX augment

ENaC activity. According to our experiments, lipids are strongly suggested to directly interact with the ENaC channels via the binding sites in the ENaC molecular structure. The defined ENaC activity will be determined by lipid affinity to ENaCs and spatial distribution of different AA metabolism enzymes. In A6 cells, application of aristolochic acid from the apical membrane increases trans-epithelial Na^+ current whereas application from the basolateral side reduces Na^+ transport (Worrell, Bao et al. 2001). Basolateral application of ibuprofen mimics the effects of inhibition of cPLA via basolateral application of aristolochic acid, suggesting that the distribution of COX in A6 is likely close to the basolateral membrane.

This study provides direct evidence to reveal the direct effects of common cytoplasmic lipids on ENaC activity, because membrane patches in inside-out recordings containing channels are physically isolated from the rest of the cell and cytoplasmic factors are absent. This evidence will be conducive in understanding the regulation mechanisms of ENaC by lipids, though many issues remain to be resolved.

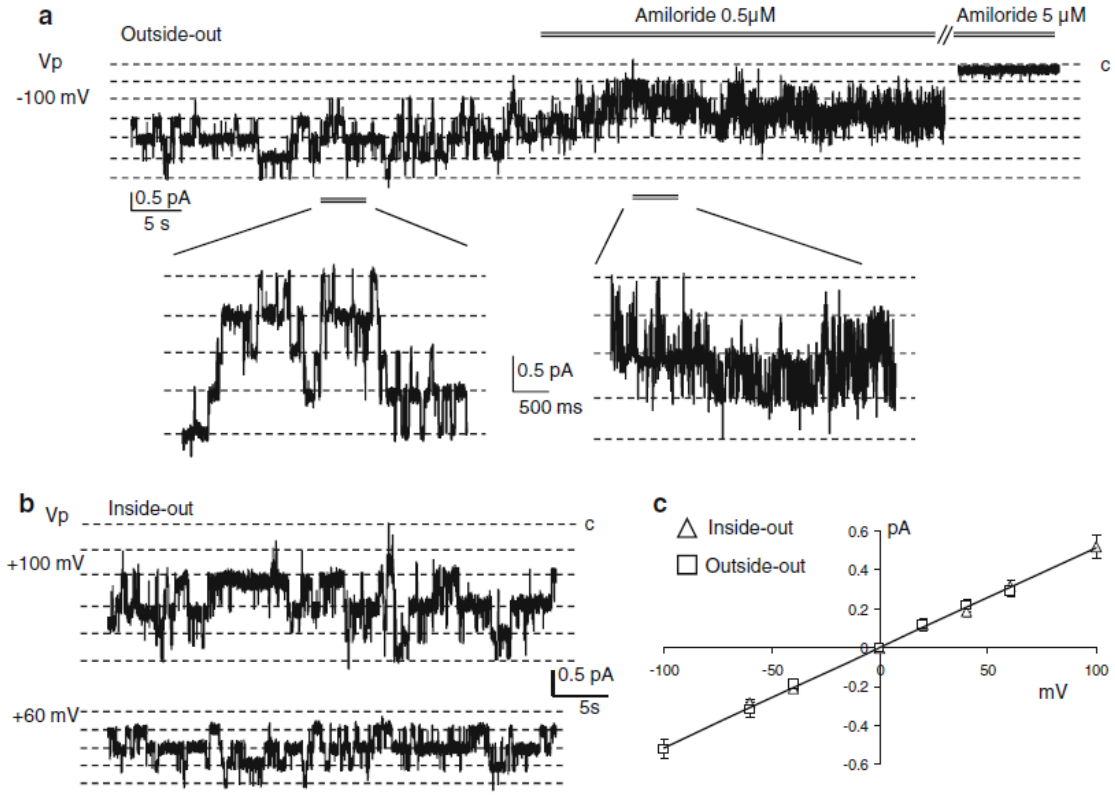
a
Outside-out
Amiloride 0.5μM
Amiloride 5 μM
Vp
-100 mV
c
0.5 pA
5 s
0.5 pA
500 ms
b
Vp
Inside-out
c
+100 mV
0.5 pA
5s
+60 mV
c
Inside-out
Outside-out
pA
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-100
-50
0
50
100
mV

Fig. 3.1

Fig. 3.1 The single channel currents of ENaC in M1 cells.

a. In an outside-out recording, the currents were monitored when the pipette voltage was held at -100 mV. The bath application of 0.5 μM amiloride significantly reduced the ENaC open probability and 5 μM amiloride almost abolished the ENaC currents. b. In an inside-out recording, single channel currents were detected when the pipette voltage was held at +100 mV and +60 mV. c. Currents corresponding to the voltages from inside-out (n=60) and outside-out recordings (n=15) were plotted. The points were fitted by a line (γ^2=0.99).

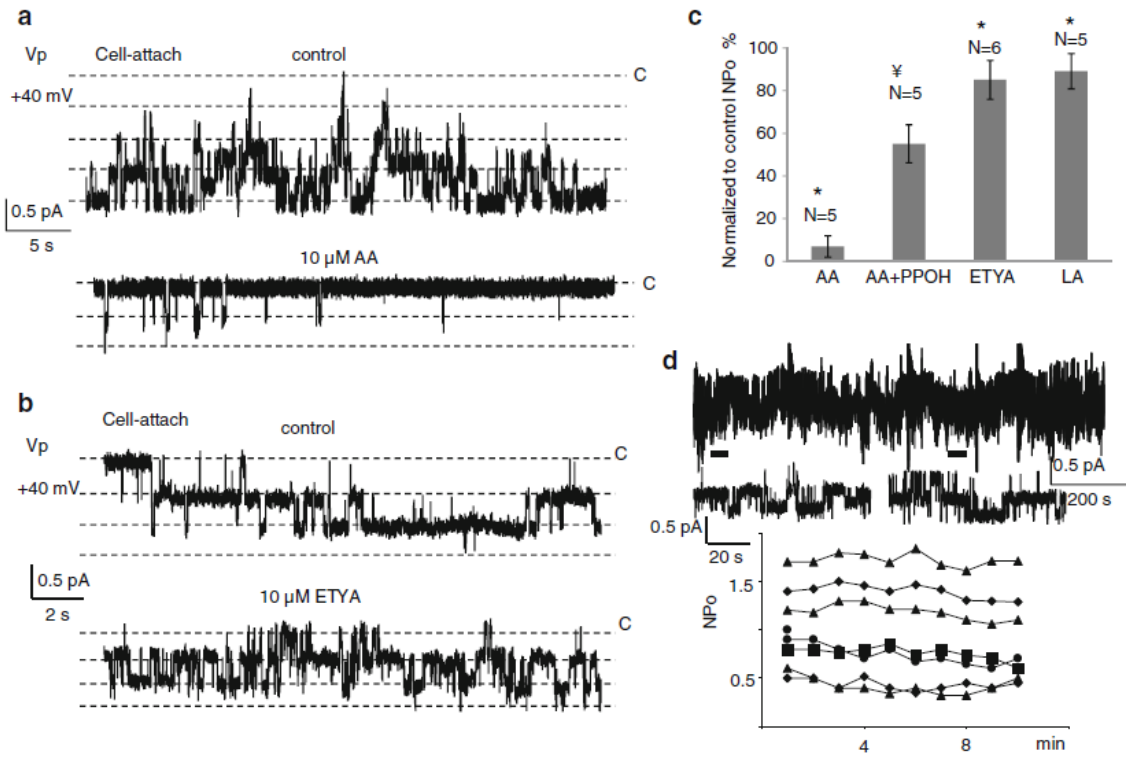
a
Vp
Cell-attach
control
+40 mV
C
0.5 pA
5 s
10 μM AA
C
b
Cell-attach
control
Vp
+40 mV
C
0.5 pA
2 s
10 μM ETYA
C
c
Normalized to control NPo %
100
80
60
40
20
0
*
N=5
¥
N=5
*
N=6
*
N=5
AA
AA+PPOH
ETYA
LA
d
0.5 pA
200 s
0.5 pA
20 s
NPo
1.5
0.5
4
8
min

Fig 3.2

Fig.3.2. The effects of PUFA on ENaC activity in cell-attached recordings.

a. In a cell-attached recording, the bath application of 10 μM AA significantly reduced the ENaC NPo. b. In a cell-attached recording, the bath application of 10 μM ETYA slightly reduced the ENaC NPo. c. The bar figure shows the effects of extracellular PUFA on ENaC NPo. d. A trace of single channel current from an inside-out recording. Summary plots of the NPo from seven individual cells along 10 minutes after the formation of the inside-out configuration.

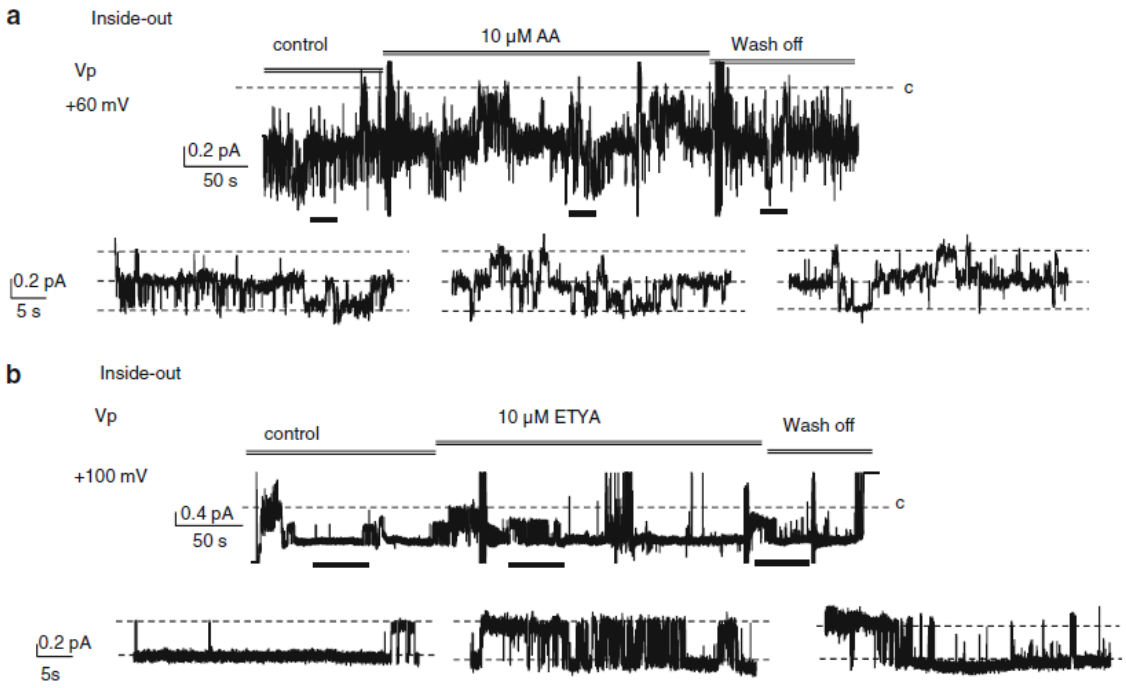
a
Inside-out
control
10 μM AA
Wash off
Vp
+60 mV
c
0.2 pA
50 s
0.2 pA
5 s
b
Inside-out
Vp
control
10 μM ETYA
Wash off
+100 mV
0.4 pA
50 s
c
0.2 pA
5s

Fig 3.3

Fig.3.3. The effects of AA and ETYA on ENaC activity in inside-out recordings.

a. In an inside-out recording, the bath application of 10 μM AA slightly reduced the ENaC open probability and this effect of AA was reversed when AA was washed away. b. In an inside-out recording, the bath application of 10 μM ETYA slightly reduced the ENaC open probability and this effect was reversed when ETYA was washed away.

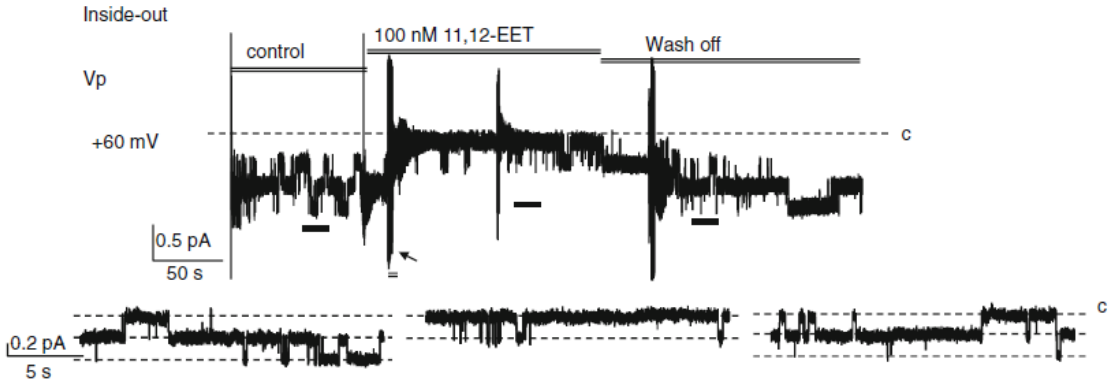
Inside-out
control
100 nM 11,12-EET
Wash off
Vp
+60 mV
c
0.5 pA
50 s
c
0.2 pA
5 s

Fig.3.4

Fig.3.4. The effects of 11, 12-EET from the cytoplasmic side on ENaC activity.

In an inside-out recording, 11, 12-EET at 100 nM significantly reduced the ENaC open probabilities. This effect was reversed when the 11, 12-EET was washed away. The arrow bar indicates the noise induced by the fluid turbulence in the recording bath.

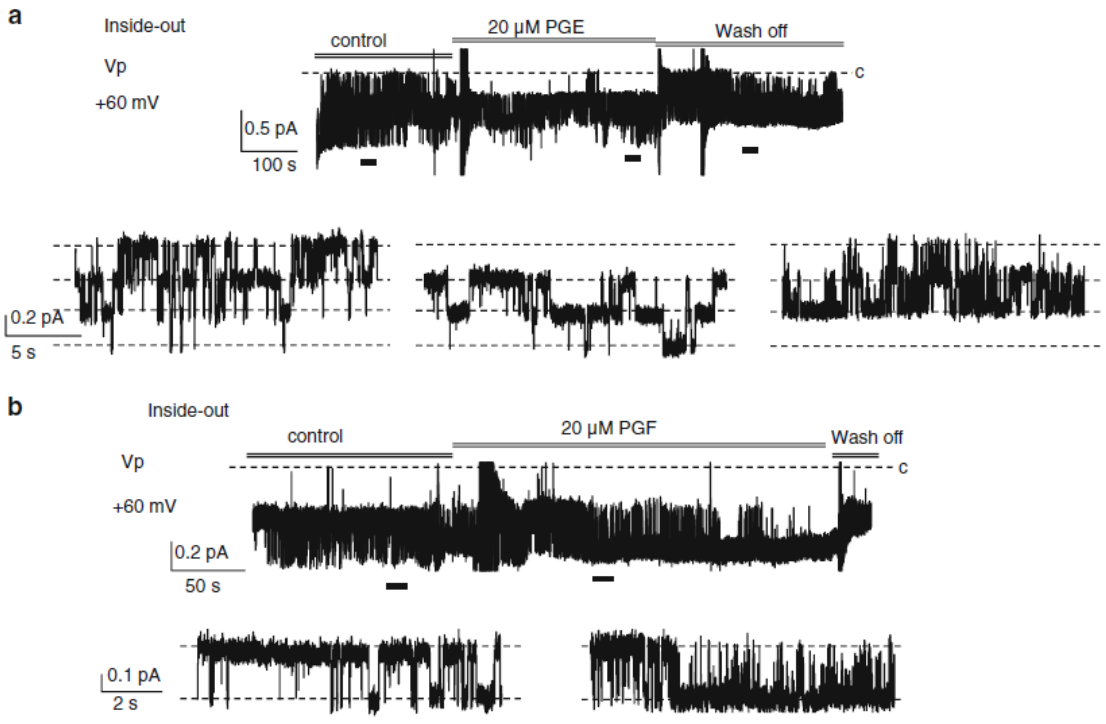
a
Inside-out
Vp
+60 mV
control
20 µM PGE
Wash off
c
0.5 pA
100 s
0.2 pA
5 s
b
Inside-out
control
20 µM PGF
Wash off
Vp
+60 mV
c
0.2 pA
50 s
0.1 pA
2 s

Fig 3.5

Fig.3.5. The effects of PGE2 and PGF2α from the cytoplasmic side on ENaC activity.

a. In an inside-out recording, the bath application of 20 μM PGE2 enhanced the ENaC open probability. b. In an inside-out recording, 20 μM PGF2α slightly enhanced the ENaC open probability. This effect was reversed when PGE2 was washed away.

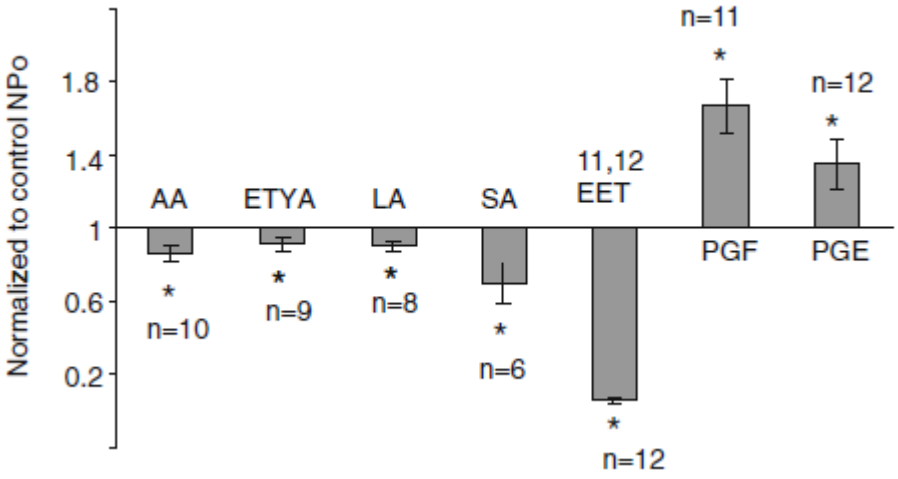
Normalized to control NPo
1.8
1.4
1
0.6
0.2
AA
ETYA
LA
SA
11,12
EET
PGF
PGE
*
n=10
*
n=9
*
n=8
*
n=6
*
n=12
n=11
*
n=12
*

Fig 3.6

Fig.3.6. The effects of lipids from the cytoplasmic side on ENaC activity.

This figure shows the analyzed results about the effects of the lipids on ENaC NPo. Data were normalized to the control.

Chapter 4

An oxygen-sensitive mechanism regulating of epithelial sodium channels (ENaCs)

4.1 Introduction

In hypoxic conditions, such as following haemorrhage or in other circumstances in which effective circulating volume is decreased, the metabolic activity of many tissues is reduced. The kidneys, however, must restore the effective circulating volume, and hence have an *increased* oxygen requirement in order to increase Na^+/K^+ ATPase activity. This would appear to be a recipe for renal failure. A number of protective mechanisms enable the kidney to increase Na^+/K^+ ATPase activity, but limit the extent of the increase. Such mechanisms include increased PGE_2 synthesis, and increased production of other arachidonate metabolites (Lote and Haylor 1989; Breyer and Breyer 2000). Alternatively, controlling the availability of Na^+ at Na^+/K^+ ATPase sites in the distal nephron epithelial cells could provide the required regulation.

Epithelial sodium channels (ENaCs), which are expressed primarily in the apical membranes of epithelia lining the distal nephron, lung airway, alveoli, and descending colon, mediate Na^+ entry into the epithelial cell. These channels control Na^+ absorption and play a critical role in maintenance of Na^+ homeostasis, blood pressure and airway fluid volume. O_2-sensitive regulation of ENaCs is thus potentially a key mechanism for regulation of epithelial Na^+-transport during hypoxia. It has long been recognized that hypoxia may affect Na^+ absorption in the distal nephron and lung epithelia (Cohen, Merkens et al. 1980; Durand, Durand-Arczynska et al. 1986; Durand, Durand-Arczynska et al. 1988; Planes, Blot-Chabaud et al. 2002; Thome, Davis et al. 2003; Jain and

Sznajder 2005) and that O_2 -dependent epithelial Na^+ transport requires the presence of haem protein (Rafii, Coutinho et al. 2000). However, the mechanism underlying these important observations remains unclear.

We employed the excised, inside-out configuration of the patch-clamp method to examine the significance of haem protein activity of ENaCs from mouse cortical collecting duct cells (M1) in which α, β and γ ENaC subunits are expressed (Chalfant, Peterson-Yantorno et al. 1996; Nakhoul, Hering-Smith et al. 1998). In this configuration the channels are physically isolated from the rest of the cell and cytoplasmic factors are absent. We report that ENaC activity is sensitive to haem at physiological concentrations and that metabolism of haem by hemeoxygenase functions as an O_2-sensitive mechanism for the regulation of ENaC activity.

4.2 Specified materials and methods

4.2.1 Single channel patch clamp recording

Single channel recordings were performed as previously described. Briefly, a coverslip or insert on which M1 grown was transferred into a recording chamber mounted on a Nikon inverted microscope (Nikon TE 2000U). A Patch pipette with the resistance of 6 MΩ was fabricated from a borosilicate glass capillary (1.5 mm od, 0.86 mm id) (Sutter) on a Sutter Puller (P97). Bath solutions contained (in mM): 110 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPEs, 5 Na-HEPEs, pH 7.2. Pipette solution contained (in mM): 110 NaCl, 4.5

KCl, 0.1 EGTA, 5 HEPEs, 5 Na-HEPEs, pH 7.2. Single channel currents were recorded with an Axon 1D amplifier and Axon clampex 9.0. The data were acquired at 20 KHz and low pass filtered at 5 kHz. During post analysis, data were further filtered at 200 Hz. Single channel events were listed and analyzed by pclampfit 9.0 (single channel search in analyze function). 50% threshold cross method was utilized to determine valid channel openings. When multiple channel events were observed in a patch the total number of functional channels (N) in the patch was determined by observing the number of peaks detected on all point amplitude histograms. NPo, the product of the number of channels and the open probability calculated as described previously (Yue, Malik et al. 2002), or the open probability (Po) itself, was used to measure the channel activity within a patch. Initial, 3-4 minute, single channel records were normally used as the control. The activity of ENaC during application of chemicals was normalised to activity during the control period to assess the effects of chemicals on ENaC activity. In some cases ENaC activity during application of chemicals was also compared to that of ENaC when chemicals were washed off. These data were used to confirm the effects of chemicals. Data are presented as means ± s.e.m. and statistical differences were compared using Student's paired *t* test, taking $P < 0.05$ as significant and represented as *.

4.2.2 Chemicals and stock solutions

Haem, PP9, MP11, $FeSO_4$, NADPH and ZnPP9 were from Sigma-Aldrich. SnPP was from

Tocris. Biliverdin was from Frontier Scientific. Stock solutions (1 mM) of these reagents in 10 mM NaOH were made weekly and kept at -20 °C in aliquots. All the solutions were thawed and immediately before use. [ruthenium$(CO)_3Cl_2]_2$ was freshly dissolved in DMSO on the day of experiments. Ruthenium (III) chloride hydrate and ruthenium $(DMSO)_4Cl_2$ were also freshly prepared accordingly. The medium containing certain concentration of CO donor is made of 30 minutes ahead of experiments. Partial pressures of medium O_2 were measured and monitored by O_2 meter (WPI ISO2). Hypoxic medium was obtained by N_2 bubbling.

4.2.3 Statistics

Data are displayed as the mean ± s.e.m. Paired t-test was used to compare channel activity before (control) and after application of drugs. Effects of different treatments were assessed by normalising activity (to pre-treatment levels) and comparing groups using one-way ANOVA (Orgin pro 7.5) and multiple comparisons. Dosage curve was fitted using Origin pro 7.5 (sigmoid fitting) according to equation Y=minimum+(maximum-minimum)/(1+(X/X0)^p).

4.3 Results

Currents mediated by ENaCs were identified by their unique conductance (≈4.9 pS in both inside-out and outside-out recordings) (1, 7, 8) and their sensitivity to 1 μM amiloride (Gu 2008). Application of haem (Fe^{3+} protoporphyrin IX chloride) at

nanomolar concentrations to the intracellular side of the channels (inside-out recordings) consistently and significantly reduced the open probability (NPo) of ENaC (figure 4.1a). Inhibition of ENaC by haem did not require any exogenously added enzyme or cofactor. Half-maximal inhibitory concentration (IC_{50}) was 23.3 nM and IC_{80} was 122 nM (fig 4.1b), considerably less than the equivalent inhibitory concentrations on BK channels (Tang, Xu et al. 2003). No change of unitary current amplitude was observed. The inhibitory effect of haem occurred immediately and outlasted its period of application. Upon washout of haem, the reversal of inhibition was slow and sometimes partial (figure 4.1a), indicating there is a stable physical interaction between haem and its binding site. The inhibitory effect on ENaC was specific to free haem. $FeSO_4$, haem precursor protoporphyrin IX (PP9) and the microperoxidase MP11 did not cause inhibition (figure 4.1c). Tin protoporphyrin IX (SnPP) caused a small but significant decrease in NPo of ENaC (figure 4.1c). Application of haem (up to 100 nM) to the extracellular face of the membrane (outside-out recordings) did not inhibit ENaC activity (figure 4.1c).

NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate ($NADP^+$), provides the reducing equivalents for biosynthetic reactions and for oxidation-reduction. Application of 1μM NADPH alone had no effect on activity of ENaC. However, when haem was co-applied with NADPH (1 μM), the inhibitory effect of haem was reversed. Upon application of 1 nM haem with NADPH NPo was increased to 149 ± 27% ($P<0.001$) of that under control conditions (figure 4.2), unitary conductance remaining unchanged.

Modulation of ENaC activity by combined application of haem and NADPH was gradual. An increase in channel activity became apparent only 10-20 s after stimulation and recovery of activity to control levels after washout was similarly slow.

In the presence of O_2 and NADPH, hemeoxygenase converts haem to biliverdin, iron and CO (Williams, Wootton et al. 2004). To examine whether the effects of haem and NADPH might be due to activity of hemeoxygenase and consequent generation of CO, we investigated the effects on activity of ENaC of a CO donor ([ruthenium$(CO)_3Cl_2]_2$). Upon application of the CO donor (100 nM), NPo was reversibly enhanced under both normoxic (pO_2 ≈ 160 mm Hg) and hypoxic conditions (pO_2 ≈ 15 mmHg) (figure 4.3). The increase in activity of ENaC occurred rapidly upon application of CO, developed progressively and reversed slowly upon washout, indicating a stable physical interaction between CO and its binding site. When the concentration of the CO donor exceeded 20 µM the channel was held permanently open, an effect that was not reversed by washing. Application of the control compounds ruthenium (III) chloride hydrate (5 µM) (Cudmore, Ahmad et al. 2007) or ruthenium $(DMSO)_4Cl_2$ (10 µM) failed to evoke a significant enhancement of NPo.

Modulation of the activity of ENaC by CO resembled that seen upon co-application of haem and NADPH, suggesting that the stimulatory effect of these agents might indeed reflect the generation of CO by hemeoxygenase. Further to investigate this, we examined the effects of co-application of haem (1 nM) and NADPH (1 µM) on the

activity of ENaC channels in patches maintained under hypoxic conditions (PO_2≈15 mm Hg), which will inhibit action of hemeoxygenase. In hypoxic medium, haem and NADPH induced a depression on ENaC activity resembling that seen upon application of haem alone (figure 4.4). This inhibitory effect was reversed to an enhancement of NPo (in the same patch) when oxygen tension was raised to normoxic levels, permitting activity of hemeoxygenase. Hypoxia alone had no effect on ENaC activity (figure 4.4a). Application of SnPP (50 nM; a blocker of hemeoxygenase) to patches containing ENaC channels caused only ≈ 8.7% inhibition of activity. However, when SnPP was present, application of 10 nM haem plus 1 μM NADPH exerted a significant inhibition on ENaC (resembling that seen with haem alone) even under normoxic conditions (figure 4.4c). Biliverdin (100 nM), which is also generated by activity of hemeoxygenase, did not affect ENaC activity (figure 4.4b).

If the increase in NPo that occurs upon co-application of haem and NADPH under normoxic conditions reflects metabolism of haem by hemeoxygenase, application of a high concentration of haem may exceed the catalytic ability of the enzyme. Fig5 shows that this is indeed the case. Co-application of 1μM NADPH and 50 nM haem resulted in a 39%, reduction of NPo. The minimum concentration of haem necessary to inhibit channel activity under these conditions was ≈40 nM (figure 4.5b). These findings suggest that haem (a substrate of hemeoxygenase) and CO (a product of hemeoxygenase) act competitively in their effects on ENaC.

4.4 Discussion

We have shown that ENaCs in M1 mouse cortical collecting duct cells were inhibited by application of nanomolar concentrations of haem to the cytoplasmic surface of the membrane. Previous studies on the usage of haem have employed 5 – 30 μM haem in extracellular medium (Kaasik and Lee 2004; Rajagopal, Rao et al. 2008) and 1 – 200 nM haem in cytoplasmic medium (Tang, Xu et al. 2003; Williams, Wootton et al. 2004; Jaggar, Li et al. 2005) to interact with ion channels. The half-maximal inhibitory concentration (IC_{50}) of 23.3 nM reported here is consistent with these values and comparable to the dissociation constant values of other haemoproteins and haem-binding proteins (Taketani, Adachi et al. 1998; Hirotsu, Abe et al. 1999; Ponka 1999; Tang, Xu et al. 2003). Thus inhibition of ENaC activity by haem will occur under conditions similar to those for other haem-protein interactions. Interestingly, SnPP also caused a much smaller, but nevertheless significant decrease in ENaC NPo (fig 1c), suggesting that, for haem's inhibitory effect on ENaC, other metals may be able partially to substitute for iron.

Exact concentrations of haem in the cytoplasm are not known but the evidence suggests that cytoplasmic haem reaches levels more than sufficient to elicit the effects reported here. Haem is synthesized on the matrix side of the inner mitochondrial membrane (Rajagopal, Rao et al. 2008) and is exported to distinct intracellular compartments (Hamza 2006), most being used for the formation of hemoproteins

including haemoglobin, myoglobin, cytochromes and nitric oxide synthase (Ponka 1999). Haem is also liberated after cellular injury (hemolysis, trauma, and crush syndrome), hypoxia and/or stress (Dore 2002), leading to an elevation of cellular free haem that can be transported across the plasma membrane (Goldman, Beck et al. 1998; Worthington, Cohn et al. 2001). Intracellular haem may increase 10-fold when 30 μM haem is present in the extracellular medium (Kirschner-Zilber, Laufer et al. 1989). Reported values for total cellular haem vary considerably. For spleen cells the reported value is 13 nmol/mg protein (equivalent to ≈163 μM) whereas in olfactory receptor neurons approximately 1.5 nmol/mg protein (≈18.8 μM) (Ingi, Chiang et al. 1996) was reported and in K562 cells the content was as low as 0.17 nmol/mg protein (≈2.1 μM) (Kirschner-Zilber, Laufer et al. 1989; Atamna and Frey 2004). Levels of free haem in the cell are controlled by a fine balance of biosynthesis and enzymatic catabolism (by hemeoxygenase) and are likely to be below micromolar (Kim and Dore 2005), consistent with the effective concentration reported here.

Haem present in the cell might therefore be expected tonically to inhibit ENaC activity, as occurred in inside out patches (fig 1). However, when haem was co-applied with NADPH (permitting activity of hemeoxygenase) the effect on NPo was dependent upon oxygen tension. Activity of ENaC was stimulated under normoxic conditions (when hemeoxygenase would be active) but was inhibited under conditions of hypoxia. Furthermore, application of CO, a product of hemoxygenase activity, stimulated ENaC activity, mimicking the effects of haem when co-applied with NADPH under normoxic

conditions. It has been proposed that hemeoxygenase-2 functions as an oxygen sensor in the carotid body, regulating activity of high conductance Ca^{2+} activated K^+ (BK) channels (Williams, Wootton et al. 2004). We conclude that modulation of hemoxygenase by O_2 functions similarly, providing a novel, sensitive mechanism for regulation of ENaC.

An important question is whether hemeoxygenase and ENaC are sufficiently closely associated in vivo for the interaction described here to occur *in vivo*. ENaCs are known to co-localize with caveolin-1, a protein found in lipid rafts and caveolae, membrane pits of 60-80 nm diameter (Dupree, Parton et al. 1993; Parton and Simons 2007). Recent findings have shown that ENaCs are concentrated in lipid rafts in A6 cells (Hill, An et al. 2002) and mouse cortical collecting duct cells (Hill, Butterworth et al. 2007) and disruption of caveolae will reduce abundance of ENaC by >70%. Indeed the inducible form of hemeoxygenase (hemeoxygenase-1) has been shown to be present in plasma membrane caveolae and physically to interact with caveolin-1 (Kim, Wang et al. 2004). It is therefore probable that ENaC and hemeoxygenase are both present in caveolae and closely associated with caveolin. Thus there seems little doubt that close associated between these proteins occurs, sufficient to permit O_2 sensitive regulation of ENaC by hemeoxygenase as described here.

With regard to the site on ENaC through which effects of haem are exerted, the molecular structure of ENaCs contains two transmembrane domains and a large

extracellular loop with repeated cysteine and glycosylation sites (Benos 2004). Haem inhibits ENaC when it is applied to the cytoplasmic membrane face, suggesting that interaction with the channel involves the N- and/or C- termini. Histidine, methionine and cysteine residues are known to provide sites of interaction with haem iron. Previous work on BK (Slo) channels has demonstrated a conserved haem binding motif containing the amino acid sequence CXXCH (where X represents any amino acid) located between the two putative 'regulator of K^+ conductance' domains (RCK) (Tang, Xu et al. 2003). However, this motif is not present in any subunits of ENaC so binding must involve a previously unknown haem-binding domain or possibly a site formed by the allosteric structure of the three ENaC subunits. In addition, other proteins (such as caveolin-1 and hemeoxygenase) which are spatially close to ENaC may provide a platform for haem binding which results in modification of ENaC gating. Similarly, haem may interact with the β and γ subunits of ENaCs in M1 cells which are not pore-forming but exert regulatory effects (Canessa, Schild et al. 1994). Further systematic investigation by will require investigation of different combinations of ENaC subunits combined with truncation, replacement or mutation of regions within the subunits.

Regulation of renal ENaC channels by O_2-hemeoxygenase, as described here, may play a significant role in a number of processes. It is consistent with previous reports (Vivona, Matthay et al. 2001; Carpenter, Schomberg et al. 2003) that hypoxia reduces epithelial Na^+ transport to compensate reduced Na^+/K^+ATPase during metabolic stress and it may also underlie the role of hemeoxygenase in ameliorating renal injury during ischemia

followed by reperfusion of the rat kidney (Akagi, Takahashi et al. 2005). Furthermore, increased expression of hemeoxygenase provides a mechanism that modulates inflammation and promotes wound closure (Patil, Bellner et al. 2008). ENaCs are also of immense importance in functioning of the mammalian lung. Malfunction or impaired expression of ENaCs is implicated in a number of important and common pathologies including pulmonary edema (Jain and Sznajder 2005; Factor, Mutlu et al. 2007). Control of ENaC in the alveolar epithelium is particularly intriguing. Na^+ flux through ENaC is crucial for clearance of alveolar edema (Factor, Mutlu et al. 2007) yet, apparently paradoxically, activity of channel is reduced under hypoxic conditions (Jain and Sznajder 2005) . However, inhibition of ENaC under hypoxic conditions, leading to an increase in airway surface liquid may be beneficial for clearance of mucus (Taguchi, Niisato et al. 2005; Mall 2008). Thus activity of ENaC, a channel pivotal to lung function, may be 'regulated' by PO_2 and hemeoxygenase, a process that may be crucial to survival of lung injury or infection. In addition to the direct regulation of ENaC through haem and haem metabolism described here, other less direct effects may occur, such as activation soluble guanylate cyclase by CO (Rodgers 1999), regulating ENaC cycling and activity. More fundamental effects may also occur through effects of haem on processing of micro RNAs. Such effects can regulate expression of a large number of protein-coding genes (Faller, Matsunaga et al. 2007) and could modulate transcription of ENaC.

In summary, I have shown a novel mechanism for regulation of ENaC by which the

O_2-dependent activity of hemeoxygenase and the generation of CO controls channel activity. This novel mechanism provides a mechanistic explanation for previous observations and sheds new light on the role(s) and functioning of ENaCs, particularly in fluid clearance and functioning of renal and lung epithelium.

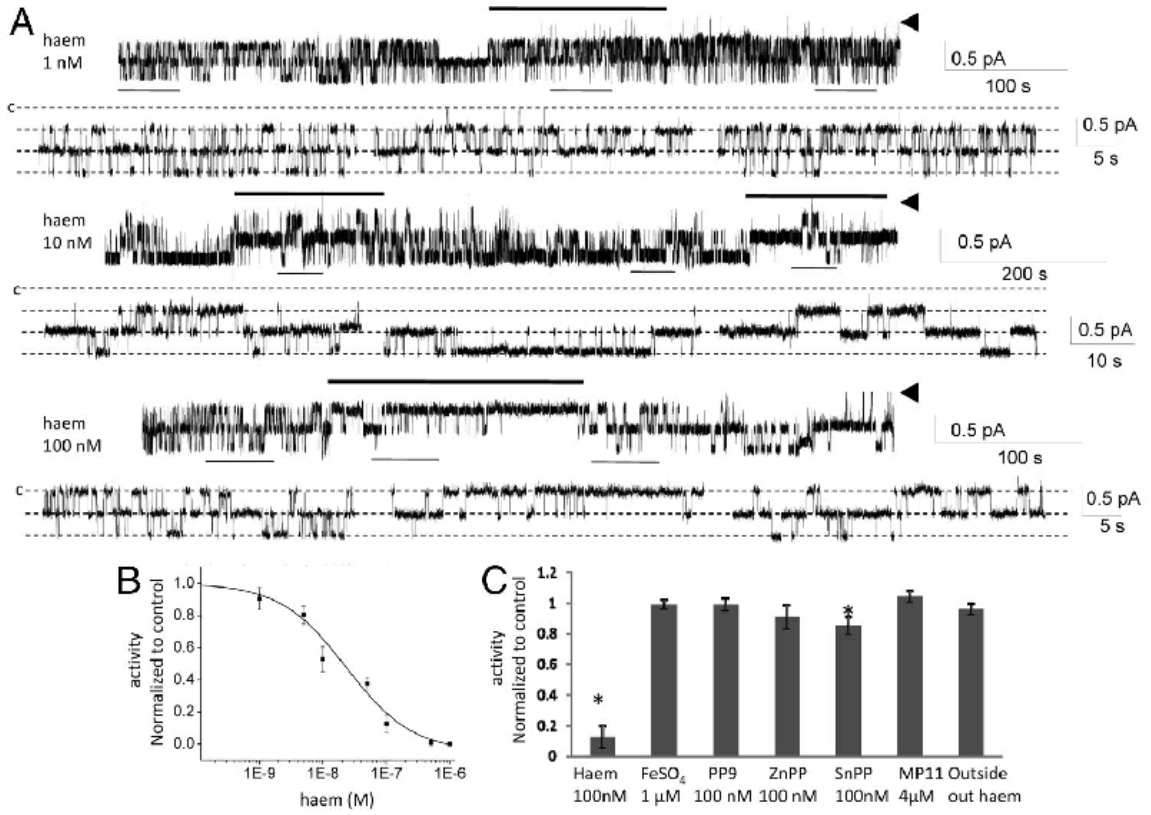
A
haem
1 nM
0.5 pA
100 s
c
0.5 pA
5 s
haem
10 nM
0.5 pA
200 s
c
0.5 pA
10 s
haem
100 nM
0.5 pA
100 s
c
0.5 pA
5 s
B
activity
Normalized to control
1.0
0.8
0.6
0.4
0.2
0.0
1E-9
1E-8
1E-7
1E-6
haem (M)
C
activity
Normalized to control
1.2
1
0.8
0.6
0.4
0.2
0
Haem 100nM
FeSO4 1 µM
PP9 100 nM
ZnPP 100 nM
SnPP 100nM
MP11 4µM
Outside out haem

Fig 4.1

Fig.4.1. Haem inhibits the opening of ENaC.

a: Inhibitory effect of 1 nM (upper panel), 10 nM (middle panel) and 100 mM (lower panel) haem on ENaC open probability (NPo). Traces were obtained in inside out configuration with a pipette voltage of +80 mV. Application of haem is shown by the bold bar above the upper current trace in each panel. Selected parts of currents (marked by bars below the upper trace) are shown with an expanded time scale in the lower trace of each panel. Arrowhead and 'c' besides the current traces indicate the closed state of ENaC currents. b: Concentration dependent inhibition by haem of ENaC activity. Each point was averaged from 6-8 cells. Data were fitted with a sigmoidal dose-effect curve based on the equation: Y=minimum+(maximum-minimum)/(1+(X/X0)^p). IC_{50} = 23.3 nM and IC_{80} = 122 nM. Hill coefficient is 0.998. c: Effects of haem related chemicals on ENaC activity. PP9 represents protoporphyrin IX. Both PP9 and Zn PP9 had no effect on ENaC activity. Asterisk, $P<0.05$ versus control. Each column shows the mean (±SEM) of 5-6 cells, except ZnPP (9 cells) Asterisk indicates $p<0.05$ compared to paired control (before application of drug). Application of one-way ANOVA showed that the haem group and SnPP group were significant different from other groups($P<0.05$) and haem group was different from SnPP group ($P<0.05$) stabilize

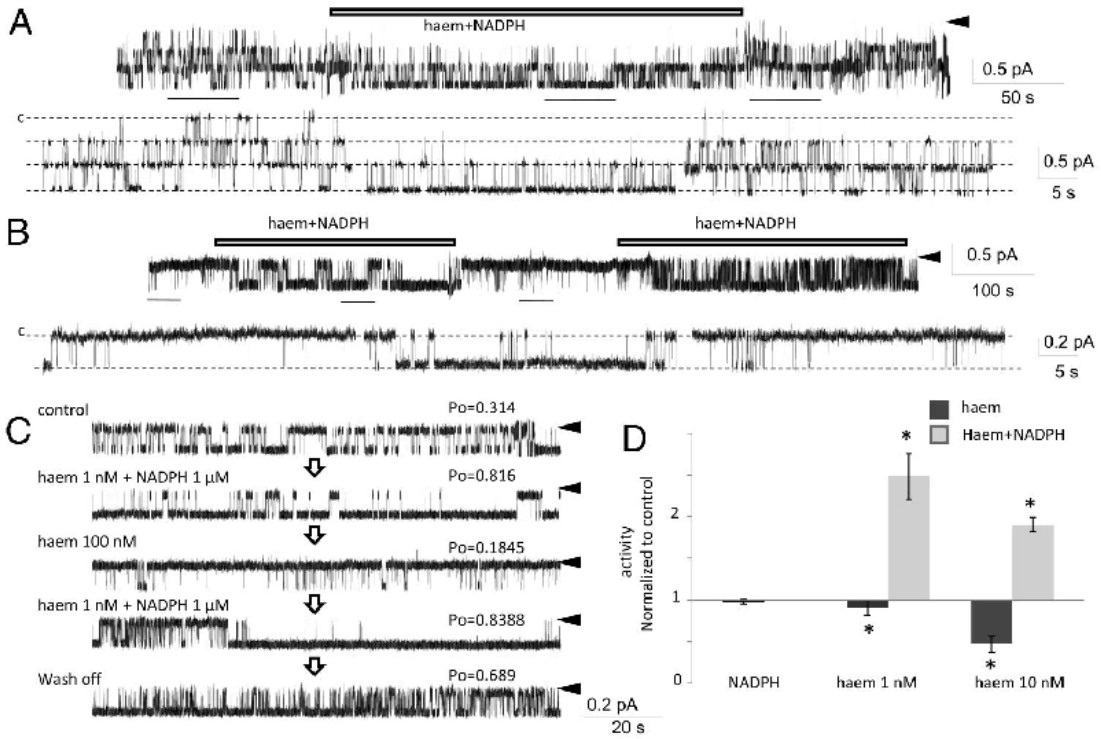
A
haem+NADPH
0.5 pA
50 s
c
0.5 pA
5 s
B
haem+NADPH
haem+NADPH
0.5 pA
100 s
c
0.2 pA
5 s
C
control
Po=0.314
haem 1 nM + NADPH 1 µM
Po=0.816
haem 100 nM
Po=0.1845
haem 1 nM + NADPH 1 µM
Po=0.8388
Wash off
Po=0.689
0.2 pA
20 s
D
haem
Haem+NADPH
activity
Normalized to control
2
1
0
NADPH
haem 1 nM
haem 10 nM

Fig 4.2

Fig.4.2. Haem and NADPH augment ENaC activity under normoxia (pO_2 ≈ 160 mmHg).

The augmented effect of haem and NADPH on ENaC was observed both with an inherent high (a) and low (b) ENaC activity. In both a: and b: application of haem+NADPH is shown by the bar above the upper current trace and selected parts of currents (marked by bars below the upper trace) are shown with an expanded time scale in the lower trace. c: The stimulatory effect of haem+NADPH and the inhibitory effect of haem alone during prolonged recording from a single patch. The 5 displayed traces were selected from 1 recording in the sequence shown by the arrows. d. summary of effects of haem and NADPH. Each column represented the mean (±SEM) of 5-6 cells. Asterisk indicates $p<0.05$ compared to paired control (before application of drug). Application of one-way ANOVA and cross-group statistical comparison showed that there was a significant difference between the effects of 1 nM and 10 nM haem, both in the presence and absence of NADPH.

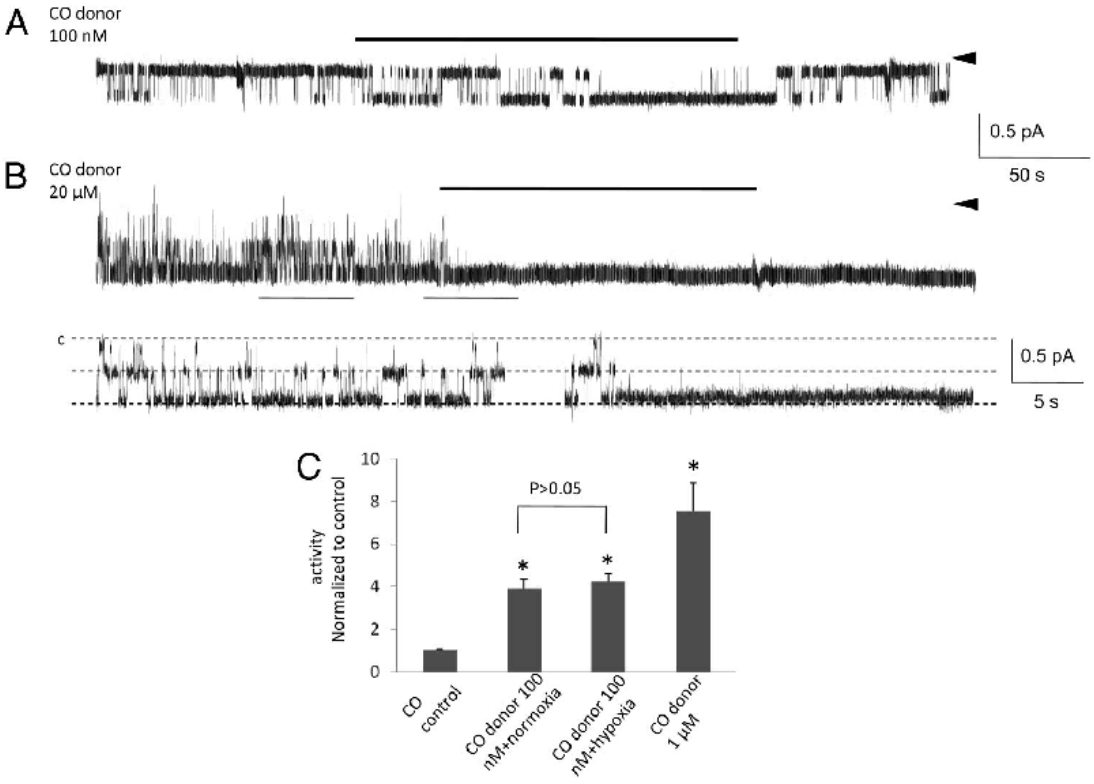
A
CO donor
100 nM
0.5 pA
50 s
B
CO donor
20 µM
c
0.5 pA
5 s
C
activity
Normalized to control
10
8
6
4
2
0
P>0.05
*
*
*
CO control
CO donor 100 nM+normoxia
CO donor 100 nM+hypoxia
CO donor 1 µM

Fig.4.3

Fig.4.3. Stimulation of ENaC activity by CO.

a: 100 nM CO donor significantly enhances ENaC activity. b: 20 µM CO donor holds ENaC constantly open. c: CO donor significantly enhances ENaC activity under both hypoxic and normoxic conditions. Each column shows mean (±SEM) of 6-8 cells. Asterisk indicates $p<0.05$ compared to paired control (before application of drug). Application of one-way ANOVA and cross-group statistical comparison showed that there were significant differences ($P<0.05$) between the CO control group, the 100 nM CO donor groups (which were not significantly different from each other) and the 1µM CO donor group.

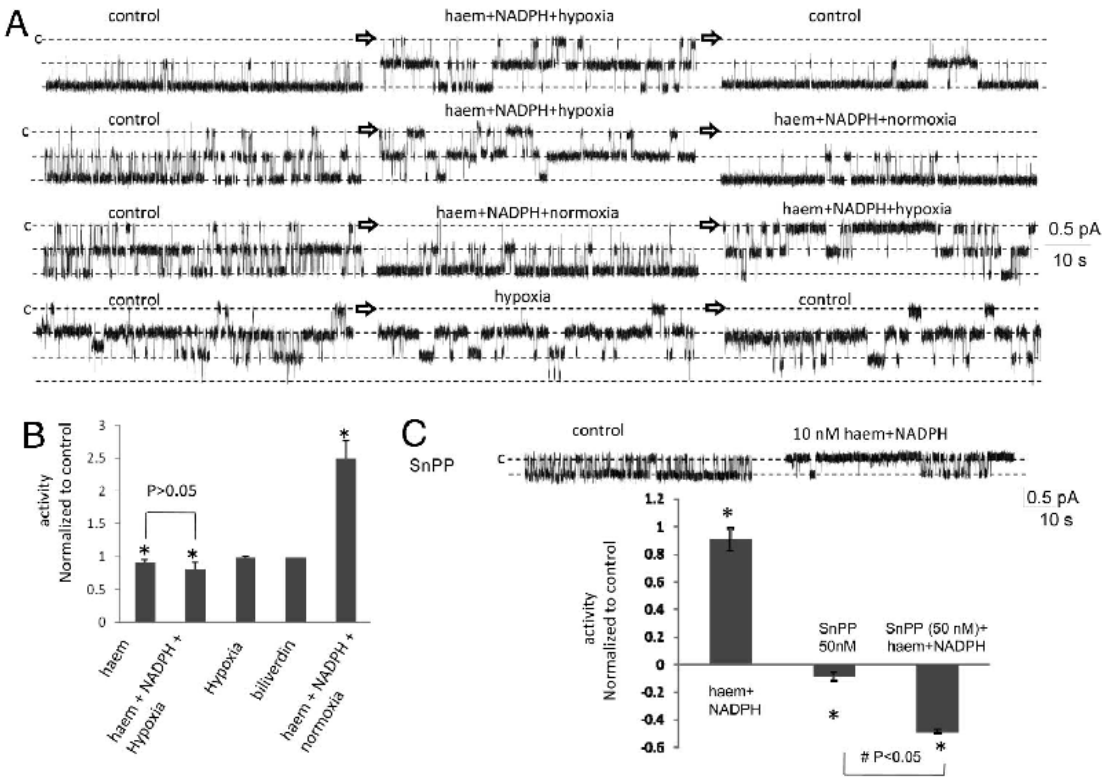
A
control
haem+NADPH+hypoxia
control
haem+NADPH+normoxia
haem+NADPH+hypoxia
hypoxia
0.5 pA
10 s
B
activity
Normalized to control
P>0.05
haem
haem + NADPH + Hypoxia
Hypoxia
biliverdin
haem + NADPH + normoxia
C
SnPP
10 nM haem+NADPH
SnPP 50nM
SnPP (50 nM)+ haem+NADPH
haem+ NADPH
# P<0.05

Fig 4.4

Fig.4.4. O_2 is required to maintain the stimulatory effect of haem (1 nM) and NADPH (1 μM).

a: Upper panel shows reversible inhibitory effect of haem and NADPH applied under hypoxic conditions (pO_2≈15 mmHg), 2nd panel shows inhibition by haem and NADPH under hypoxic conditions followed by stimulation of activity (in the same patch) when applied under normoxia (pO_2 ≈ 160 mmHg). 3rd panel shows stimulation by haem and NADPH under normoxic conditions followed by inhibition of activity (in the same patch) when applied under hypoxia. Bottom panel shows the lack of effect of hypoxia alone on ENaC activity. b: Effects of haem and NADPH on activity of ENaC. Each column shows mean (±SEM) of 5-6 cells. Asterisk indicates $p<0.05$ compared to paired control (before application of drug). Application of one-way ANOVA showed that there was a significant difference between groups ($P<0.05$). Cross-group statistical comparison showed that the haem + NADPH + normoxia group was significantly different from the other groups. There was no significant difference between effects of haem and haem with NADPH under hypoxia. c: Effect on ENaC activity of haem plus NADPH during inhibition of hemeoxygenase. Asterisk indicates $p<0.05$ compared to paired control (before application of drug). Application of one-way ANOVA showed that there was a significant difference between groups ($P<0.05$). Cross-group statistical comparison showed significant differences between all three groups. Inset shows inhibitory effect of 10 nM haem plus NADPH on ENaC activity when applied in presence of 50 nM SnPP.

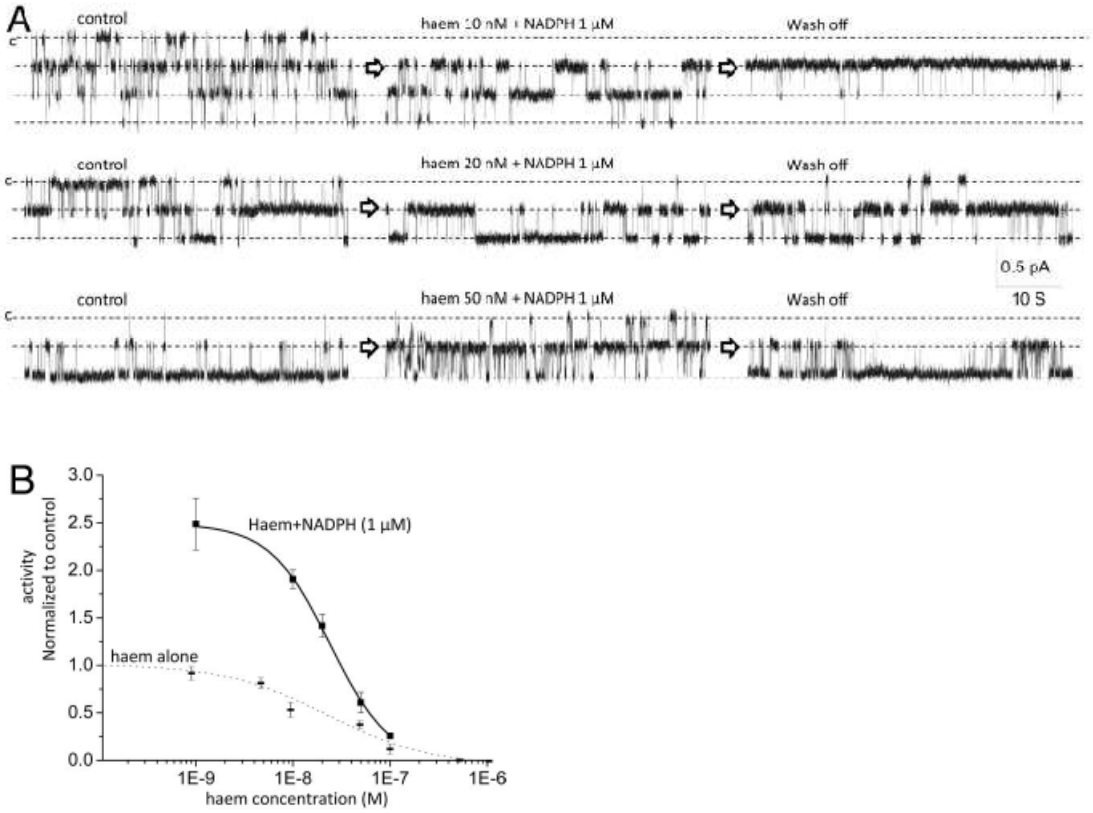
A
control
haem 10 nM + NADPH 1 µM
Wash off
control
haem 20 nM + NADPH 1 µM
Wash off
0.5 pA
control
haem 50 nM + NADPH 1 µM
Wash off
10 S
B
activity
Normalized to control
Haem+NADPH (1 µM)
haem alone
haem concentration (M)

Fig 4.5

Fig.4.5. Concentration dependent effects of haem on ENaC activity when co=applied with NADPH.

a: Effects of 10 nM (upper panel), 20 nM (middle panel) and 50 nM (lower panel) haem, co-applied with 1 M NADPH, on ENaC activity. In each panel an example trace is shown before (left) during (centre) and after (right) exposure. b: Data were fitted with a sigmoidal dose-effect curve. Hill coefficient is 1.0. based on the equation: Y=minimum+(maximum-minimum)/(1+(X/X0)^p). Each point was averaged from 6-8 cells. Dashed line represents effect of haem alone (from data shown in figure 1.). Minimum concentration of haem necessary to induce inhibition of NPo was ≈40 nM.

Chapter 5

Functional ENaC channels expressed in endothelial cells: a new candidate for mediating shear force

5.1 Introduction

The endothelium is a thin layer of cells lining the interior surface of all blood vessels, forming an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells (ECs) are constantly exposed to blood flow and are capable of sensing the blood flow pattern and shear stress. The "immediate response" of ECs to alteration of blood flow occurs in a range of seconds and is mediated by a variety of ion channels. Several types of ion channels have been suggested to mediate the initial shear force signalling events, including stretch-activated cation channels (Popp, Hoyer et al. 1992; Hoyer, Kohler et al. 1997; Yao, Kwan et al. 2002), P2X4 (Yamamoto, Korenaga et al. 2000; Sim, Chaumont et al. 2006), TRPM7 (Oancea, Wolfe et al. 2006), TRPV (Nilius, Droogmans et al. 2003), TRPC (Nilius and Droogmans 2001; Wei, Freichel et al. 2001), K^+ channels (Hoger, Ilyin et al. 2002; Brakemeier, Kersten et al. 2003; Fang, Schram et al. 2005; Chatterjee, Levitan et al. 2006; Fang, Mohler et al. 2006), Ca^{2+}-activated Cl^- channels (Maertens, Wei et al. 2000; Hisadome, Koyama et al. 2002) and volume-regulated anion channels (VRAC) (Maertens, Wei et al. 1999; Vennekens, Trouet et al. 1999). The activations of shear force-sensitive ion channels eventually lead to alterations of membrane potential with or without $[Ca^{2+}]_i$ elevation.

Epithelial Na^+ channels (ENaCs) were initially identified in the epithelium of the distal nephron, colon and lung, and are related to Na^+ uptake and trans-epithelial water movement. They contain only two trans-membrane domains and a large extracellular

region with cysteine-rich domains and glycosylation sites. The ENaC superfamily has been demonstrated to be able to sense mechanical stress (Adams, Anderson et al. 1998; Hong, Mano et al. 2000) and transduce the stress into electrical events, resulting in membrane depolarization (Snitsarev, Whiteis et al. 2002). ENaCs are comprised of three basic subunits, α, β, γ, which have tertiary structures and much amino acid sequence homology. Either the α or δ subunit of ENaCs can form the pore structure to permeate Na^+. Most of the ENaC regulators, including PIP2 (Ma and Eaton 2005), PI3K (Staruschenko, Nichols et al. 2004; Staruschenko, Patel et al. 2004; Pochynyuk, Tong et al. 2007), G-protein (Yue, Malik et al. 2002) and $[Cl^-]_i$ (Bachhuber, Konig et al. 2005), have been revealed to interact with the β and γ subunits of ENaC. Administration of amiloride and benzamil, both antagonists of ENaCs, results in blocking the myogenic constriction of blood vessels (Jernigan and Drummond 2005), suggesting a potential role of ENaCs in mediating vascular vessel tone. Changes in the plasma Na^+ concentration affect EC function and thus control vascular tone via NO (Oberleithner, Riethmuller et al. 2007). Additionally, as a stimulator of ENaCs, aldosterone has been shown to cause HUVEC swelling (Oberleithner, Schneider et al. 2003) and surface insertion of ENaC in ECs (Oberleithner, Riethmuller et al. 2006; Kusche-Vihrog, Sobczak et al. 2007; Kusche-Vihrog, Sobczak et al. 2008). However, many previous efforts have failed to detect ENaC activity in ECs (Nilius and Droogmans 2001), generating questions about its function in ECs. In this study, we sought to determine whether ENaCs is functionally expressed in ECs and what mechanisms regulate ENaCs in ECs.

5.2 Materials and methods

5.2.1. ECs and artery

Human dermal microvascular endothelial cells (HMEC) and primary cultured human dermal microvascular endothelial cells (pHMEC) were kindly donated by Prof A Ahmed (University of Birmingham, UK). These cells were maintained as previously described (Ades, Candal et al. 1992). pHMECs were used within five passages. The aorta and arteries from femoral, pulmonary and tail regions were dissected from anesthetized rats or rats killed by cervical dislocation. Vessels were placed in ice cold Hanks' solution containing (in mmol/L): NaCl 137, KCl 5.4, $CaCl_2$ 0.01, NaH_2PO_4 0.34, K_2HPO_4 0.44, D-glucose 8, and HEPES 5, and were carefully prepared by removing connective tissue, fat and lateral branches under a dissecting microscope. Endothelia from aorta and pulmonary arteries were removed by air bubble flushes as described in previous reports. All blood vessels were immediately placed either in liquid nitrogen to isolate protein or at 4ºC to isolate mRNA.

5.2.2. RT-PCR and western blots

RT-PCR experiments followed standard protocols. Primers against human (Ji, Su et al. 2006) and rat ENaCs were used as previously described. A 406-bp product of human α-ENaC was amplified by the primers: 5-AAC AAA TCG GAC TGC TTC TAC-3 (sense) and 5-AGC CAC CAT CAT CCA TAA A-3 (antisense); a 440-bp product of human β-ENaC was amplified by the primers: 5-GGG ACC AAA GCA CCA AT-3 (sense) and

5-CAG ACG CAG GGA GTC ATAG-3 (antisense); a 325-bp product of human γ ENaC was amplified by the primers: 5-GCA CCG TTC GCC ACC TTC TA-3 (sense) and 5-AGG TCA CCA GCA GCT CCT CA-3 (antisense); a 456-bp product of human ζ-ENaC was amplified by the primers: 5-CGA AGC ATG GAC GGG AGA ATG-3 (sense) and 5-GGT GCC AGT GAC GCT CAA AGA-3 (antisense). Thirty cycles of 94°C for 0.5 min, 56°C for 1 min, 72°C for 1.5 min were performed, followed by a single 10-min cycle at 72°C for extension.

A 168-bp product of rat α-ENaC was amplified by the following primers: 5-TGG TAC CGC TTC CAT TAC AT-3 (sense) and 5- GTA GCA GTT CCC GTA CAT GG-3 (antisense). A 378-bp product of rat β-ENaC was amplified by the primers 5-TGG CAA CTG CTA CAT CTT CA-3 (sense) and 5- ATG TGG TCT TGG AAA CAG GA -3 (antisense). A 302-bp product of rat r-ENac was amplified by the primers 5-CGC CCT CCT CGT CTT CTC TTT C-3 (sense) and 5-TGG CCT TTC CTT TCT CGT TCT C-3 (antisense). Thirty cycles of 94ºC for 0.5 min, 57ºC for 1 min, 72ºC for 1 min were performed, followed by a single 10 min cycle at 72ºC for extension.

A 329 bp product of human CYP 2C was amplified by the following primers: 5'-GCT AAA GTC CAG GAA GAG ATT G-3' (forward) and 5'-GCT GAG AAA GGC ATG AAG TA-3' (reverse) (Fujitaka, Oguri et al. 2001). Thirty five cycles of 94ºC for 0.5 min, 51ºC for 0.5 min, 72ºC for 1 min were performed, followed by a single 10 min cycle at 72ºC for extension.

For western blotting, arteries were quickly frozen in liquid nitrogen and homogenized

with CelLytic M lysis/extraction reagent and Protease Inhibitor cocktail (Sigma). Protein concentrations were determined by the Bradford Assay method. Equal amounts of total protein were loaded into the SDS-PAGE gel wells. β-actin was used as the inner marker to calibrate the protein quantity. Samples prepared with 5x loading buffer, were separated on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes, which were rinsed with Tris-Buffered Saline Tween-20 (TBS-T) and then blocked in TBS-T containing 5% milk for 1 hour (at room temperature). The membrane was incubated with the first antibody, α-ENaC antibody (1:500, CHEMICON/1:500 Santa Cruz Biotechnology sc-21012) or eNOS antibody (1:2500, BD BIOSCIENCES, PRODUCT:610 297) overnight at 4°C, followed by washes in TBS-T and incubation with the secondary antibody (1:10000, HRP, BioRad) for 1 hour at room temperature. Membranes were washed with TBS-T and blots were detected by ECL plus.

5.2.3. Knockdown experiments

Expression of the α subunit of ENaC was decreased using validated on-Targetplus siRNA (smart pool ENaC channel, Cat No. L-006504-00) from Thermo Fisher Scientific (Dharmacon). Negative controls were employed using a negative control siRNA (non-targeting pool, Dharmacon) and vehicle alone. DharmaFectin 1(T-2001-01) transfection reagent was used for transfection. The knockdown experiments were carried out according to the manufacturer's instructions. Knockdown effects were examined by RT-PCR and western blot analysis after 24 and 48 hours.

5.2.4. Electrophysiology

Cells were seeded onto a coverslip at approximately 30% confluence. The coverslip was used within 72 hours after preparation. After a high resistance seal (over 2 GΩ) was obtained, cell-attached recording was performed immediately. Subsequent to cell-attached recording, an inside-out or outside-out configuration was formed according to the standard procedure. The membrane resistance was monitored regularly to ensure the quality of recording. The pipette was pulled by a two stage vertical puller (Narishige PP-83). The resistance of the pipette in bath medium was around 5.8 MΩ.

Currents were recorded with an Axon 1D amplifier and Axon clampex 9.0. The data was acquired at 20 kHz and filtered by a 5 kHz low pass filter. The channel events were analyzed by pclampfit 9.0 (single channel search in analyze function). Data was further filtered at 200 Hz before analysis. A 50% threshold cross method was utilized to determine valid channel openings. When multiple channel events were observed in a patch; the total number of functional channels (N) in the patch was determined by observing the number of peaks detected on all point amplitude histograms. NPo, the product of the number of channels and the open probability, or the open probability (Po) itself, was used to measure the channel activity within a patch. The NPo was calculated according to previous descriptions (Yue, Malik et al. 2002). Because the recording membrane patch usually contained multiple channels, the changes in NPo (not Po) were directly observed and compared in most cases. Due to the variance of channel open probability, the first two-three minute single channel

recording (inside out recording) in normal bath medium was normally used as the control. The open probability (NPo) of ENaC during applications of modulators was directly compared with the NPo of the control. The ratio thus obtained was employed to determine the effects of modulators on ENaC activity. The data are presented as means ± S.E.M., and the statistical differences were compared using Student's paired *t* test, taking $P < 0.05$ as significant.

Bath solutions contained (in mM): 115 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPEs, 5 Na-HEPEs, pH 7.2. The pipette solution contained (in mM): 115 NaCl, 4.5 KCl, 0.1 EGTA, 5 HEPEs, 5 Na-HEPEs, pH 7.2. In some experiments, the low Na^+ bath medium contained (in mM): KCl 120, $MgCl_2$ 1, 5 Na-HEPEs, 5 HEPEs, 0.2 EGTA, pH 7.2. Stock solutions of PPOH (Cayman), DDMS (Cayman), 11,12-EET (Biomol) and amiloride (Biomol) were made up according to the chemical data sheet. All recording solutions were made on the day of experiments. The solvent at the same dilution was tested alone in controls and had no effect.

5.2.5. Flow setup

After the outside-out configuration was obtained, the patch pipette was moved toward a perfusion tube (ID 0.5 mm) to a distance within 200 μm. A syringe pump (Harvard Apparatus, Harvard, Mass) connected through tubing to the glass pipette (ID 0.5 mm) provided fluid flow. The flow value was set to 1 ml/min, which resulted in a laminar shear force of 1.5 dynes/cm^2 in the tube according to equations described previously (Oancea, Wolfe et al. 2006). Although the shear force decayed

exponentially as fluid left the tube, we assumed the drop to be minimal at a distance of 200 μm. The single channel currents obtained by outside-out recordings were compared between static bath and flow stimulation.

5.3 Results

5.3.1. Expression of ENaC channels in cultured ECs and arteries

Desired products of RT-PCR revealed that the α-subunit of ENaCs, but not the β, γ and δ subunits, was expressed in cultured HMEC and HUVEC (figure 5.1a). Positive bands for the β, γ and δ subunits were obtained in A549 cells (data not shown). In order to elucidate whether ENaCs were also present in ECs *in situ*, several rat arteries from aorta, femoral and tail were examined with PCR primers against the α, β and γ subunits of ENaC. The positive products with expected sizes indicated that the α, β and γ subunits of ENaC (figure 5.1b) were expressed in rat blood vessels. Consistent with the PCR results, the protein of the α-subunit of ENaC was found in a variety of blood vessels e.g. aorta, pulmonary, femoral and tail (figure 5.1c), and the kidney.

Previous studies (Drummond, Gebremedhin et al. 2004; Grifoni, Gannon et al. 2006) have demonstrated that the β and γ subunits of ENaC are present in the smooth muscle of cerebral artery and are involved in smooth muscle cell migration. To determine the location of the α-subunit of ENaC, the blood vessels with or without ECs were investigated to check the protein level of α-subunit of ENaC. The protein level of eNOS was utilized as a marker to quantify the ECs in the artery segments

(figure 5.1d). The semi-quantification was performed by the intensity of targeted protein in calibration with β-actin of distinct samples. In parallel to the decrease of 32.5 ± 13.3 % of eNOS in endothelium stripped vessels (n=4) , the protein level of the α-subunit of ENaC channel also decreased by 21.1 ± 5.1%, suggesting the presence of an α-subunit of ENaC in ECs of blood vessels.

5.3.2 ENaC currents were detected in HMEC-1 and pHDMEC.

The α subunit of ENaC alone can form the pore structure and permeates amiloride sensitive Na^+ currents (Canessa, Schild et al. 1994). We utilized 120 mM Na^+ saline in both pipette and solutions, which gave symmetrical Na^+ across the membrane in the excised recordings. Prior to excised-membrane recording, cell-attached recording was performed when a tight seal was achieved. It was hard to detect ENaC currents in cell-attached recordings. Six recordings from 340 cells exhibited small conductance of channels (4.72 ± 0.52 pS as calculated from the slope between Vp= +0 mV and Vp=+40 mV). The currents exhibited flickering and short opening times. However, when excised outside-out configurations were formed after termination of cell-attached recording, an inward current with a conductance of 4.83 pS was found in 153 of 340 cells. The currents were further identified by their conductance and by their amiloride sensitivity. Figure 5.2a shows a recording of ENaC activity in an outside-out patch clamped at -100, -80 and -40 mV, respectively. The average channel slope conductance was 4.83 ± 0.29 pS (n=120) between -100 and -40 mV. In outside-out recordings, amiloride at a concentration of 0.5 or 5 μM was applied into the bath to confirm that the currents were mediated by ENaC channels. 0.5 μM

amiloride induced flickering current and reduced the open probability (Po) (figure 5.2b). Amiloride at a concentration of 5 μM almost abolished ENaC currents (n=12). Inward currents of similar conductance were also observed in inside-out recordings. IV curves obtained from either inside-out or outside-out exhibited a similar linear slope (conductance) (figure 5.2c). When 120 mM Na^+ medium was replaced with low Na^+ medium (5 mM), ENaC currents exhibited longer opening times. The I-V curve (each point represented the analysis of data from six to eight cells was linearly fit, γ^2 = 0.99) was shifted to the right (figure 5.2c), suggesting a Na^+ selectivity for this channel. Recordings were carried out in both HMEC and pHMEC.

Inconsistent with a previous study (Kelly, Lin et al. 2003), the conductance of ENaC in HMEC was around 4.8 pS. Specific knockdown experiments against the α subunit of ENaC were therefore performed to confirm these small conductance inward currents were mediated by ENaC. The knockdown effect was examined by RT-PCR and Western blot analysis. After 24 hours, mRNA of the α subunit of ENaC was significantly decreased, resulting in a negative PCR product after 20 PCR cycles, whereas a positive PCR product was shown in knockdown control cells (Fig3a). Consistent with the expression result of ENaC, protein levels of the α subunit of ENaC were significantly decreased after 48 hours (figure 5.3b). In parallel with the decrease of ENaC protein in HMEC, the probability (17 out of 200 recordings) of detecting such conductance currents (around 0.3 pA at Vp=60 mV and 0.4 pA at Vp=80 mV) in the inside-out recording from the ENaC knockdown groups (N=3) was also reduced, when compared to the control groups trasfected by the negative control siRNA (80 out of 180 recordings) (N=3).

Our data show that functional ENaCs are expressed in HMEC-1 and pHMEC. However, the significant differences in channel open probability between the cell-attached and excised membrane recordings suggest that membrane ENaCs in intact cells are strongly inhibited by intracellular molecules.

5.3.3 ENaC currents were inhibited by 11, 12-epoxyeicosatrienoic acid (EET)

Low ENaC activity was observed in cell-attached recordings, but high ENaC activity was detected in excised membranes, suggesting the presence of cellular inhibition of ENaC. EET was proposed as one of the candidate inhibitors, because of the predominant expression of CYP in blood vessels (Elbekai and El-Kadi 2006; Fleming and Busse 2006). To determine the effect of EET on ENaC currents in endothelial cells, 11,12-EET at the concentration of 200 nM was applied to the bath, after either the inside-out or outside-out configurations were formed. Figure 4 demonstrates that 11,12-EET almost abolished all ENaC activity and reduced NPo from 0.67 ± 0.12 to 0.08 ± 0.05 (n=4). This inhibition was reversed when EET was washed off. It was apparent that EET, as the product of cytochrome P450 (CYP) –epoxygenase, could effectively inhibit ENaC.

MMS-PPOH (15 μM), an antagonist of CYP4502C, was incubated with cells for 12 hours prior to experiments. Sixty cells were examined. ENaC currents were detected in 21 of 60 excised patch recordings and in six cells of 60 cells by cell-attached recordings. The rate of detected ENaC currents in MMS-PPOH treated cells (6 out of 21recordings) significantly increased, compared to the control (6 out of

153 recordings) by two-tailed binomial test ($p=0.013<0.05$). However, treatment with DDMS (3 out 90 recordings) did not exert significant effect ($p>0.05$). The expression of CYP450 2C in pHMEC and HMEC was shown by positive RT-PCR products (figure 5.4c). The results also suggested the unknown cellular molecular in addition to 11,12-EET exerted strong inhibition on ENaCs.

5.3.4 ENaC activities enhanced by flow

To assess the effect of shear force on ENaC activity, the outside-out recording was employed as a standard protocol. In the outside-out configuration, the extracellular loop of ENaC faces the fluid flow and it resembles the situation in the intact cells, when channels are activated by shear force. ENaC currents were detected in an excised outside-out recording in the static bath. In the outside-out recording configuration, the open probability (Po) of ENaC was increased from 0.325 ± 0.17 to 1.1 ± 0.3 ($n=4$) ($p<0.05$) by fluid flow (figure 5.5a). However, when 1μM amiloride was present in the fluid, the flow failed to enhance the Po ($n=4$). This result suggested that ENaCs can change their open probability directly in response to flow.

5.4 Discussion

Our data show that the α subunit of ENaC was functionally expressed in cultured ECs and ECs in blood vessels. However, the activities of ENaCs in intact ECs, such as HMEC and pHMEC, were strongly inhibited by intracellular molecule, including 11,12-EET. ENaC in ECs mediated Na^+ currents, only when intracellular inhibitors were removed.

In outside-out recording mode the channel open probability of ENaC was significantly enhanced by flow, indicating the capability of ENaCs to respond to the shear force. Our results suggest that ENaC may play an important role in vascular physiology as a new candidate in mediating shear force.

The α subunit of ENaC alone can form the pore structure and permeate amiloride sensitive Na^+ currents (Canessa, Schild et al. 1994). For maximal ENaC activity, each subunit is required in equal numbers to form the proper stoichiometry (Staruschenko, Adams et al. 2005). Although the PCR results demonstrate the presence of only an α subunit in cultured ECs, there may be a difference in channel composition between cultured ECs and intact ECs *in vivo*. We observed the conductance of ENaC in HMEC and pHMEC to be much smaller than previously reported (Kelly, Lin et al. 2003). One possible explanation for this conductance difference is distinct channel complexes in different cells, where these include co-localized proteins, ion channels and cytoskeleton filaments. When short actin filaments are present on the presumptive cytoplasmic surface of the channel, the single channel conductance of ENaC is decreased by half from 13 to 6 pS (Jovov, Tousson et al. 1999). In the absence of CFTR, ENaC currents with 13 pS conductance are predominant. However, a shift of ENaC gating into its 40 pS conduction state is observed in bilayer experiments when CFTR is present (Ismailov, Awayda et al. 1996). In bilayers, the α, β and γ components of ENaC from rabbit reticulocyte cell lysate display three subconductive states of 13, 26 and 39 pS (Ismailov, Berdiev et al. 1997), with a characteristic gating pattern. The bovine ENaC-α exhibits a main transition state of 39 pS with a very rare 13 pS, whereas the rat renal ENaC-α has a main transition state of 13 pS (Fuller, Ismailov et

al. 1996). In contrast, α,δ, and γ of rat colon ENaC obtained from transfected sf9 cells possess amiloride sensitive currents with a conductance of 6 pS (Rao, Mehdi et al. 2000) in bilayers. The conductance of rat ENaC measured in *oocytes* is around 5 pS (Fuller, Ismailov et al. 1996). Taken together; these reports strongly suggest that ENaC kinetics are varied, likely due to the nature of the channel complex. Our observations were based on experiments in the native endothelial cells, while the other work was carried out in expression systems and bilayers. The difference in cytoskeleton, co-localized ion channels and other complex proteins might lead to the different channel conductances and channel gating kinetics. In addition, our results were further supported by gene knockdown experiments.

Laminar shear stress induces a reversible increase in benzamil sensitive Na^+ currents in oocytes transfected by ENaC (Carattino, Sheng et al. 2004; Carattino, Sheng et al. 2005). Our results and those of previous study (Althaus, Bogdan et al. 2007) have shown that laminar flow led to an increased open probability of ENaC without affecting the number of active channels. ENaC could serve as the mechanical sensor (Drummond, Gebremedhin et al. 2004) in response to flow. Na^+ entry via ENaC into ECs will depolarize ECs to change the membrane potential. The enhanced intracellular Na^+ could subsequently reverse the Na^+-Ca^{2+} exchanger to increase $[Ca^{2+}]_i$ (Sedova and Blatter 1999) (Teubl, Groschner et al. 1999) and facilitate activity of the Na^+-K^+ pump in the basolateral membrane of ECs to reduce the interstitial K^+, which exerts its effect on smooth muscle. In addition, the electrogenic absorption of Na^+ via ENaC creates trans-epithelial osmotic gradients that might represent the main driving force for water movement via aquaporins expressed in ECs (Schnitzer

and Oh 1996; Levick 1997). However, ENaC in intact ECs remains silent. The above speculations on the role of ENaC might only be relevant when intracellular inhibitors are compromised, for example in inflammation. They therefore suggest that ENaC might significantly alter the response of ECs to shear forces under specific conditions. Influx of Na^+ mediated by ENaC might then directly depolarize ECs or facilitate membrane depolarization.

The activity of ENaC in ECs is inhibited by intracellular molecules such as 11,12-EET, suggesting a complex regulation of ENaC in blood vessels. EET is derived from CYP4502C, which is abundant in the endothelium, where it mediates cell proliferation and regulates blood vessel tone (Pritchard, Tota et al. 1990; VanRollins, Kaduce et al. 1993; Mombouli, Holzmann et al. 1999; Zou, Yang et al. 2001). It has been demonstrated to be regulated by laminar flow (Spector and Norris 2007) (Brooks, Lelkes et al. 2002) and PPAR (Liu, Zhang et al. 2005; Pozzi, Ibanez et al. 2007). The dynamic processes of generation and degradation of EET in ECs is predicted to alter the activity of ENaC. Although it has been reported that the expression of CYP-2C decreases following cell isolation (Fleming, Michaelis et al. 2001; Michaelis, Fisslthaler et al. 2005), expression and activity of CYP-2C are determined by many factors including the isolation protocol, culture condition (Pozzi, Ibanez et al. 2007; Michaelis, Xia et al. 2008), mechanical (Fisslthaler, Popp et al. 2001) and chemical stimulus (Fisslthaler, Hinsch et al. 2000). Intracellular generation of EETs by CYP is profoundly regulated by mechanical forces and other stimuli. In addition, recent studies have suggested that PPARα (Spector and Norris 2007) and γ (Liu, Zhang et al. 2005) can be activated under the flow and exert an anti-inflammatory effect.

Activation of PPARγ has been shown to up-regulate the expression of the α (Hong, Lockhart et al. 2003) and γ (Guan, Hao et al. 2005) subunits of ENaC and to increase the activity of ENaC via the serum and glucocorticoid regulated kinase pathway (Guan, Hao et al. 2005; Vallon and Lang 2005). The presence and activity of ENaC in ECs may therefore be profoundly regulated by multiple known and unknown factors, resulting in dynamic responses to shear forces. In addition, blockade of EET generation fails to completely reverse inhibition of ENaC, suggesting additional cellular molecular inhibitors. Further study is required to identify these inhibitors. Nevertheless, this finding implies that ENaC channels in endothelial cells are tonically inhibited and only activated when endogenous intracellular inhibitors are removed under specific conditions e.g. inflammation.

It has been established that P2X4 receptors mediate shear-induced Ca^{2+} influx in bovine and human endothelium, in the presence of extracellular ATP (Yamamoto, Korenaga et al. 2000). The ECs in P2X4 (P2X4 $^{-/-}$) knockout mice do not have a normal response to flow, such as the influx of Ca^{2+} and subsequent production of NO, resulting in high blood pressure (Yamamoto, Korenaga et al. 2000). A key element in mediating P2X4 induced-shear response is the release of ATP from cells to serve the paracrine stimulation. CFTR and volume regulated anion channels, as endogenous ATP release pathways (Reigada and Mitchell 2005), are typically co-localized with ENaC (Yang, Ajonuma et al. 2004; Choi, Son et al. 2006; Leroy, Prive et al. 2006) and reciprocally interact with ENaC in term of their respective functions (Schreiber, Konig et al. 2003; Bachhuber, Konig et al. 2005; Carattino, Edinger et al. 2005; Nagel, Barbry et al. 2005). Another role of ENaC might be to coordinate amount of ATP released by

CFTR and VRAC in ECs.

In summary, the α subunit of ENaC is widely expressed in ECs, but the ENaC activity is strongly inhibited by cellular molecules. When inhibition is removed, ENaC can sense flow directly by changes in channel open probability, thereby modulating Na^+ entry, resulting in membrane depolarization. In coordination with other channels and transporters, ENaC may affect $[K^+]_o$ in the vicinity of nearby smooth muscle cell, and may modulate the release of ATP from ECs. It may also be involved in the regulation of vascular tone. As a novel candidate for sensing shear force, ENaC might play an important role in vascular biology.

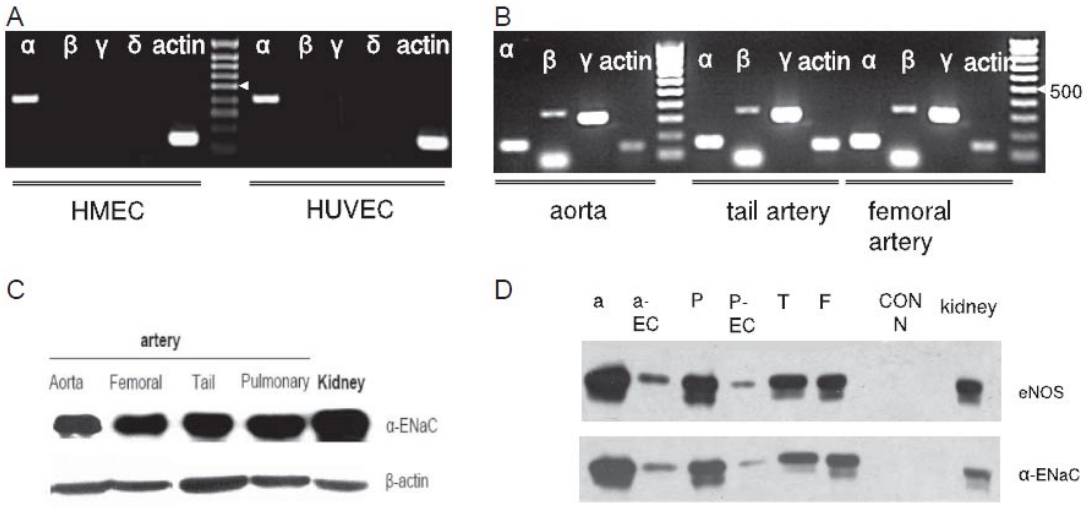
A
α β γ δ actin α β γ δ actin
HMEC
HUVEC
B
α β γ actin α β γ actin α β γ actin
500
aorta
tail artery
femoral artery
C
artery
Aorta Femoral Tail Pulmonary Kidney
α-ENaC
β-actin
D
a a-EC P P-EC T F CON N kidney
eNOS
α-ENaC

Fig 5.1

Fig.5.1. ENaC expression in ECs.

A) Transcripts for the α subunit of ENaC, but not the β, γ or ζ subunits, were detected in cultured human ECs. B) Transcripts for α, β and γ subunits of ENaC were found in isolated arteries including aorta, tail artery and femoral artery. The transcript for actin was used as the sample control. C) The α subunit of ENaC protein was detected in aorta, femoral, tail and pulmonary artery. Rat kidney was used as the positive control. D) Aorta and pulmonary arteries stripped of ECs possessed less eNOS protein and α subunit protein, in comparison with normal aorta and pulmonary artery (white arrow bar in A, B indicates 500 bp).

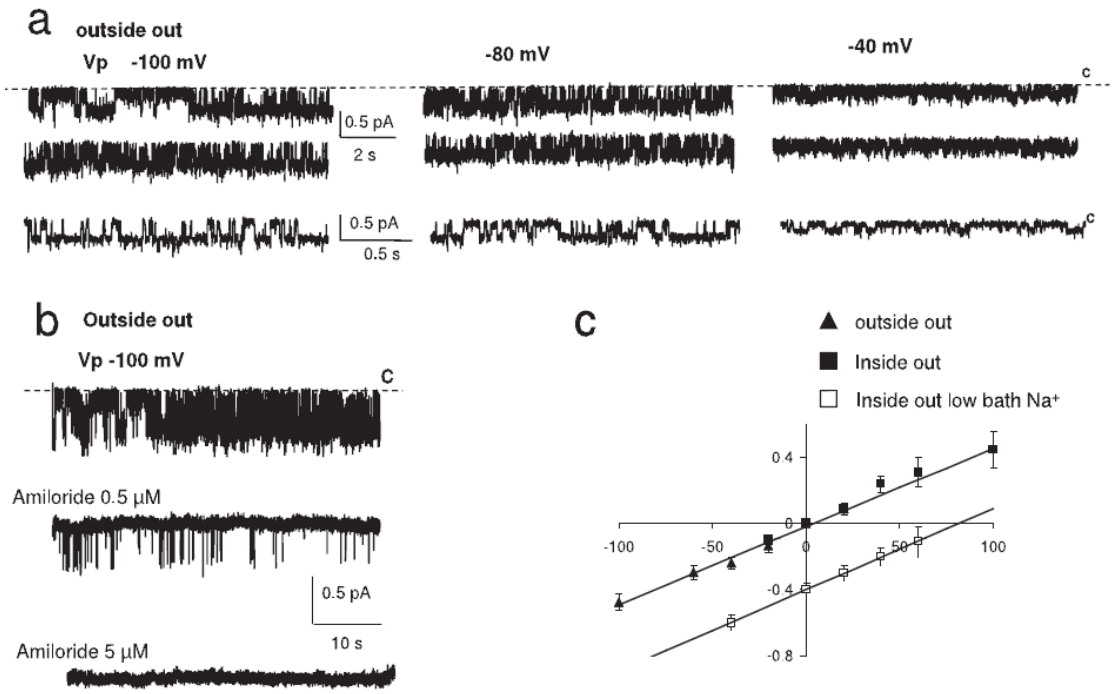
a
outside out
Vp -100 mV
-80 mV
-40 mV
c
0.5 pA
2 s
0.5 pA
0.5 s
c
b
Outside out
Vp -100 mV
c
Amiloride 0.5 μM
0.5 pA
10 s
Amiloride 5 μM
c
outside out
Inside out
Inside out low bath Na+
0.4
0
-0.4
-0.8
-100
-50
0
50
100

Fig 5.2

Fig.5.2. ENaC currents detected in excised membrane recordings.

A) Small conductance currents were elicited in an outside-out recording held at -100, -80 and -40 mV respectively. The lower trace in each panel represented the current fluctuations was expanded in a smaller time scale. B) In an outside-out recording, bath application of amiloride at a concentration of 0.5 μM significantly reduced the NPo of small conductance currents detected in ECs. 5 μM amiloride almost abolished the small conductance currents detected in ECs. C) Summary plots of I-V curves obtained in excised membrane recordings. Each point represents data from six to eight cells.

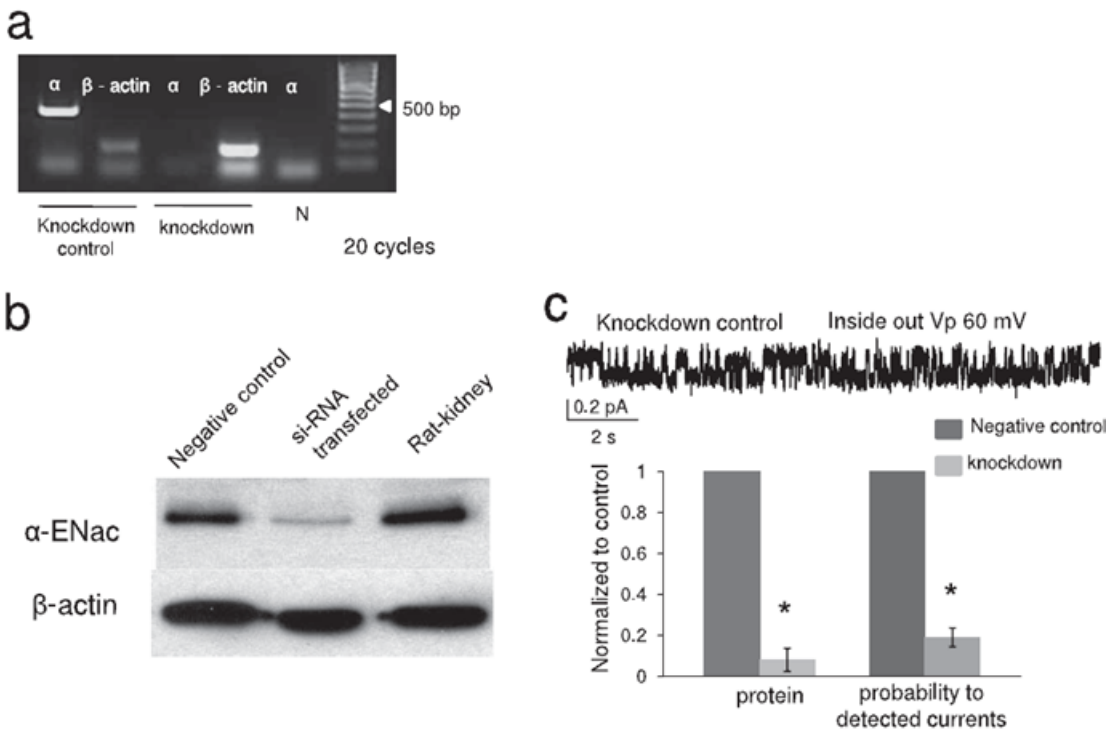
a
α
β - actin
α
β - actin
α
500 bp
Knockdown control
knockdown
N
20 cycles
b
Negative control
si-RNA transfected
Rat-kidney
α-ENac
β-actin
c
Knockdown control
Inside out Vp 60 mV
0.2 pA
2 s
Negative control
knockdown
Normalized to control
1
0.8
0.6
0.4
0.2
0
*
*
protein
probability to detected currents

Fig 5.3

Fig.5.3. Knockdown of ENaC significantly reduced the possibility of detecting currents.

A) A positive PCR result of the α subunit of ENaC was only detected in control groups after 20 cycles of PCR. B) A significant decrease in the α subunit of ENaC was revealed by Western blot analysis. C) The top trace represents an example recording of inward current revealed in knockdown and control cells when the pipette was held at +60 mV in the inside-out recording. In conjunction with a decrease in expression of α subunit of ENaC, the probability of detecting ENaC currents was reduced as shown in the bar graph. * represents significance $P<0.05$. (white arrow bar in A, B indicates 500 bp).

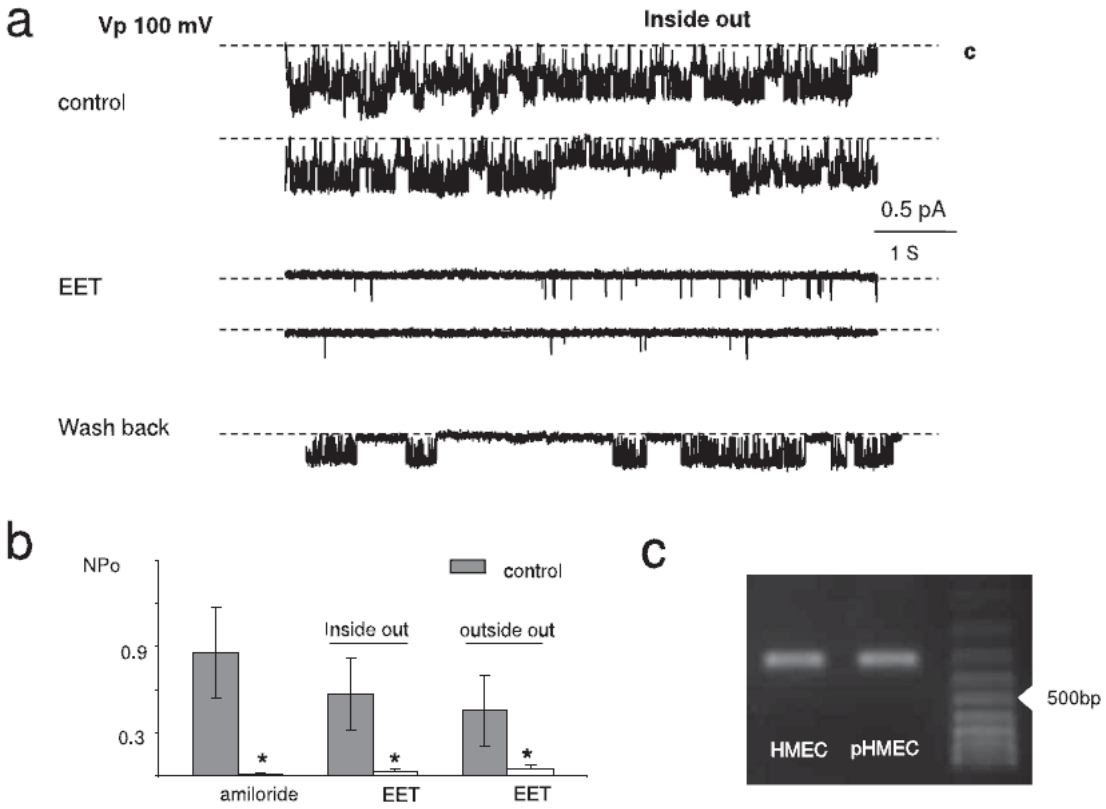
a
Vp 100 mV
Inside out
c
control
0.5 pA
1 S
EET
Wash back
b
NPo
control
Inside out
outside out
0.9
0.3
amiloride
EET
EET
c
500bp
HMEC
pHMEC

Fig 5.4

Fig.5.4. ENaC currents in ECs sensitive to 11,12-EET.

In an inside-out recording, bath application of 11,12-EET at a concentration of 200 nM almost abolished ENaC currents. ENaC currents partly recovered subsequent to removal of 11,12-EET. A). The bar graph shows the effects of amiloride and 11,12-EET on NPo of ENaCs from ECs. B) 11,12-EET exerted a remarked inhibition on ENaC from either outside or inside the membrane. *, represents significance $P<0.05$. C) Transcripts for the CYP 2C were detected in pHMEC and HMEC.

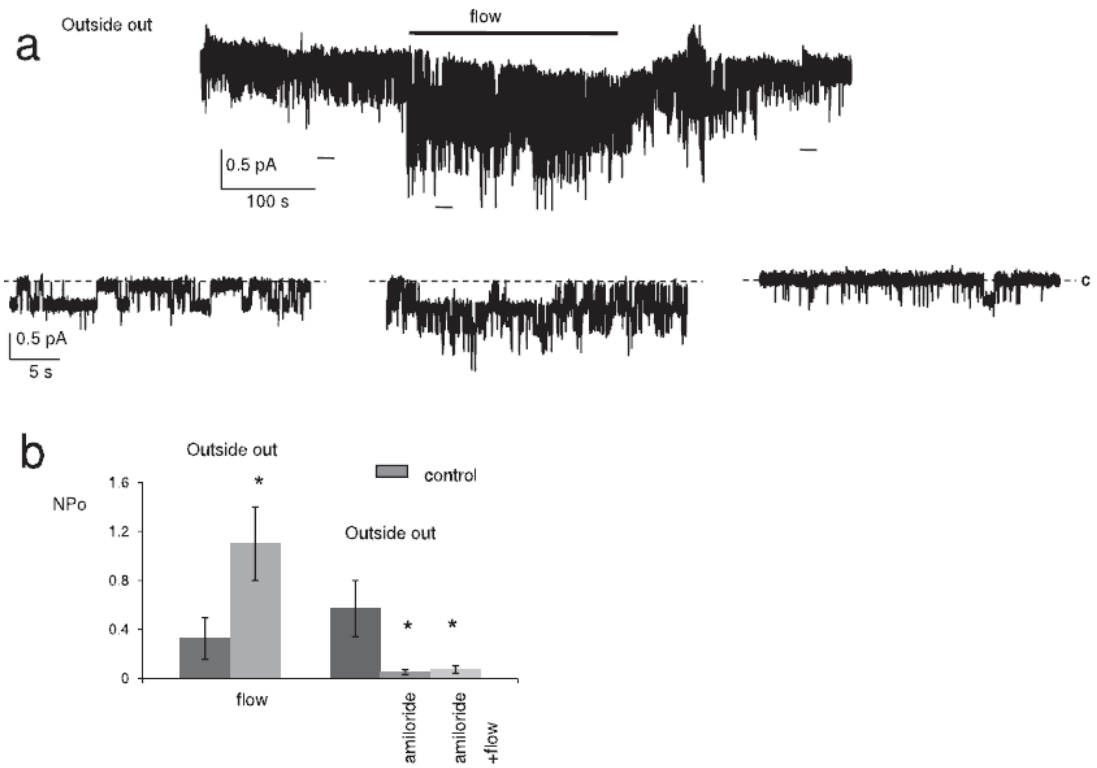
a
Outside out
flow
0.5 pA
100 s
0.5 pA
5 s
c
b
Outside out
control
NPo
1.6
1.2
0.8
0.4
0
Outside out
*
*
*
flow
amiloride
amiloride
+flow

Fig 5.5

Fig.5.5. Flow enhanced NPo of ENaC from ECs.

A) In an outside-out recording, laminar flow significantly enhanced the NPo of ENaC in comparison to the static bath. Lower panel figures show the current fluctuations on an expanded time scale. B) The bar graph showed the effects of flow on NPo of ENaCs. Each column represents the data from 4-6 cells. *, represents significance $P<0.05$.

Chapter 6

Role of Epithelial sodium channels (ENaC) in endothelium function

6.1 Introduction

Vascular endothelial cells (ECs), which form a monolayer lining the luminal surface of all blood vessel walls, not only provide a selective barrier between circulating blood elements and underlying tissues but also serves a number of important vascular functions. One of the most important EC functions is that ECs act as a sensing interface which converts mechanical stimuli such as shear stress resulting from the blood flow into biochemical signals that initiates various vascular responses. This aspect of EC function is perhaps best exhibited by the endothelial-dependent vessel dilatation in response to increased blood flow rate. The precise tuning of vessel-motion by ECs is crucial to control the blood pressure and protects blood vessels from the adverse effects of various pathogenetic factors. Among the many putative mechano-transducers, endothelial ion channels are considered to be a particularly important candidate that mediates the first setting of EC responses (in a range of seconds and minutes) to changes in blood flow. Intensive studies have been launched to identify the potential shear force-sensing ion channels in ECs and an increasing number of channels have been suggested to participate in the process (Nilius and Droogmans 2001). We have identified that the epithelial Na^+ channel is one of the mechano-transducing ion channels in human endothelial cells and its activity is tightly and actively regulated by various endogenous vascular signaling molecules including heme, HO-1 and CO. however, the overall impact of the ENaC mediated blood flow sensing is negative, since hyperactivity of the channel resulting

from systemic inflammation is a potent pro-atherosclerosis factor which causes endothelial cell dysfunction manifested by a decreased NO bioactivity and impaired shearforce sensitivity, and eventually leads to the initiation and progression of vascular diseases.

Epithelial sodium channels (ENaCs) are of immense importance, controlling Na^+ transport across epithelia and thus playing a central role in all aspects of fluid clearance as well as numerous other functions. Accumulated evidence has suggested that ENaCs are expressed in a variety of endothelial cells. Administration of amiloride and benzamil, both antagonists of ENaCs, results in blocking the myogenic constriction of blood vessels (Jernigan and Drummond 2005), suggesting a potential role of ENaCs in mediating vascular vessel tone. Changes in the plasma Na^+ concentration affect EC function and thus control vascular tone via NO (Oberleithner, Riethmuller et al. 2007). Additionally, as a stimulator of ENaCs, aldosterone has been shown to cause HUVEC swelling (Oberleithner, Schneider et al. 2003) and surface insertion of ENaC in ECs (Oberleithner, Riethmuller et al. 2006; Kusche-Vihrog, Sobczak et al. 2008). However, role of ENaC in endothelium remains unclear. In hypertensive subjects with hyperaldosteronism, endothelium dependent flow-mediated vasodilatation is impaired (Nishizaka, Zaman et al. 2004). A decreased L-arginine conversion to NO are shown in these hypertensive patients after salt loading (Ni and Vaziri 2001). In addition, HO and its metabolic products e.g. carbon monoxide (CO) have been implicated in regulation of basal tone and the initial level

of blood pressure (Johnson, Lavesa et al. 1996; Johnson, Colombari et al. 1997; Kozma, Johnson et al. 1997; Johnson, Kozma et al. 1999; Sabaawy, Zhang et al. 2001). However, the mechanisms remain unclear.

6.2 Specific methods and materials

6.2.1 HUVEC cells

Primary cultured HUVEC cells were from Lonza and Promocell ltd. Cells were grown in the HUVEC medium (Lonza) in a 37C and 5% CO_2 incubator. Cells were used within 5 passages.

6.2.2 Electrophysiology recording

Single channel recordings were performed as previously described. Briefly, a coverslip on which HUVECs were grown was transferred into a recording chamber mounted on a Nikon inverted microscope (Nikon TE 2000U). Patch pipettes with a resistance of 6 MΩ was fabricated from a borosilicate glass capillary (1.5 mm od, 0.86 mm id) (Sutter) on a Sutter Puller (P97). Bath solutions contained (in mM): 110 NaCl, 4.5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 HEPEs, 5 Na-HEPEs, pH 7.2. Pipette solution contained (in mM): 110 NaCl, 4.5 KCl, 0.1 EGTA, 5 HEPEs, 5 Na-HEPEs, pH 7.2. Single channel currents were recorded with an Axon 1D amplifier and Axon clampex 9.0. The data were acquired at 20 kHz and low pass filtered at 5 kHz. During post-experiment analysis, data were

further filtered at 200 Hz. Single channel events were listed and analyzed by pclampfit 9.0 (single channel search in analyze function). 50% threshold cross method was utilized to determine valid channel openings. When multiple channel events were observed in a patch, the total number of functional channels (N) in the patch was determined by observing the number of peaks detected on all point amplitude histograms. NPo, the product of the number of channels and the open probability calculated as described previously (Yue, Malik et al. 2002), or the open probability (Po) itself, was used to measure the channel activity within a patch. Initial, 3-4 minute, single channel records were normally used as the control. The activity of ENaC during application of chemicals was normalized to the activity during the control period to assess the effects of chemicals on ENaC. In some cases ENaC activity during application of chemicals was also compared to that of ENaC after chemicals were washed off. These data were used to confirm the effects of chemicals. Data are presented as means ± s.e.m. and statistical differences were compared using Student's paired *t* test, taking $P < 0.05$ as significant and marked by *.

Patch pipettes with the resistance of 6 MΩ were fabricated from borosilicate glass capillary (1.5 mm od, 0.86 mm id) (Sutter) on a Sutter Puller (P97). Conventional whole cell recording was performed as previously descriped. The bath solution contained (in mM): NaCl 110, KCl 4.5, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10, HEPEs 5, HEPE-Na 5. The pipette solution contained (in mM): KCl 40, K-gluconate 100, $MgCl_2$ 1, $CaCl_2$ 1, EGTA 0.1, Na2ATP 4, Glucose 10, HEPEs 10, GTP 2. pH by KOH to 7.2. In the

case of perforated whole-cell recording, the normal pipette solution contained 10-20 μg/ml amphotericin B. On the day of experiment, 0.1 g amphotericin B (Sigma) was weighted and dissolved with 0.1-0.5 ml DMSO to make a stock solution. 0.5 μl stock solution was added into 1 ml pipette solution to make up final pipette solution, which containing amphotercin B 10-20 μg/ml. DMSO as a vehicle in same dilution within the pipette solution was tested. No effect was observed.

6.2.3 Western blot analysis and PCR

For western blotting, arteries were quickly frozen in liquid nitrogen and homogenized with CelLytic M lysis/extraction reagent and Protease Inhibitor cocktail (Sigma). Protein concentrations were determined by the Bradford Assay method. Equal amounts of total protein were loaded into the SDS-PAGE gel wells. β-actin was used as the inner marker to calibrate the protein quantity. Samples prepared with 5x loading buffer, were separated on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes, which were rinsed with Tris-Buffered Saline Tween-20 (TBS-T) and then blocked in TBS-T containing 5% milk for 1 hour (at room temperature). The membrane was incubated with the first antibody, α-ENaC antibody (1:500, CHEMICON/1:500 Santa Cruz Biotechnology sc-21012) or HO-1 antibody (AB-cam), HO-2 antibody (AB-cam) and caveolin-1 antibody (AB-cam) overnight at 4°C, followed by washes in TBS-T and incubation with the secondary antibody (1:10000, HRP, BioRad) for 1 hour at room temperature. Membranes were

washed with TBS-T and blots were detected by ECL plus.

RT-PCR experiments followed standard protocols. Primers against human (Ji, Su et al. 2006) ENaCs were used as previously described. A 406-bp product of human α-ENaC was amplified by the primers: 5-AAC AAA TCG GAC TGC TTC TAC-3 (sense) and 5-AGC CAC CAT CAT CCA TAA A-3 (antisense).

6.2.4 Knockdown experiments

Expression of the α subunit of ENaC was decreased using validated on-Targetplus siRNA (smart pool ENaC channel, Cat No. L-006504-00) from Thermo Fisher Scientific (Dharmacon). Negative controls were employed using a negative control siRNA (non-targeting pool, Dharmacon) and vehicle alone. DharmaFectin 1(T-2001-01) transfection reagent was used for transfection. The knockdown experiments were carried out according to the manufacturer's instructions. Knockdown effects were examined by RT-PCR and western blot analysis after 24 and 48 hours.

6.2.5 Isolation of caveolae compartment

HUVEC were grown to confluence in 75ml culture flasks and used for fractionation. The homogenates in MBS (25 mM 2-(N-morpholino)-ethanesulfonic acid (MES), pH 6.5, 0.15 M NaCl) containing 1% Triton X-100 were adjusted to 40% sucrose by the addition of 2 mL of 80% sucrose prepared in MBS and placed at the bottom of an

ultracentrifuge tube. A 5–30% discontinuous sucrose gradient was formed above (4 mL of 5% sucrose/4 mL of 30% sucrose, both in MBS lacking detergent) and centrifuged at 39,000 rpm for 18 h in an SW41 rotor (Beckman Instruments, Palo Alto, CA, USA). A light-scattering band at the 5–30% sucrose interface was collected or fractionated into 12 subfractions(32). A 100 µL aliquot of each fraction was used to measure alkaline phosphatase activity. After direct addition of substrate solution (p-nitro-phenyl phosphate, R&D systems, Minneapolis, MN, USA), the absorbance at 405 nm was measured.

6.2.6 Flow setup

After the outside-out configuration was obtained, the patch pipette was moved toward a perfusion tube to a distance within 160 µm. A syringe pump (Harvard Apparatus, Harvard, Mass) connected through tubing to the glass pipette provided fluid flow (figure 2a).

The shear force applied upon cell surface was calculated according to equation

(Oancea, Wolfe et al. 2006):

$$T = \frac{\rho V^2}{2} X \frac{0.664}{\sqrt{R_x}}$$

Where: **ρ** is the density of water)

V is The fluid velocity and calculated by **V** = Q/A, where **A**=πd^2/4 (d is the flow application pipette diameter). **Q** is the flow rate generated by the syringe pump measured in m^3/s

$\mathbf{R_x}$=V**X*/μ where **X** is the distance between pipette and cell, $\boldsymbol{\mu}$ is the kinematic viscosity of the water

In our setup, d was approx 120 µm and X about 160 µm. ρ=1025kg/m^3 and μ=1.139 X 10^{-6}m^2/s. Accordingly, 1 ml/min of flow generated 0.49 dyne stress; 3 ml/min of flow was 3.67 dyne; 5 ml/min was 8.42 dyne; 8 ml/min was 17 dyne.

6.2.7 Chemicals and stock solutions

Benzamil and amiloride were purchased from Biomol International. Haem, PP9, MP11, $FeSO_4$, NADPH and ZnPP9 were from Sigma-Aldrich. SnPP was from Tocris. Biliverdin was from Frontier Scientific. Stock solutions (1 mM) of these reagents in 10 mM NaOH were made weekly and kept at -20 °C in aliquots. All the solutions were thawed and immediately before use. [ruthenium$(CO)_3Cl_2]_2$ was freshly dissolved in DMSO on the day of experiments. Ruthenium (III) chloride hydrate and ruthenium $(DMSO)_4Cl_2$ were also freshly prepared accordingly. The medium containing certain concentration of CO donor is made of 30 minutes ahead of experiments. Partial pressures of medium O_2 were measured and monitored by O_2 meter (WPI ISO2).

Hypoxic medium was obtained by N_2 bubbling.

6.3 Results

6.3.1 ENaCs expressed in HUVEC were functional

Consistent with our previous work (Wang, Meng et al. 2009), small conductance inward currents were detected in HUVEC. In the inside-out recordings, a range of currents were elicited by different voltages (figure 6.1a). Analysis of these results concluded the conductance of this channel was 4.8 pS (figure 6.1b), which is similar to that of ENaC founded in renal tubular cells (Wei, Lin et al. 2004). With respect to the conventional blockers for ENaC, e.g. amiloride and benzamil, ENaC activity was significantly reduced when 1μM amiloride was included in the pipette solution (ismilov) (figure 6.1c). To ensure that this small conductance currents were mediated by ENaCs, knockdown experiments against the human α subunit of ENaC were therefore performed. The knockdown effect was examined by RT-PCR and Western blot analysis. After 24 hours, mRNA of the human α subunit of ENaC was significantly decreased, resulting in a negative PCR product after 20 PCR cycles, whereas a positive PCR product was shown in knockdown control cells (not shown). Consistent with the RT-PCR results, western blot analysis indicated that protein levels of the human α subunit of ENaC were significantly decreased after 48 hours (Figure 6.1d). In parallel with the decrease of ENaC protein in HUVEC, the probability (60 out of 110 recordings) of detecting such conductance currents (around 0.3 pA at Vp=60 mV and

0.4 pA at Vp=80 mV) in the inside-out recording from the ENaC knockdown groups (N=3) was also reduced, when compared to the control groups trasfected by the negative control siRNA (80 out of 180 recordings) (N=3).

6.3.2 ENaCs were activated by flow.

The ENaC superfamily has been demonstrated to be able to sense mechanical stress (Adams, Anderson et al. 1998; Hong, Mano et al. 2000) and transduce the stress into electrical events, resulting in membrane depolarization (Snitsarev, Whiteis et al. 2002). Structurally, ENaCs contain only two trans-membrane domains and a large extracellular region with cysteine-rich domains and glycosylation sites, which will be rationally anticipated to sense flow. In a conventional whole-cell recording by rupturing the membrane to form whole-cell configuration, flow at the speed of 5 ml/min (equivalent to 8.42 dyne) significantly augmented inward currents mediated by ENaC, which was completely reversed by benzamil (10μM) (figure 6.2a). Shear stress on stimulated the ENaC mediated whole-cell current on a dose dependent manner (figure 6.2c).

6.3.3 ENaCs were activated by stretch

Alternatively, the ENaCs were also activated by stretch/pressure. To mimic the pressure of blood flow on endothelium, a positive pressure was applied through the patching pipette to apical membrane of HUVEC in inside-out recordings. Pressure (10

mmHg) significantly increased ENaC activity (figure 6.3a) almost 1.5 fold in a repeated manner. Intriguingly, flow also stimulated ENaCs even when channels were covered within the patching pipette in cell-attach recordings (figure 6.3b). It suggested there is an unknown mechanism through which ENaCs are indirectly activated by flow. The candidates might include the molecules co-localized with ENaCs.

6.3.4 ENaCs co-localized with caveolin-1, HO-1 and HO-2.

Previous reports have demonstrated that ENaCs are present within caveolae(Hill, Butterworth et al. 2007) and regulated by lipid rafts (Hill, An et al. 2002). Accordingly (Hill, Butterworth et al. 2007), HUVEC were lysed and membranes separated by density on sucrose gradients at 39,000 rpm, SW41 rotor for 18 hours (Beckman Instruments, Palo Alto, CA, USA). Total 12 fractions were obtained after 18 hours. Each fraction was precipitated and then immune-blotted with antibodies against α-subunit of ENaC, caveolin-1, hemeoxygenase-1 (HO-1) and hemeoxygenase-2 (HO-2). Low density membranes which correspond to lipid rafts are found near the top of the gradient in fractions of 4 and 5 while non-raft markers are found at higher sucrose densities in fraction 8-12. Caveolin-1 was found in fractions 4 and 5. Meanwhile, α-subunit of ENaC, HO-1 and HO-2 were also detected in these fractions (figure 6.3c), suggesting ENaCs are expressed within caveolae. It might co-localize with caveolin-1, HO-1 and HO-2. Flow mechanically deforms the endothelial cell

membrane. The deformation of apical membrane will affect caveolae structure, resulting in modulation of ENaC activity. It will be manifested by the observation that flow indirectly activates ENaCs.

6.3.5 Regulation of ENaC by Haem and CO

Application of haem at nanomolar concentration to the intracellular side of the channels (inside-out recordings) consistently and significantly reduced the open probability (NPo) of ENaCs (figure 6.4a, b). In inhibition of ENaCs by haem did not require any exogenously added enzyme and cofactor. No change of unitary current amplitude was observed. Upon washout of haem, the reversal of inhibition was slow and sometimes partial.

Application of 1 nM NADPH alone had no effect on activity of ENaC. In the presence of O_2 and NADPH, hemeoxygenase converts haem to biliverdin, iron and CO (Williams, Wootton et al. 2004). Modulation of the activity of ENaC by CO resembled that seen upon co-application of haem and NADPH (figure 6.4a, b), suggesting that the stimulatory effect of these agents might indeed reflect the generation of CO by hemeoxygenase. However, when haem exceeding the concentration of 60 nM, reduction of NPo of ENaC occurred, suggesting that haem and CO act competitively in their effects on ENaC expressed in HUVEC.

Activity and density of HO appear the determinant factor in the regulation of ENaC activity. In HO-1 knock down HUVEC, 20 nM haem plus NADPH exerted a similar inhibitory effect on ENaC as 20 nM haem. However, ENaCs exhibited more active in presence of 20 nM haem plus NADPH when HO-1 was over-expressed in HUVEC (figure 6.4c).

6.3.6 Regulation of HO expression and ENaC activity by TPA

TPA (phorbol 12-myristate 13-acetate) is a potent tumor promoter and it can activate protein kinase C in vivo and vitro (DeRiemer, Strong et al. 1985; Sagi-Eisenberg, Lieman et al. 1985). In addition, it is also regarded as a common inflammatory factor. In this study, TPA at a concentration of 100 ng/ml was added into the growing medium 24 hours prior to experiments. A western blot analysis showed that HO-1 expression was significantly increased after 24 hours stimulation of TPA, whereas caveolin-1, HO-2 and α subunit of ENaC remained unchanged (figure 6.5a). TNF-α exerted similar stimulation effects on caveolin-1, HO-1, HO-2 and ENaCs.

Over-expression of HO-1 leads to higher amount of HO products e.g. CO, resulting in increase of ENaC activity. To avoid the dialysis of cellular messengers by patching pipette solution, perforated whole-cell recording was therefore employed. Inward currents mediated by ENaCs were bigger and exhibited a bigger increase in TPA stimulated HUVEC, comparing with the control HUVEC (figure 6.5b).

6.3.7 Increase of $[Na^+]_i$ impeded cationic amino acid transporter (CAT) activity

In hypertensive subjects with hyperaldosteronism, endothelium dependent flow-mediated vasodilatation is impaired (Nishizaka, Zaman et al. 2004). A decreased L-arginine conversion to NO are shown in these hypertensive patients after salt loading (Ni and Vaziri 2001). eNOS requires L-arginine as a substrate to generate NO. L-arginine transport across the membrane is via CAT, which utilizes the Na^+ gradient across cell membrane as a driving force. Increase of $[Na^+]_i$ reduced the activity of CAT evoked by extracellular L-arginine in a dose-dependent manner (figure 6.5c). It suggested that activation of ENaC might exert a complex effect on vascular regulation in response to flow, likely negative and passive effect.

6.3.8 Inhibition of inward rectified K (IRK) channels by CO

IRKs are predominantly expressed in endothelial cells and generally regarded as a flow sensor. Shear stress activates IRK, channels, resulting in membrane hyperpolarization in endothelial cells, consequent enhancement of Ca^{2+} influx and stimulation of eNOS. Since increased flow apparently activates ENaC channels though regulation of HO-1, we investigated whether HO activity (generation of CO) might contribute to the regulation of IRK channels in HUVECs. IRK-like currents in HUVECs were activated by flow (figure 6.5a) and identified by their I-V curve, reversal potential and sensitivity to Ba^{2+} and TEA. When cells were exposed to CO in the flow medium (CO donor at a concentration of 20 μM) the IRK currents were significantly

attenuated. To clarify our observation, we transfected HEK cells with IRK 2.3 plasmid. Typical IRK currents were then observed and they are reversed at -60 mV and sensitive to Barium. 1 mM Co donor also induced a significant blockage on IRK currents as shown in igure 6.6

6.4 Discussion

In this study, we demonstrated that ENaC expressed in HUVEC are functional. ENaCs in HUVEC could be activated directly and indirectly by flow and stretch. ENaCs, HO-1 and HO-2 were expressed within caveolae on apical membrane of HUVEC. Deformation of membrane also stimulated ENaC via caveolae. However, activity of ENaC in an intact cell remained low due to the tonic inhibition of haem. HO acts as a modulator and O_2 sensor, its substrate (haem) and product (CO) inhibiting and stimulating (respectively) the activity of ENaC. TPA as well as other inflammatory factors could increase expression of HO-1, which leading to high activity of ENaC and their sensitivity to flow. Nevertheless, the increase of $[Na^+]_i$ impeded the activity of CAT, resulting in reduction of NO in response to flow. In addition, ENaC may mediate the toxic effect of CO on HUVEC.

Laminar flow increases the open probability of ENaC without affecting the conductance of ENaCs. ENaC can therefore serve as a mechanical sensor (Drummond, Gebremedhin et al. 2004) in response to flow. Activation of ENaC will permeate Na^+

entry, subsequently depolarize ECs. The increase of $[Na^+]_i$ will facilitate the Na^+-K^+ pump in the basolateral membrane of ECs to reduce the interstitial K^+, which results in hyperpolarization of smooth muscles (Jiang, Nuttall et al. 2005; Pritchard, Parvatiyar et al. 2007) which partly in control of vascular tone. However, it is complex in NO production by activation of ENaC. The increase of $[Na^+]_i$ will reverse the Na^+-Ca^{2+} exchanger (NCX) to augment $[Ca^{2+}]_i$ (Sedova and Blatter 1999) (Teubl, Groschner et al. 1999), which is in favour of NO generation. On the other hand, eNOS requires L-arginine as a substrate to generate NO. L-arginine across membrane is via cationic amino acid transport (CAT), which utilizes Na^+ gradient across cell membrane as a driving force. Increase of $[Na^+]_i$ will impair L-arginine transport, resulting in reduction of NO generation. Nonetheless, the significant effect of ENaC appears to mediate NO-independent vessel dilation, which is due to SMC relaxation. The increase in intracellular Na^+ results in basolateral Na^+-K^+ATPase hyperactive, causing K^+ content decrease in the myo-endothelial fluid, which hyperpolarize smooth muscle cells. However, the ENaC mediated NO-independent vessel-dilation is weak to adapt rapid change in blood flow, resulting in high pressure when high flow passes through narrow vessels. It is rational that vessels exert dilation in response to the high speed flow. The vessel dilation is mainly induced by NO released from endothelium and smooth muscle cell (SMC) relaxation due to SMC hyperpolarization. Between these two factors, effect of NO takes a major account on vessel dilation. In conclusion, ENaCs may cause SMC hyperpolarization and hance slight vessel dilation, but it reduces NO production, which is pathlogical. Moreover, the slight vessel

dilation might not be sufficient to adapt the increase of flow, resulting in high pressure.

TNF has been shown to stimulate Na^+ uptake in distal tubule cells isolated from diabetic rats (DiPetrillo, Coutermarsh et al. 2003; DiPetrillo, Coutermarsh et al. 2004). Activity and expression of HO determine CO. Expression of HO could be regulated by many factors, majorly inflammatory factors. High expression of HO occurs in many chronic diseases and vascular diseases. In these situations, ENaC participates in endothelial shear force sensing. Consequently, vessels may exhibit good dilation initially in response to flow (Sabaawy, Zhang et al. 2001), but appear slight dilation in a long term. The mismatch between the flow and diameter of vessel will lead to high blood pressure and or damage of endothelium, resulting in the other vascular diseases related to endothelium dysfunction. Interestingly, recent reports have shown that plasma sodium in the high physiological range stiffens endothelial cells and reduces the release of NO, whereas potassium softens vascular endothelium and increases NO release (Oberleithner, Callies et al. 2009). In addition, accumulated cellular Na^+ mediated by ENaC appeared to initiate or activate cellular necrosis, which appeared blisters on cell membrane and membrane damage.

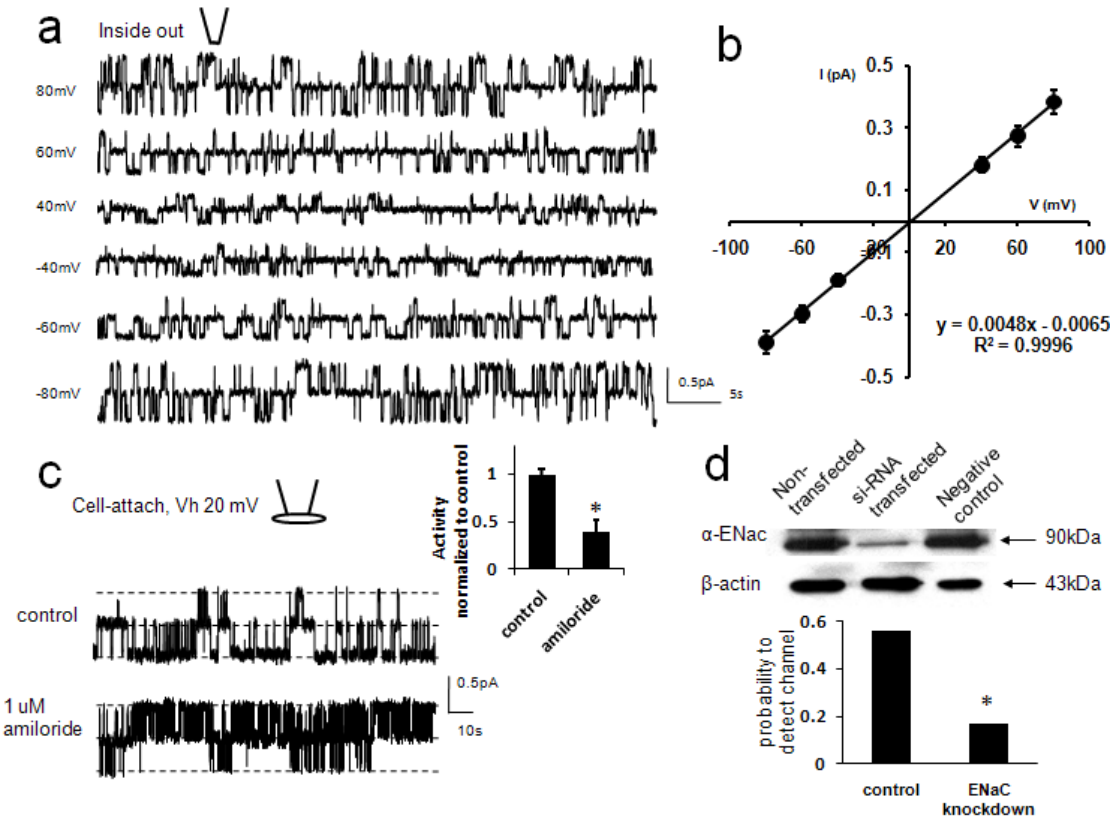
a
Inside out
80mV
60mV
40mV
-40mV
-60mV
-80mV
0.5pA
5s
b
I (pA)
0.5
0.3
0.1
V (mV)
-100
-60
-20
20
60
100
-0.3
-0.5
y = 0.0048x - 0.0065
R² = 0.9996
c
Cell-attach, Vh 20 mV
control
1 uM amiloride
0.5pA
10s
Activity normalized to control
1
0.5
0
*
control
amiloride
d
Non-transfected
si-RNA transfected
Negative control
α-ENac
β-actin
90kDa
43kDa
probability to detect channel
0.6
0.4
0.2
0
*
control
ENaC knockdown

Figure 6.1

Figure 6.1 ENaCs expressed in HUVEC were functional. a, examples of current traces elicited at different voltages in inside out recordings. The voltage on the left to the current traces represented the pipette command voltages. b. I-V curved constructed from the results of inside-out recordings. The conductance was approx 4.8 pS. Each point represented the mean (±SEM) of 8-10 cells. c. 1 μM amiloride significantly reduced the channel open probability but not conductance. Example traces of currents detected in the cell-attach recording with or without 1μM amiloride in the pipette solution are shown in the left panel. Extracellular amiloride significantly inhibited channel activity (right panel). d. knockdown of ENaC in HUVEC significantly attenuated the probability to detect currents with this conductance. In parallel to reduction of ENaC in HUVEC by si-RNA against human ENaC (top panel), probability to detect such currents was significantly attenuated from 60 out of 110 to 18 out of 110.

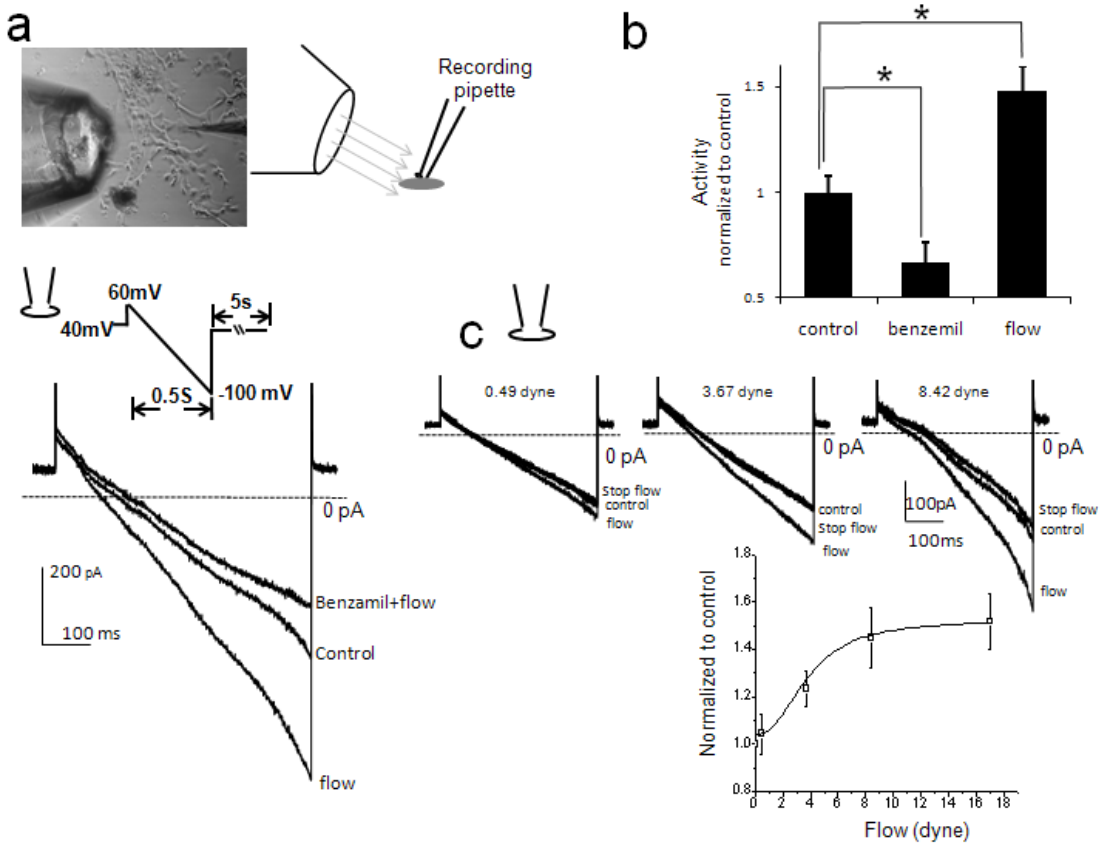
a
Recording pipette
60mV
40mV
5s
0.5S
-100 mV
0 pA
200 pA
100 ms
Benzamil+flow
Control
flow
b
Activity normalized to control
control
benzemil
flow
c
0.49 dyne
3.67 dyne
8.42 dyne
0 pA
Stop flow
control
flow
100pA
100ms
Normalized to control
Flow (dyne)

Figure 6.2

Figure 6.2 Flow activated ENaCs. a. In a conventional whole cell recording, ENaC currents were elicited by a voltage-ramp protocol (as shown in inset of a). Cells were hold at 40 mV. A voltage ramp started from +60 mV and ended at -100 mV in a period of 500 mS (Tong, Gamper et al. 2004). The ramp protocol was repeated in every 5 S. Flow at a speed of 5 ml/min, which is equitant to 8.42 dyne, significantly enhanced the inward currents mediated by ENaC, which was reversed and blocked by benzamil (10 µM). b. Flow significantly increased the whole cell currents mediated by ENaC, but failed to do so when benzamil were present in medium. Each column represented the mean (±SEM) of 6-8 cells. c. Flow enhanced ENaC activity. X axis represented shear stress against on cell membrane and y axis represented the increase ratio of whole cell current density carried by ENaC. Each point was averaged from 4-5 cells.

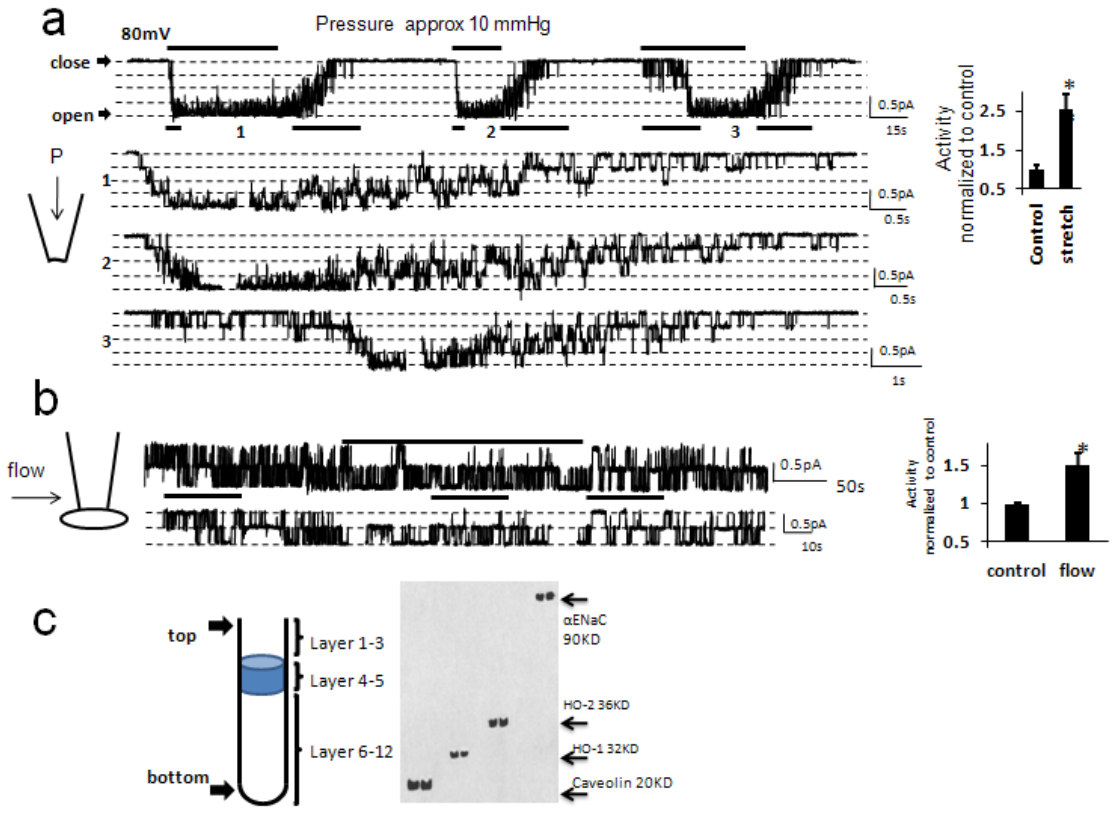
a
80mV
Pressure approx 10 mmHg
close
open
1
2
3
0.5pA
15s
P
0.5pA
0.5s
0.5pA
0.5s
0.5pA
1s
Activity normalized to control
2.5
1.5
0.5
Control
stretch
b
flow
0.5pA
50s
0.5pA
10s
Activity normalized to control
1.5
1
0.5
control
flow
c
top
bottom
Layer 1-3
Layer 4-5
Layer 6-12
αENaC 90KD
HO-2 36KD
HO-1 32KD
Caveolin 20KD

Figure 6.3

Figure 6.3 Stretch activated ENaCs. a. In an inside-out recording, positive pressure (10 mmHg) via the patch pipette on outside membrane of HUVEC activated ENaC currents in a repeatable manner. Traces were obtained when pipette voltage was held at 80 mV. Application of pressure is shown by the grey bars above the upper current trace. Selected parts of currents (marked by bars below the upper trace) are shown with an expanded time scale in the lower panel as in the numeric traces. b. Stretch significantly increased ENaC activity by 1.5 fold. Each column represented the mean (±SEM) of 8 cells. c. Flow indirectly activated ENaCs. In a cell-attach recording, flow significantly increased ENaC open probability, suggesting the present of an indirect mechanism. Application of flow is shown by the grey bars above the upper current trace. Selected parts of currents (marked by bars below the upper trace) are shown with an expanded time scale in the lower traces. Each column represented the mean (±SEM) of 5 cells. c. Caveolae compartmentalization of ENaC, HO-1 and HO-2 in HUVEC. Total cell lystaes were loaded on the discontinuous sucrose gradients for 18 hours (39,000 rpm, SW41 rotor). 12 fractions were obtained and subjected to Western blot analysis. Caveolin-1 was concentrated in fractions 4-5, where ENaC, HO-1 and HO-2 were also present.

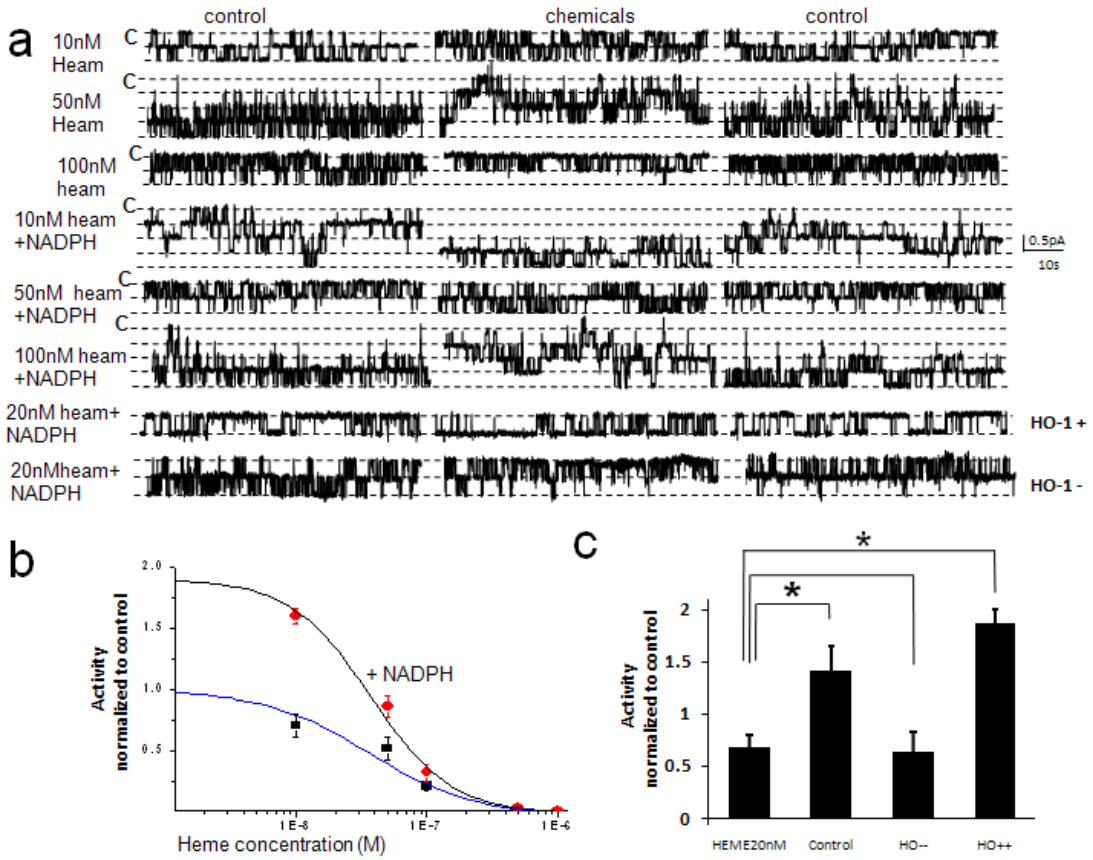
a
control
chemicals
control
10nM Heam
50nM Heam
100nM heam
10nM heam +NADPH
50nM heam +NADPH
100nM heam +NADPH
20nM heam+ NADPH
20nMheam+ NADPH
C
0.5pA
10s
HO-1 +
HO-1 -
b
Activity normalized to control
+ NADPH
Heme concentration (M)
1E-8
1E-7
1E-6
c
Activity normalized to control
HEME20nM
Control
HO--
HO++
*

Figure 6.4

Figure 6.4 Haem alone inhibited ENaC whereas Haem plus NADPH activated ENaC in HUVEC. a. Traces were obtained in the inside out configuration with a pipette voltage of +80 mV. 2^{nd} panel shows effects of haem or haem plus NADPH (1μM) on ENaCs. Bottom two traces represents effects of 20 nM haem plus NADPH on ENaCs from HUVEC of HO-1 overexperssion ($HO\text{-}1^{+}$) or HO-1 knockdown ($HO\text{-}1^{-}$), respectively. b. Concentration dependent effects of haem or haem plus NADPH on ENaC activity. Data were fitted with a sigmoidal dose-effect curve. Hill coefficient is 0.99, based on the equation: Y=minimum+(maximum-minimum)/(1+(X/X0)^p). Each point was averaged from 6-8 cells. Blue line with black square symbols represents effect of haem alone. c. Haem (20 nM) plus NADPH significantly increased ENaC activity whereas haem at this concentration inhibited ENaC. In presence of Haem (20 nM) plus NADPH, ENaC from HUVEC with knockdown of HO-1 were inhibited whereas ENaC from HUVEC with overexperssion of HO-1 were further activated. Each column represented the mean (±SEM) of 10 cells.

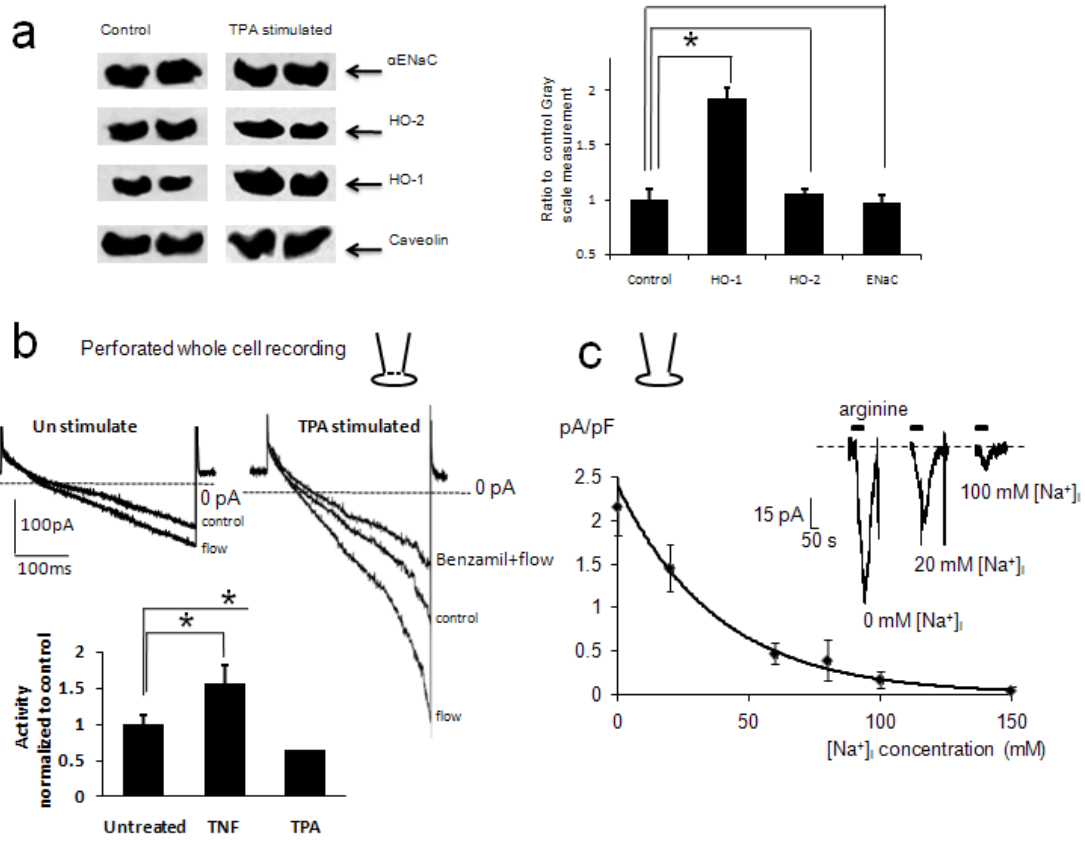
a
Control
TPA stimulated
αENaC
HO-2
HO-1
Caveolin
Ratio to control Gray scale measurement
Control
HO-1
HO-2
ENaC
b
Perforated whole cell recording
Un stimulate
TPA stimulated
0 pA
100pA
100ms
control
flow
Benzamil+flow
Activity normalized to control
Untreated
TNF
TPA
c
pA/pF
arginine
15 pA
50 s
100 mM [Na+]i
20 mM [Na+]i
0 mM [Na+]i
[Na+]i concentration (mM)

Figure 6.5

Figure 6.5 TPA facilitated flow response mediated by ENaCs in HUVEC. a. Incubation HUVEC with TPA (100 ng/ml) in medium significantly augmented HO-1 expression but not HO-2 and ENaC in 36 hours. b. stimulation of TPA significantly enhanced ENaC whole cell currents and flow response mediated by ENaC. Perforated whole cell recordings were applied in this section. In the perforated whole-cell recordings, stimulation of either TPA or TNF significantly increased ENaC whole cell current. Each column represented the mean (±SEM) of 10 cells. c. Increase of $[Na^+]_i$ attenuated the activity of CAT. The inward current evoked by extracellular L-arginine were significantly reversed by increase of $[Na^+]_i$ in a manner of dosage dependence. Each point represented the mean (±SEM) of 4-6 cells. The curve was fitted by Exp-decay (first) on the equation $y=A1*exp^{(-x/t1)}+y0$.

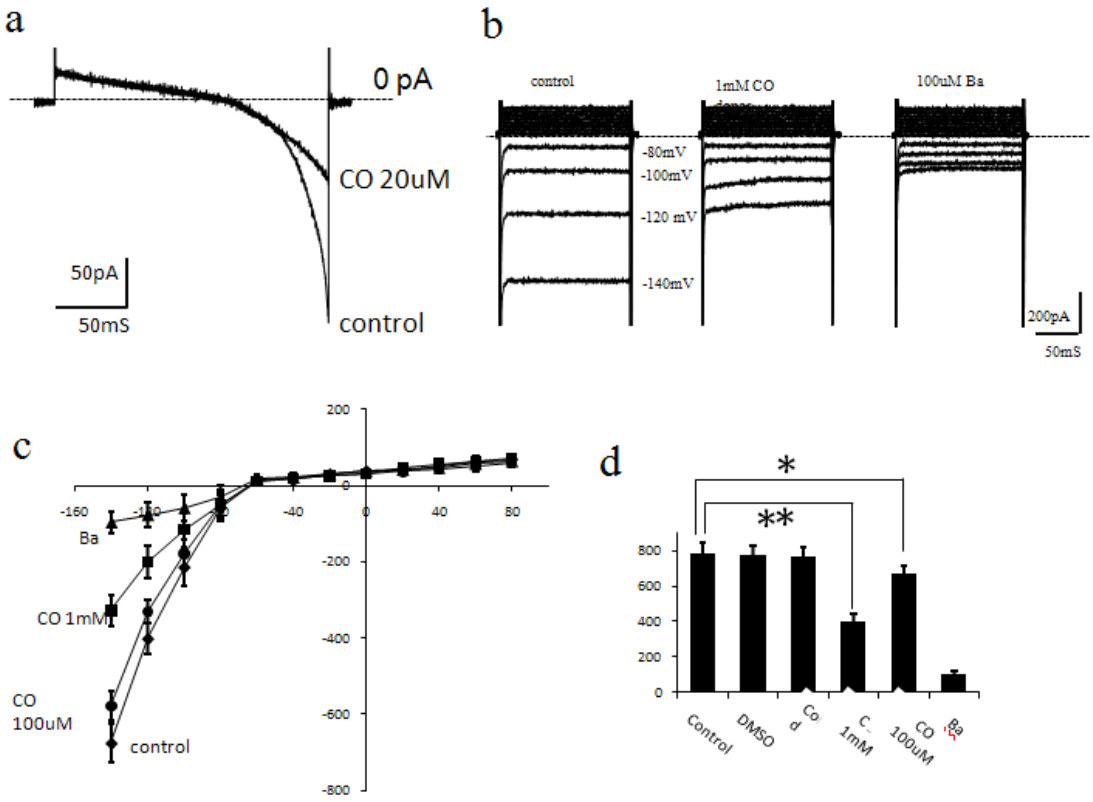
a
0 pA
CO 20uM
50pA
50mS
control
b
control
1mM CO
100uM Ba
-80mV
-100mV
-120 mV
-140mV
200pA
50mS
c
200
0
-200
-400
-600
-800
-160
-40
0
40
80
Ba
CO 1mM
CO
100uM
control
d
*
**
800
600
400
200
0
Control
DMSO
Co
C_
1mM
CO
100uM
Ba

Figure 6.6

Figure 6.6 CO inhibited IRK. a. In HUVEC cell, 20 μM CO donor significantly inhibited IRK component. b. In HEKs transfected with IRK, 1 mM CO donor significantly attenuated IRK currents and 100 μM Ba2+ further caused a reduction of IRK currents. c. different effects of 1 mM CO donor, 100μM CO donor and 100 μM Ba2+ on IRK currents. d. 1 mM CO donor, 100μM CO donor and 100 μM Ba2+ on IRK currents significant reduced IRK currents in comparing with the control and vehicle group.

Chapter 7

Discussion

7.1 The effect of lipids on ENaC

The bath application of AA in cell-attach configuration significantly reduced the ENaC open probability whereas bath application of ETYA under the same condition slightly reduced the ENaC NPo. Bath application of LA also reduced the ENaC NPo slightly. However, in the excised membrane recording configuration, where the ion channel loses its intracellular environment and AA cannot be metabolized, intracellular application of AA only induced a slight inhibitory effect on ENaC. The extent of this inhibition effect is similar to that of the both extracellular and intracellular application of ETYA and LA. This finding is consistent with the previous result that the blockade of CYP-epoxygenase pathway of AA abolished its inhibition effects over ENaC(Wei, Lin et al. 2004; Wei, Sun et al. 2006), suggesting the metabolites of AA rather than AA itself are responsible for the strong inhibition of ENaC. The slight inhibition of unchanged AA, ETYA and LA over ENaC indicate that these lipids exert only nonspecific effects on channel activity. Previous studies demonstrated that AA and LA are capable of directly modulating ion channels such as TRP (Chyb, Raghu et al. 1999), BK (Denson, Wang et al. 2000) and K^+ channels(Kim and Clapham 1989; Ordway, Walsh et al. 1989; Oliver, Lien et al. 2004), and these regulations are not resulted from alterations of the membrane fluidity. The potential explanation for the result that ENaC is not under direct regulation by these lipids in this study is perhaps due to the channel structural property.

7.2 The direct effect of PGs on ENaC

Bath application of PGE2 significantly enhanced the ENaC NPo in inside-out recordings and the effect was reversible as channel NPo returned to control level when PGE2 was washed away. Intracellular application of $PGF2_{\alpha}$ also enhanced the channel activity without affecting ENaC conductance. In addition, PGE2 at a physiological concentration of 2μM increased channel activity by over 50%. The recordings were acquired from channels that were physically isolated from intracellular environment and E-prostanoid receptor antagonists were included in the pipette solution during the recordings. Thus it is reasonable to conclude that the up-regulation of ENaC by PGs observed here are direct.

PGF2 and PGE2 are the major products of cyclooxygenase-initiated arachidonic acid metabolism in the kidney and is synthesized at high rates along the nephron (Bonvalet, Pradelles et al. 1987; Farman, Pradelles et al. 1987). PGE_2 is the major prostaglandin produced along the collecting duct and potently regulates the Na^+ and water homeostasis in kidney. Previous studies have extensively investigated their effects on epithelial solute and water transport through various E-prostanoid receptors (EP-receptors). Our results that both PGE2 and $PGF2_{\alpha}$ are able to directly stimulate ENaC activities have provided new explanation for the distinct effects of PGs on the renal Na^+ reabsorption. The dual effects of PGE2 on the epithelial solute and water transport are generally considered the results of the activation of different

EP receptors. In addition to this, PGF2 and PGE2 are also able to stimulate ENaC channel when they are present in the luminal space because of their membrane permeability. Thus, it is clear that the direct modulation of ENaC by PGs contributed to their distinct overall effects on renal epithelial reabsorption. Our finding may contributes to further explaining the following previous experiments; infusion PGs in renal filtrates increases Na^+ reabsorption (Hornych, Bedrossian et al. 1975; Lazzeri, Barbanti et al. 1995; Breyer and Harris 2001).

7.3 11,12-EET is a potent inhibitor of ENaC – both in kidney and vessels

EET inhibits Na^+ reabsorption in kidney. Both intracellular and extracellular application of 11,12-EET significantly inhibited ENaC activity and reduced channel NPo. This inhibition was reversible as channel activity returned to normal when 11,12-EET was washed off. Thus, it is apparent that EET, a product of AA metabolism by cytochrome P450 (CYP)-epoxygenase, is one potent inhibitor of ENaC and the significant inhibition effects of AA on ENaC activity is mediated by its metabolite 11,12-EET. Previous studies indicated that EETs are important regulators in vascular and renal systems. EETs have been shown to inhibit the amiloride-sensitive ion transport in renal epithelial cells and modulate Na^+ transport in kidney(Staudinger, Escalante et al. 1994; Escalante, Staudinger et al. 1995; Sakairi, Jacobson et al. 1995). Previous studies also suggested that EETs regulate Na^+ transport primarily by regulating the membrane-bound transport proteins or by interacting with other

intracellular factors such as prostaglandin synthesis pathway(Sakairi, Jacobson et al. 1995; Nusing, Schweer et al. 2007). Our finding demonstrated that EET is able to directly modulate the ENaC activity and this direct regulation is contributed to EET's overall effects on epithelial Na^+ reabsorption in kidney.

EET protect ECs by inhibiting ENaC. The inhibition of ENaC by EETs in endothelial cells underlines the diverse effects of EETs in vascular system. We found that 11,12-EET is able to inhibit the activity of ENaCs expressed in endothelial cells(HMEC). Bath application of 11,12-EET significantly decreased channel NPo in both inside-out and outside-out recording configurations, and these inhibitory effects are reversible since channel activity returned to control levels when EET was washed off. EETs are primarily synthesized in endothelial cells by CYP epoxygenases(Rosolowsky and Campbell 1996). Our subsequent studies demonstrated that ENaC is functionally expressed in endothelial cells but they are only active when intracellular environment is absent. Thus, it is reasonable to conclude that the inactivity of ENaC on endothelial cells at least partially results from intracellular inhibitors such as EETs.

One physiological role of this EETs-mediated ENaC inhibition is perhaps to maintain intracellular homeostasis. The endothelial cells are polarized cells but there is no evidence indicating their Na^+ reabsorption function. On the contrary, the endothelium is in fact function as barrier between the blood and underlying tissues. The apical Na^+ influx in endothelial cells may induce intracellular Na^+ build up and

disturb the ion composition in the interstitial space, resulting smooth muscle cells disorder or edema. Excessive intracellular Na^+ produces various pathological impacts such as disturbed cellular osmotic pressure and irregular changes in membrane potential, and the most importantly, excessive intracellular Ca^{2+} build up. One of the important roles of ion channels in endothelial cells is the fine-tuning of the electrochemical gradient for Ca^{2+}. The Na^+- Ca^{2+} exchanger, one of the diverse Ca^{2+} entry pathways, extrudes 1 Ca^{2+} ion out from the cell in exchange for 3 Na^+ entry down its electrochemical gradient. Thus a desirable Na^+ gradient is vitally important in maintaining the cellular free Ca^{2+}; a decreased Na^+ gradient induced by abnormal Na^+ influx would increase intracellular Ca^{2+}. Chronic imbalance in ion homeostasis may result in cellular dysfunction or even endothelial cell damage. Thus, under physiological condition, Na^+ channel is constantly inhibited. Furthermore, EETs have been repeatedly suggested as an EDHF (endothelial derived hyperpolarize factor) in the vascular system(Hu and Kim 1993; Harder, Campbell et al. 1995; Campbell, Gebremedhin et al. 1996; Pratt, Rosolowsky et al. 1996; Fisslthaler, Popp et al. 1999; Kroetz and Zeldin 2002; Sun, Sui et al. 2002), causing vascular relaxation in various vascular beds. Previous studies demonstrated that this EDHF effect of EETs is mainly mediated by their ability to activate BK_{Ca} channels either directly or indirectly, causing smooth muscle cell hyperpolarization which in turn produces vaso-dilation. Our finding that EETs are potent inhibitors of ENaC may help to further understand the above regulation pathway since ENaC is also suggested to participate in the relaxation of artery smooth muscle cells(Drummond, Gebremedhin et al. 2004;

Jernigan and Drummond 2005; Jernigan and Drummond 2006; Guan, Pollock et al. 2009; Jernigan, Speed et al. 2009; VanLandingham, Gannon et al. 2009). However, the interaction between ENaC, EETs and other smooth muscle ion channels need to be further investigated.

7.4 Heme is a potent inhibitor of ENaC – both in kidney and vessel

We have demonstrated that nanomolar concentrations of haem applied to the cytoplasmic surface of the membrane inhibited ENaCs in both M1 and HUVEC. Different concentrations of haem from 1nM to 10μM were applied to cytoplasmic medium to interact with ENaCs and a half-maximal inhibition concentration of 23.3nM and 51.2nM were determined respectively in M1 and HUVEC cells, and the inhibitory effects are comparable to the dissociation constant values of other hemoproteins and heme-binding proteins. Although reported values for total cellular heme vary considerably and exact concentrations of heme in the cytoplasm are not known, the evidence suggests that cytoplasmic heme reaches levels more than sufficient to elicit the effects shown in our work; Intracellular haem may increase 10-fold when 30 μM heme is present in the extracellular medium. The ubiquitous existence of haem in the circulation and renal system permit great physiology relevance to its regulation over ENaC activity. Heme is synthesized on the matrix side of the inner mitochondrial membrane and is exported to distinct intracellular compartments, most being used for the formation of haemoproteins including

hemoglobin, myoglobin, cytochromes and nitric oxide synthesis. However, heme is also pathologically liberated from heme proteins such as myoglobin and hemoglobin after cellular injury, hypoxia and/or stress, leading to an elevated content of free cellular heme, which causes various cell damages as seen in rhabdomyolysis induced myohemoglobinuric acute renal failure.

A self-protective mechanism during heme induced tubular injury. Our finding that cellular HEME inhibits ENaC activity have revealed a negative feedback self-protective mechanism by which tubular epithelial cells could alleviate the damage caused by pathologically elevated intraluminal heme content. Previous investigations in the pathology of the heme protein induced renal failure revealed that the endocytic reabsorption of heme protein by renal tubular epithelial cells is the main cause of tubular epithelia damage. The increased concentration of intraluminal heme initiated the heme protein endocytosis, inducing cellular heme elevation in tubular epithelial cells. However, as we have demonstrated, ENaCs are extremely sensitive to intracellular heme build-up since nanomolar concentration of which could cause channel open probability decrease. Therefore, it is obviously that the initial heme entry, even a small amount, impairs the Na^+ and water reabsorption, resulting in a reduced amount of total filtered load reabsorption including heme protein and an accelerated toxic substance secretion. Furthermore, the ENaC mediated diuresis is able to shorten the circulating half life of heme proteins within renal tubules, reducing the time period of intraluminal heme presence. The ultimate

goal of all these diuresis induced effects is to minimize the risk of further entry of heme into tubular epithelial cells. It is therefore rational to conclude that the tubular epithelial cells are able to protect themselves from the heme toxicity by limiting heme entry, and this protection mechanism is working in a “feedback” fashion since it can only be triggered by a small elevation of intracellular heme. This proposed feedback protection has been perhaps best proved by the observations that when the kidney GFR remains undisturbed, the nephrotoxic effects of heme are minimized. It is well established that decreased GFR either induced by intravascular volume depletion or by renal vasoconstriction is the prerequisite condition of heme induced acute tubule necrosis as seen in rhabdomyolysis induced acute renal failure. Diuresis also has been proved to be crucial in the clinical prevention and treatment of the heme protein induced acute renal failure. Volume expansion therapy, one of the most effective early treatments, is working by promoting GFR to enhance the diuresis and dilute out the intraluminal heme proteins.

Heme protects endothelial cells by inhibiting ENaC. The inhibitory effect of heme over ENaC in endothelial cells is similar to that of the EETs as described in 7.3. However, according to our results, endothelial ENaCs seem to be more sensitive to intracellular heme than it to 11,12-EET, suggesting the former is perhaps more potent in inactivating ENaC under physiology conditions in the vascular system. Thus, the physiology role of this heme mediated inhibition in vascular endothelium is perhaps also similar to that of the EETs, to maintain a desirable intracellular environment.

7.5 An oxygen-sensitive regulation mechanism of ENaC

As we have demonstrated, intracellular heme presence tonically inhibits ENaC activity. However, we have also found that when heme co-existed with NADPH and oxygen on the cytoplasmic surface of the cell membrane, its effect on channel NPo was dramatically reversed; ENaC activity was significantly stimulated. Furthermore, this stimulation was mimicked by the CO, a product of hemeoxygenase activity. Modulation of the activity of ENaC by CO resembled that seen upon co-application of heme and NADPH, suggesting that the stimulatory effect of these agents indeed reflect the generation of CO by hemeoxygenase. However, this stimulation effect did not occur under hypoxic conditions due to the fact that activation of hemeoxygenase requires adequate oxygen supply. To further investigate this regulation pathway, we performed HO-1 over-expression and knockdown experiments while keep other participators and reaction conditions unchanged. The ENaC open probability exhibited a strict HO-1 dependent manner. Thus, this regulation mechanism has been clearly present, heme together with hemeoxygenase are two potent regulators of ENaC activity both in endothelial cells and renal epithelial cells, and this regulation is strictly dependent upon oxygen tension. Our finding provided a new insight into the pathology of renal dysfunctions/failures induced by hypoxia and ischemia.

The oxygen-sensitive regulation pathway helps to protect tubular cells during hypoxia. According to our findings, the oxygen-sensitive regulation pathway may

help to sustain the intracellular ion homeostasis and cellular osmotic pressure by inhibiting the apical Na^+ entry during hypoxia induced renal dysfunctions. It is well demonstrated that hypoxia induced renal failure is associated with a significant decrease in tubular Na^+ reabsorption with undisturbed GFR or in some cases increased GFR and increased urine flow. Studies of mammalian and amphibian renal function during hypoxia indicated that when adequate oxygen supply is deprived (Kakuta, Namba et al. 1992; Cock, Wlodek et al. 1996; Peters, Tijsen et al. 1998), fractional reabsorption of Na^+ in renal tubule is significantly decreased, while both GFR and urine flow remaining stable or increased. This hypoxic inhibition of Na^+ reabsorption is in a dose dependent manner and is reversible as normoxia restored the renal function to control level (Kakuta, Namba et al. 1992; Cock, Wlodek et al. 1996). Thus it is clear that oxygen deprivation impairs the renal tubular reabsorption capacity and exerts a diuretic effect upon the kidney. Subsequent studies indicated that hypoxia induced renal reabsorption dysfunction is due to the inability of renal epithelial cells to sustain cellular ion gradients following depletion of intracellular ATP (Allis, Endre et al. 1989; Schonefeld, Noble et al. 1996). Na^+- K^+-ATPase activity relies heavily on oxidative phosphorylation for energy supply, so deprivation of adequate oxygen supply results in cellular ATP depletion which in turn down-regulates or even completely impedes Na^+- K^+-ATPase activity. The impaired Na^+- K^+-ATPase transport across the basolateral membrane of epithelial cells therefore reduces the trans-epithelial Na^+ movement (Spencer, LeFurgey et al. 1991). However, Na^+ reabsorption across the epithelial cells occurs via a two-step mechanism; apical Na^+

entry through ENaCs and basolateral extrusion by Na^+ pump. The apical Na^+ entry is the rate limiting step of the transepithelial Na^+ transport as its activity is tightly controlled. Thus, based on our finding that hypoxia down-regulates ENaC activity, we conclude that inadequate oxygen supply decreases renal Na^+ reabsorption, which at least partially contributes to limiting apical Na^+ entry. Furthermore, the slowed rate of Na^+ intake is crucial to sustain the intracellular ion homeostasis especially during prolonged hypoxic conditions. It is imaginable that if the Na^+ entry is maintained while its extrusion is strictly limited, the intracellular Na^+ would be dramatically increased in a short period of time. This abnormal intracellular Na^+ build-up induces various pathologic consequences such as cellular ion imbalance and a hypertonic cytoplasm environment which would finally cause membrane rupture. Thus, it is clear that during hypoxic conditions, a limited Na^+ entry is vitally important to protect tubular cells from further damages. In conclusion, the oxygen-sensitivity of ENaC at least partially contributes to the overall oxygen-sensitivity of tubular epithelial cell and in fact protects the intracellular environment from Na^+ overloading during hypoxia.

The role of oxygen-sensitive regulation pathway in the protection of HO-1 against reperfusion injury: Another potential role of the oxygen-sensitive regulation is indicated in the protective role of HO-1 against ischemic acute renal failure. Ischemic acute renal failure, which is due to the reperfusion injury of the kidney, accompanies acute tubular epithelial cell injure, and it is the major form of ARF. The reperfusion

injury caused ARF after ischemia is likely due to the reactive oxygen species (ROS) generated by reperfusion as a result of the rapid release of heme from microsomal cytochrome P450 (Paller, Hoidal et al. 1984). Specifically, renal tubular epithelial cells are the target in the reperfusion injury. Previous studies demonstrated that kidney adapts to oxidative stress by inducing heme oxygenase activity, mainly HO-1 (Ishizuka, Nagashima et al. 1997; Shiraishi, Curtis et al. 2000; Baan, Peeters et al. 2004; Poole, Wang et al. 2005; Goncalves, Cenedeze et al. 2006; Goncalves, Cenedeze et al. 2006). HO-1 expression is increased in the cortex or in the outer medulla immediately following reperfusion and reaches a maximum in hours (Horikawa, Ito et al. 1998; Akagi, Takahashi et al. 2005). Substantial evidence indicates that the induction of HO-1 effectively alleviates reperfusion injury whereas inhibition of HO worsens it. Since HO-1 is the rate limiting step in heme degeneration, it is generally regarded to contain cell demerge by eliminating microsomal free heme content in the tissue. According to our finding, another outcome of HO-1 mediated heme degeneration is to significantly enhance ENaC activity, which restores the crucial renal function and reduces the tissue oedema by potentating surface fluid reabsorption.

Oxygen-sensitive regulation pathway is crucial in the ENaC mediated fluid clearance in lung epithelium during perinatal period: The oxygen sensitive regulation pathway is likely to play an important role in the rapid removal of lung liquid during the perinatal period, since the process requires transient and significant increases in epithelial Na^+ conductance. Previous studies demonstrated that fetal lung is

transiently and permanently switched from a secretory to an absorptive phenotype tissue only during the very last stages of gestation and birth is associated with a fall in lung water content which begins immediately after birth and is largely complete within 2 hours. The changes in the oxygen environments are implied to be the key triggers that initiate the ENaC mediated liquid absorption process, and oxygen is a key factor that responsible for the permanent switch of lung epithelial function from secretory to absorptive. In previous studies, cAMP-mediated increase in Na^+ absorption was considered to be the main contributor of the spontaneous absorption of lung liquid during labour (O'Brodovich, Hannam et al. 1990; Walters, Ramsden et al. 1990). Thyroid hormone (T3), adrenaline and glucocorticoid hormones were also believed to be involved in the process. Other hormones that are able to slow secretion or accelerate absorption in the fetal lung at birth by regulating ENaC include vasopressin PACAP, serotonin and glucagon. However, none of the hormonal regulation mentioned above could comprehensively account for the rapid epithelial permeability increase after birth. Since the fetal lung fluid absorption is a very swift process, which happens immediately after birth and finish within hours, both hormone-mediated elevations in ENaC subunit mRNA and surface expression are unlikely to account for such dramatic and transient increase in Na^+ transport. Physiological increase in PO_2 can only activate ENaC subunit promoter after PO_2 has been raised for 24-48 hours (Baines, Ramminger et al. 2001). Furthermore, accumulating evidence indicates that the oxygen-evoked increase in Na^+ conductance happens before any elevation in ENaC mRNA abundance or in some cases with no

change in ENaC mRNA abundance in fetal distil lung endothelial cells (Pitkanen, Tanswell et al. 1996; Richard, Ramminger et al. 2003). Nevertheless, oxygen was able to stimulate Na^+ conductance in hormone free medium (Richard, Ramminger et al. 2003). In conclusion, all these results suggest a mechanism which is able to enhance the Na^+ conductance immediately and directly before the hormonal-mediated effects when there is a change in the ambient oxygen content. Based on our finding that HO-1 and heme can significantly increase ENaC activity only during the presence of oxygen, it is rational to propose that the oxygen, heme and HO-1 regulation pathway is the underline mechanism by which oxygen, at least partially, initiated the transient and significant increase in Na^+ transport during the perinatal period. To further substantiate the hypothesis, the availability of the other two factors, namely heme and HO-1 in lung epithelial cells need to be evaluated. The expression of HO-1 in lung has been well investigated; it is expressed in various cells in lung such as alveolar type II cells and its expression is highly inducible. The exact cellular concentration of heme in lung epithelial cells is unknown, but it is still reasonable to assume that the cytoplasmic heme reaches the level to permit the reaction because; the ubiquitous presence of heme in body and the extreme low quantity of heme required for the reaction. However, further works are required to completely validate the hypothesis such as determining the exact expression rate of HO-1 and heme in lung epithelial cells during perinatal period and investigating the lung ENaCs activity under different ambient oxygen contents.

7.6 Identify ENaCs in endothelial cells

ENaC is functionally expressed in ECs. α- but not β- and γ- ENaC subunit expression were detected in cultured endothelial cells. This homolegous ENaC channel composed by only α-subunits exhibited similar biophysical characterizations as seen in other tissues where all ENaC subunits are expressed. ENaC single channel currents were examined and the current and voltage relation for this channel has been determined. Current-voltage (I-V) curves obtained from either inside-out and outside-out exhibited a linear slope and when 120 mM of Na^+ medium was replaced with low Na^+ medium (5 mM) in bath the I-V curve was shifted to the right which displayed a $[Na^+]_i$ dependence of channel conductance. The channel in cultured endothelial cells possess a conductance approximately 4.83 pS which is similar to that of the channels found in the mouse collecting duct cells where all α-, β- and γ-ENaC are expressed. To further indentify this channel, pharmacological experiments were performed. Extracellularly applied amiloride at the concentration of 0.5μM induced flickering current and reduced the Po, whereas 5μM bath amiloride almost abolished ENaC currents. Thus it is clearly that ENaCs expressed in endothelial cells exhibit similar biophysical properties; high selectivity for sodium over potassium, high-sensitivity to amiloride, a unitary conductance of 4.7 pS and voltage independence of both the open probability and conductance. However, the currents mediated by these channels exhibited slightly more flickering pattern and shorter dwell time in compare to the currents obtained from mouse collecting duct

cells. To further determine whether the amiloride sensitive currents were mediated by ENaC channels, specific knockdown experiments against the α-subunit were performed. Consistent with the significant decrease in mRNA and protein expression, the probability to detect the current with such conductance dropped nearly 8-fold, indicating that these small conductance inward currents were indeed mediated by ENaC.

ENaC is constantly inhibited in ECs. When investigating channel activity by patch clamping, ENaC currents were rarely acquired in cell-attached configuration. However, after excising the patch of cell membrane enclosed in the pipette tip from the cell, ENaC currents were immediately observed in most of the cases. The explanation for this phenomenon is that certain intracellular molecules are constantly exerting inhibitory effects on the channel. According to our previous results, heme and 11,12-EET are such intracellular inhibitors since HO-1 induced heme degradation completely liberated ENaC from inhibition.

We concluded that this inhibition of ENaC in ECs is essential to protect ECs from various damages induced by cellular ion imbalance (detailed discussion in 7.3). For example, intracellular Na^+ elevation induces Na^+-K^+ ATPase hyperactivity, causing excessive potassium lost in the interstitial space between smooth muscle cells and endothelial cells. Furthermore, high cellular Na^+ content interfere with membrane-bound transporters which utilize Na^+ entry as driving force such as Ca^{2+}

pump. Thus, ENaC inhibition is important in maintaining intracellular ion homeostasis.

7.7 Investigations of the machano-sensitivity of ENaC in endothelial cells

The DEG/ENaC superfamily members have long been known for their mechano-transducer ability. However, there is no direct evidence that native ENaCs can be activated by mechano-stimulation. We have thoroughly investigated the ENaC mechano-sensitivity and we found that various mechano-stimulation, including membrane stretch, shear force and perhaps membrane deformation are able to activate channel. Pressure and shear stress are the main hemodynamic forces which are directly induced by blood flow and are responsible for the triggering of the endothelium-derived vasodilatation. Thus the stretch test performed by applying positive pressure was conducted to evaluate the effect of blood pressure on ENaCs and the flow test was performed to varify the effects of shear stress on ENaCs. By performing this intensive investigation, we have acquired the most direct and comprehensive evidence that ENaC expressed in endothelial cells are activated by both mechanical forces. As we have demonstrated in HMEC cells and HUVEC cells, when the intracellular inhibitors were absent, flow and membrane stretch are able to either directly or indirectly enhance the open probability of ENaC without affecting channel conductance. In HMEC cells, single ENaC channel open probability in outside-out configuration was significantly increased by exposure to the shear flow.

In the HUVEC cells, single ENaC channel open probability was significantly increased by mechanical stretch and exposure of the intracellular membrane to shear flow. It is noticeable that the flow induced increase in channel NPo was obtained from cell-attach recording configuration where target ENaC channels were in fact isolated from the bath environment and there is no direct contact between the channels and flow (mechano stimulation).

The molecular basis of the mechano-sensitivity of ENaC: Based on our results that ENaC is mechano-sensitive in the excised cell membrane, we believe that ENaC can be, at least, activated by cell membrane tension and curvature.

MEC channel in C. elegans touch receptor neurons, which is composed of DEG subunits MEC-10 and MEC-4, was the first recognized mechano-transducing channels in animal cells. Although exact gating mechanism of the MEC channel still remains unclear, most of the researchers believe that this channel is gated by a “tethering” mechanism in which the ion channel binds either to the cytoskeleton or the extracellular matrix, thus any disturbance of this network would stress the structure and hence gate the channel. Several associated proteins have been indentified on both the intracellular and extracellular side of the membrane and are believed to either directly or indirectly interact with the channel core complex. These proteins are required to maintain the localization of the channel on the plasma membrane, and also participate in the channel gating during mechanical stimulation. The

associated protein is responsible for stressing the channel pore structure and hence gating the channel during mechano-stimulation (Markin and Hudspeth 1995; Markin and Hudspeth 1995; Garcia-Anoveros and Corey 1997; Bianchi 2007). Although the fact that DEG subunits share a common topology with ENaC subunits was the first hint of the mechano-sensitivity of ENaC, the molecular basis of their mechano-transduction mechanism is unlikely to be same, as there is no evidence that ENaC has requires associated protein as the MEC channel does. Another possible scheme is that releasing of signal molecules from other cells or cellular organelles during mechano-stimulation activates ENaC (Welsh, Price et al. 2002). However, this model is unequivocally rejected in this case since ENaC in the excised membrane exhibits mechanical-sensitivity. In addition, this hypothesis is also excluded by the rapid response of ENaC to mechano-stimulation both in whole cell and cell attach configuration. Thus, the most possible explanation would be the cell membrane tension and/or curvature caused by mechano-forces directly controls the channel gating.

The evidence supporting the idea that membrane tension activates ENaC can be summarized as: 1), the biophysical characterizations of lipid bilayers; 2) the observation that ENaC is symmetrically activated by both negative pressure and positive pressure in membrane patching; 3) the fast activation and slow inactivation during mechano-stimulation 4) evidence that reconstituted ENaC in lipid bilayers exhibits mechano-sensitivity. Accumulating evidence suggests that the lipid bilayers

are capable of rapidly developing tension during mechano-stimulation and the bilayers tension exerts stress on the inserted ion channels. The stress transduced by the cell membrane may affect the channel structure and hence gate the channel. Furthermore, the flexibility of lipid bilayers can be described as fast expansion but slow restoration, which means the membrane tension is quickly developed but slowly dispelled. In vivo, this characterization is manifested by the fast activation and slow inactivation of channel, as seen in our results (chapter 6). Based on all this evidence and reasoning above, we conclude that ENaC is gated by membrane tension.

The understanding of mechano-sensitivity of ENaC in endothelial cells can help to elucidate the role of this channel in other mechano-transducing tissues. For example, β- and γ- ENaC subunits are found to be expressed in the mechano-transduction site in baroreceptor nerve terminals and are suggested to be components of the baroreceptor mechano-transducer as an amiloride analog could block the baroreceptor nerve activity and baroreflex control of bold pressure (Drummond, Welsh et al. 2001; Drummond, Gebremedhin et al. 2004). RT-PCR results indicated that α-, β- and γ- ENaC subunits are expressed in rat trigeminal ganglia and immunocytochemistry results demonstrated that α-, β- and γ- ENaC are localized in the perikarya of the trigeminal neurons and in a minor fraction of the neurons termination site in the vibrissal follicle-sinus complex (Fricke, Lints et al. 2000), suggesting a possible role of ENaC in mechano-transduction in these neurons.

However, the mechano-transduction is poorly understood on the molecular level. There is no consensus in the gating of mechano-sensitive ion channel and different channel seems to have different gating mechanism. Thus, this area still needs extensive studies in future.

7.8 ENaCs are localized within caveolae and co-localized with caveolin, HO-1 and HO-2

Our results that ENaC channels are co-localized with caveolin, HO-1 and HO-2 indicates that HO-1 and ENaC are sufficiently closely associated in vivo to enable the oxygen regulation pathway to function and this co-localization may provide a novel insight into the role of CO as an important endogenous gaseous signal molecule.

The close association of ENaC and HO-1 further validates the oxygen-sensitive regulation pathway. The spacial distribution of HO-1 in cell membrane is an important factor in validating the HO-1 and oxygen regulation pathway since ENaC is activated by the byproduct of the HO-1 mediated heme degradation. The close spacial association of HO-1 and ENaC grants immediate and reliable regulation.

The generation site of second messengers is important in its downstream regulation, especially under the circumstances that the production rate of certain signal molecules is extremely low or their biophysical properties are unstable, for example

Ca^{2+}. Transient and dramatic regional Ca^{2+} elevation is believe to be crucial to initiate many high-threshold Ca^{2+} processes when the cellular free Ca^{2+} level remains constant in the rest of the intracellular space. In the heme degradation case, with the close association of ENaC and HO-1, a transient and regional generation of CO could lead to immediate and dramatic ENaC activation.

ENaC is one of the most immediate recipients of CO. CO, an endogenous gaseous signaling molecule has drawn more and more attentions in the most recent decade. It has been suggested to play important physiological roles in many tissues. Our finding that ENaC is closely located to the CO generation site and ENaCs are potently regulated by CO indicated that ENaC, at least in vascular system, is one of the most immediate recipients of this gaseous messenger. This finding contributes to elucidating the diverse functions of CO and the spatial distribution determination approach could be an effective research strategy in identifying the potential CO recipient ion channels.

7.9 ENaCs are acting as mechano-transducers when inflammatory factors are present.

Inflammatory factors release ENaCs from inhibition. To further validate the physiology role of ENaC mediated shear force sensing, we found that TPA as a common inflammatory factor, is able to release ENaC from inhibition by enhancing

HO-1 expression. The original hypothesis is that HO over-expression as a result of TPA stimulation leads to an increase in CO production, which may compensate the inhibitory effects of both heme and 11,12-EET. The experimental results confirmed the hypothesis. The western blot results indicate that the expression level of HO-1 and HO-2 were significantly enhanced. The ENaC mediated Na^+ currents were observed both in whole cell and cell attach configuration, suggesting that ENaC is activated when the intracellular environment remains undisturbed. In addition, the whole cell currents obtained from the cells deprived of intracellular medium exhibited a similar increase. Thus, it is clear that inflammatory factor induced HO-1 neutralized the effects of the cellular inhibitory molecules. In other words, under certain conditions, ENaCs are released from inactivation and start to act as mechanotransducers.

ENaC serves as a mechano-transducer when inflammatory factors are present. Whole cell recordings were performed to evaluate the effects of flow-induced shear force on ENaCs under physiological conditions. HUVECs were incubated in inflammatory factors. The results demonstrated that ENaC activity was stimulated by shear flow application in a dosage dependent manner, suggesting that native ENaC is able to sense flow-induced mechano-stimulation. The mechano-transduction process in this case can be described as flow-induced shear force is converted to Na^+ influx by ENaC channel. This Na^+ influx, however, can cause many cellular responses as listed in the following sections.

7.10 $[Na^+]_i$ elevation impairs cationic amino acid transporter (CAT) activity

We have demonstrated that the ENaC mediated Na^+ influx impairs the CAT capacity, causing a decreased L-arginine transportation and a subsequent decrease in NO generation as eNOS requires L-arginine as a substrate. This observation may underly the pathogenesis of endothelial dysfunction seen in the presence of proatherogenic factors such as seen in inflammation and hypertension. It also suggests that the overall impact of the activation of endothelial ENaC is likely to be negative.

It is well established that NO is not only a potent vasodilator but also inhibits platelet aggregation, smooth muscle cell migration and proliferation, monocyte adhesion and adhesion molecule expression, thus protecting the vessel wall against the development of atherosclerosis and thrombosis (Busse, Luckhoff et al. 1987; Botting and Vane 1989; Furchgott and Vanhoutte 1989; Mugge, Forstermann et al. 1989; Faint 1992; Simon, Stamler et al. 1993; Vanhoutte 1993; Vanhoutte, Boulanger et al. 1995). The incompetence of endothelial cells to produce sufficient NO may lead to the initiation of vascular deseases such as atherosclerosis. There is strong evidence that endothelial dysfunction is closely releted to inflammation and hypertension. For example, in hypertensive subjects with hyperaldosteronism, endothelium dependent flow-mediated vasodilatation is impaired (Nishizaka, Zaman et al. 2004). A decreased L-arginine conversion to NO is shown in these hypertensive patients after salt loading (Ni and Vaziri 2001). There is also convincing experimental evidence

suggesting that proinflammatory factors lead to a reduced bioavailability of endothelium-derived NO (Bhagat, Moss et al. 1996; Hingorani, Cross et al. 2000; Kharbanda, Walton et al. 2002). In addition, human studies have demonstrated that acute proinflammatory stimuli cause short-term impairment of endothelium-dependent dilatation in arteries and resistance vessels (Hingorani, Cross et al. 2000). The following studies further demonstrated that high-dose aspirin treatment prevents the development of endothelial dysfunction induced by such stimuli (Kharbanda, Walton et al. 2002). Previously, the dysfunction of ECs manifested by a decreased NO production was mainly believed to be as a result of the downregulated expression of eNOS (Vallance, Collier et al. 1997; Verma and Anderson 2002). However, based on our findings, the reduced L-ariginine transport resulted from the activation of ENaC contributes, at least partially, to the EC dysfunction under the pathological conditions. Therefore, we suggest that activation of endothelial ENaC exerts complicated effects on the vascular responses to flow; however, the overall outcome is more likely to be negative.

7.11 A possible role of ENaC in myogenitic tone

The ENaC's effects on endothelial cells are in favor of vasoconstriction. Thus it is likely that the ENaC may contribute to maintain the myogenic tone. Myogenic tone is a major contributor to vascular resistance to blood flow and blood pressure. Previous studies indicated that various vasoconstrictors as well as mechano-stimulations are

able to cause smooth muscle cells depolarization and hence vasoconstriction. The vasoconstriction could be both endothelium-derived and endothelium-independent, for example, hormones such as vasopressin cause vasoconstriction through endothelial cells whereas intravascular pressure increase induced vasoconstriction is endothelium-independent (Meininger & Davis, 1992). Either mechanism exerts their effects by increasing the intracellular Ca^{2+} content in smooth muscle cells. ENaC mediated Na^{+} increase in endothelial cells, as we have demonstrated, impairs both NO and EDHF production, resulting in a depolarized smooth muscle cells and an increased intracellular free Ca^{2+} concentration. Thus it is reasonable to presume that ENaC may contribute to maintain the vessel tone and blood pressure.

7.12 CO, the by-product of heme degradation inhibits inward-rectifying K^{+} channel (IRK)

We have demonstrated that CO inhibited IRK current in HUVECs and in HEK cells expressed with IRK. This finding may help to understand the role of inflammation in the initiation, complication and progression of atherosclerosis. Inflammation has long been recognized as a pivotal factor in the pathogenesis of atherosclerosis. The presence of inflammation factors not only leads to an increased expression of cell adhesion molecules, cytokines and chemokines, which facilitate leukocyte attachment to the endothelium and transmigration into the extracellular matrix, but also cause endothelial dysfunction, resulting in an impaired endothelial-dependent

vasodilatation (Bhagat, Moss et al. 1996; Davis, Yeung et al. 1996; Vallance, Collier et al. 1997; Hingorani, Cross et al. 2000; Kharbanda, Walton et al. 2002; Stocker and Keaney 2004; Pepys, Hawkins et al. 2005). This incompetence of the endothelial cells to initiate dilatation was previously considered to result from the reduction in NO generation as described in 7.10. However, recent NOS knockout experiments in mice suggest that endothelium-derived hyperpolarizing factor (EDHF) is also one important instrument by which ECs induce SMC relaxation since the EDHF-mediated vessel dilation compensated for the absence of endothelial NO. Furthermore, following studies suggest that EDHF rather than NO plays an increasingly prominent role in the vascular response to shear force as arterial diameter decreases; especially in resistance vessels where NO-independent vasodilation plays a dominant role. Therefore, insufficient NO production alone can not account for the impaired endothelial-dependent vasodilatation.

Our finding suggest that the inflammation induced endothelial dysfunction is a collective result of insufficient NO production and impaired EDHF generation and release. Earlier studies have demonstrated that an increase in K^+ conductance is among the most immediate cellular responses to shear force which in turn triggers the generation and release of the EDHF by ECs. This increasing in K^+ conductance has been proved as a result of the activation of IRK channels because of the observations that shear stress activates IRK current in aortic endothelial cells and in oocytes and HEK cells expressed with exogenous endothelial Kir2.1 channels. Furthermore, we

have demonstrated that proinflammatory factors upregulate HO expression, which in turn accelerates heme degradation and increases CO production. The excessive CO generation inhibits KIR and subsequently blunts the shearforce sensing, causing an impaired EDHF function in endothelial cells. Thus, we conclude that inflammation impair the endothelial-dependent vasodilation by disrupting both the endothelial-derived NO and EDHF action.

7.13 A comprehensive discussion about an ENaC mediated shear force sensing mechanism in endothelial cells and its physiological impact

7.13.1 A summary of experiments results

1, ENaCs are expressed in vascular endothelial cells.

2, ENaCs are active only when their intracellular environment is absent

3, 11,12-EET and heme potently inhibit ENaC from the intracellular membrane

4, ENaC is mechano-sensitive

5, HO-1 induction neutralize the inhibition of ENaC

6, HO-1 can be induced by inflammatory factors

7, endothelial expressed ENaC can serve as mechano-transducers when inflammatory factors are present.

8, CO, the byproduct of heme degradation by heme-oxygenase, inhibits inward-rectifying potassium channel.

7.13.2 Inflammation shifts the endothelial cell shear force sensing from IRK-mediated to ENaC-mediated

7.13.2.1 Inflammation impairs the IRK-mediated shear force sensing

Earlier studies have demonstrated that shear stress activates the inward-rectifying K+ current and increases cell membrane permeability to K^+. Following studies have further demonstrated that shear stress causes an increase in K^+ current in oocytes and HEK cells expressing the exogenous endothelial Kir2.1 channel. These previous studies indicate that the IRK channel is one of the sensing elements for shear stress, and the IRK-mediated K^+ conductance increase is among the most immediate cellular responses to shear force. The increase of IRK mediated membrane permeability to K^+ leads to endothelial cell hyperpolarization, resulting in a facilitated Ca^{2+} entry by increase its driving force, which in turn initiates the generation and release of the endothelium-derived EDHF and EDRF, casing the underline SMC relaxation and hence vasodilatation. Based on these results, it is obvious that the activation of IRK channel is the prerequisite in the whole endothelium-dependent vasodilatation in. Therefore, the inhibition of IRK by inflammation severely desensitizes EC shear force sensing and blunt the ability of vessel to adapt flow. However, in this case, Na^+ channels start to assume the duty of shear force sensing and EDHF generation.

7.13.2.2 ENaC mediated shear force sensing replaced the IRK mechanism during inflammation

Similar to the EC expressed IRK channel, endothelial ENaC is also mechano-sensitive, and able to transfer the mechano-signal to bioelectrical signals. We have demonstrated that during the presence of inflammation factors, an increase in Na^+ conductance predominates the first setting of cellular response to flow. The ENaC mediated flow sensing can be described as follow; shear stress activates ENaC and causes an increased Na^+ entry rate. To extrude excessive in tracellular Na^+, the activity of the Na^+-K^+ exchanger is facilitated. The hyperactive basolateral Na^+-K^+ pumps extrude Na^+ into and absorb K^+ from the myo-endothelial interstitial space by a ratio of 2/3 (Na^+/K^+), causing a reduced K^+ content in the interstitial space. The interstitial fluid is the extracellular fluid for smooth muscle cells and the decrease in extracellular K^+ causes smooth muscle cells hyperpolarization and subsequently relaxation. However, this ENaC mediated NO independent vaso-dilation is incapable of adapting to the rapid changes in blood flow and is cytotoxic in the long run.

7.13.3 The overall impact of ENaC mediate shear force sensing mechanism: the first step toward atherosclerosis

The ENaC mediated inadequate vaso-dilation may serve the vascular shear force sensing temporarily, however, it causes negative consequences to endothelial cells in the long term. The excessive Na^+ influx is toxic to endothelial cells. Since the

epithelial Na^+ channel is a “leak channel”, and the electrochemical gradient across the endothelial cell membrane is in favour of Na^+ entry, the release of endothelial ENaCs from inhibition leads to an increased Na^+ influx even though the ECs are at rest. This Na^+ in flux has multiple negative consequences; 1, it may disturb the resting membrane potential of ECs, causing ECs to constantly stay at a slightly depolarized state; 2, elevated cellular Na^+ is in favour of Ca^{2+} retention as described in 7.9, high cellular Ca^{2+} may result in various negative effects including cell damage; 3, high cellular Na^+ reduce the NO generation. Previous studies have demonstrated that NO is produced by endothelial cells not only in response to stimulation but also at rest. The constant NO generation is critical in the inhibition of platelets aggregation and adhesion molecules expression, and is helpful to maintain vessels at a relatively dilated status by preventing excessivel vasoconstrictions. Once the endothelium-dependent NO production is hampered, the progression of atherosclerosis will be accelerated. Nevertheless, since the ENaC is mechano-sensitive, the presence of shear stress will only exaggerate the process and augment the reverse effects.

In conclusion, the activation of ENaC exerts multiple negative effects on endothelial cells including damage to the cell and cell function. Persistent activation of ENaC will inevitably lead to vascular diseases such as atherosclerosis.

7.14 Conclusion

Epithelial sodium channels (ENaC) are of immense importance, controlling Na^+ transport across epithelia and thus playing a critical role in all aspects of fluid clearance as well as numerous other functions. Disorders of ENaC lead to various pathological conditions such as water and electrolyte imbalance which finally results in cardiovascular diseases e.g. hypertension. By employing electrophysiology and molecular biology techniques, we have identified profound regulation mechanisms of ENaC in distal renal epithelial cells and vascular endothelial cells by lipid metabolites, heme and mechanical forces. Our results also revealed a novel O_2 sensitive regulation pathway of ENaC channels, in which hemeoxygenase acts as the O_2 sensor and the substrate and product of which either inhibits or stimulates ENaC activity. This mechanism may eventually introduce novel clinic treatments for disease such as renal failure, kidney reperfusion injury, hypertension, pulmonary edema and pre-clampsia. Additionally, I have identified the expression and the mechano-sensitivity of ENaC in endothelial cells, determined the effects of inflammatory factors on HO-1 expression, and validated the effects of the HO-1 mediated heme degradation pathway on both ENaC and Kir channels. By piecing all these findings together, a scenario clearly appeared. ENaC is expressed on endothelial cells, and this expression not only retained the channel function as a Na^+ entry pathway but also possesses mechano-sensing ability. However, the reason why the physiological role of this expression has not been noticed is that ENaCs are

constantly inhibited by certain cellular contents in ECs. ENaCs are kept silent until their environment changes, in this case, the appearance of inflammation. Pro-inflammatory factors greatly upregulate the highly inducible protein HO-1, which in turn facilitates heme degradation and hence increases CO generation. CO potently increases the bioactivity of ENaC, releasing the channel from inhibition, while on the other hand exerts a strong inhibitory effects on Kir channels. This aspect of CO's impact on ECs can be therefore described as that CO shifts the duty of mechano-sensing from Kir to ENaC. Eventually, endothelial cells start to response to shear stress by increasing the Na^+ influx rate. The ENaC mediated shearforce-sensing mechanism under inflammatory conditions, however is unfortunately not a supplement or subsidiary to the shearforce sensing of the endothelial cells; on the contrary, it acts as a bridge that "direct" the pro-pathogenesis factors such as inflammation to endothelial cell dysfunction and even damage, which eventually lead to various vascular diseases.

Appendix 1: the chapters which have been published as papers

Appendix 2: published papers not included in the chapters

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