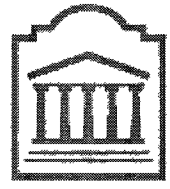

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Alternative Splicing Regulation of the Epithelial Sodium Channel (ENaC) in Dahl Rats

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Alternative splicing regulation of the epithelial sodium channel (ENaC) in Dahl rats

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Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements for the degree of

Ph.D. in Cellular and Molecular Medicine

Department of Cellular and Molecular Medicine

Faculty of Medicine

University of Ottawa

Library and Archives Canada

Published Heritage Branch

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Bibliothèque et Archives Canada

Direction du Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-74249-5
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ISBN: 978-0-494-74249-5

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Abstract

The epithelial sodium channel (ENaC) is critical in controlling the rate of renal sodium reabsorption and maintaining long term blood pressure control. ENaC activity is twice as high in kidneys of high salt-fed Dahl salt-sensitive (S) rats *versus* a sister salt-resistant strain (Dahl R), which might explain the increased blood pressure in high salt-fed Dahl S rats *versus* R rats. ENaC blockade in the brain by benzamil rescued Dahl S rats from salt-induced hypertension. The aims of the present study were: (i) To test whether Dahl S rats harbor genetic polymorphisms in the ENaC α, β, and/or γ genes that might contribute to their enhanced ENaC activity; (ii) To investigate whether α ENaC in Dahl rats' kidney is associated with alternatively spliced forms, and their corresponding mRNA levels, should they exist, in Dahl S *versus* R rats on normal and high salt diet; (iii) To examine the putative biological function of α ENaC alternatively spliced forms when co-expressed with α ENaC-wt. The first comprehensive sequence analysis of ENaC genes did not reveal any differences between Dahl S and R rats that were isogenic in the entire coding regions, exon-intron junctions, 3' and 5' flanking regions of ENaC α, β, and γ genes. Two coding (a and b) and two non coding (c and d) α ENaC alternatively spliced forms were identified whose mRNA levels were elevated in Dahl R *versus* S rats. Among the four α ENaC transcripts, the salt-sensitive α ENaC-b was highly abundant exceeding α ENaC-wt abundance by ~32 fold. The translated α ENaC-b protein sequestered α ENaC-wt and reduced α ENaC-wt expression in a dose-dependent manner. Increased ENaC activity in Dahl S *versus* R rats might be attributed to the lower abundance of α ENaC-b, a dominant negative expression regulator of α ENaC.

Authorizations for the Use of Published Material

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Biochemistry Insights (one article) – Appendix C

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List of Abbreviations

α= alpha

β= beta

BP= Blood pressure

C-terminus= carboxy terminus

cDNA= coding DNA

CHEP= Canadian Hypertension Education Program

CSF= cerebral spinal fluid

Dahl R= Dahl salt resistant

Dahl S= Dahl salt sensitive

DHPLC= Denaturing High Performance Liquid Chromatography

EDTA= ethylenediamine tetraacetic acid

ENaC= Epithelial Sodium Channel

γ = gamma

HR= Heart rate

mRNA= messenger RNA

PCR= polymerase chain reaction

Q-RT-PCR= Quantitative real-time PCR

RT-PCR= Reverse transcriptase-polymerase chain reaction

NH_2 terminus or N-terminus= amino terminus

SNP= single nucleotide polymorphism

TM= transmembrane domain

UTR= untranslated region

Acknowledgments

I sincerely thank my Master's thesis supervisor Dr. Alan Pater (Memorial University of Newfoundland) who has reviewed several components of my current thesis and generously provided me with much needed guidance and support.

From the University of Ottawa, I would like to extend my gratitude and appreciation to a number of researchers who have contributed to my doctoral thesis work. These researchers include: Drs. F. Leenen, F. Tesson, J.V. Huysse, R. Roberts, B. Tuana, A. Stewart, J-M. Renaud, S. Lee, P. Gupta, H. Wang, S. Amin, E. Reza and M. Ahmad.

From the University of Ottawa Faculty of Graduate and Postdoctoral Studies stand Drs. G. Slater, I. Makaryk, M. Moriarty, B. Vanderhyden, R. Slack as well as the Dean Faculty of Medicine Dr. J. Bradwejn for their support of my PhD thesis work.

I also would like to thank the colleagues I worked with, whose company has been inspiring and whose friendship will always stay in my heart. Pierrette Bolongo, Becky Grimwood, and Tracy Jackson – my faithful Christian friends and prayer worriers, and Allen Teng – God sent angel to guide me in the toughest stage of my thesis, and last but not least, Mireille Gervais whose enthusiasm and support will be engraved in my heart and mind forever.

My deepest gratitude goes to my sister Eng. Marian and my brother Dr. Fady for being there by my side during the many ups and downs over the last 7 years and for their expert guidance through many predicaments. You have never ceased to make me laugh when I was really down.

To my dearest Christian parents and precious jewels, Drs. Sozit and Fouad Shehata, I LOVE YOU. Thank you for not letting me quit the 100 or so times I thought about it. Your encouragement, love, kindness, generosity and faithful prayers helped me make it to this stage.

I whole-heartedly thank Dr. Faten and Julia for their prayers, advices and spiritual guidance through the whole process. I owe a debt of gratitude to their love and tremendous assistance. Thank you for all your patience, guidance and positive attitude. Your encouraging demeanor and support have been an invaluable addition to my PhD experience. Your amazing instruction and your willingness to interrupt your clinic work, church functions and business meetings to assist and guide me in various decisions pertaining to my PhD work will be engraved in my memories forever.

The past three years have been particularly tough for multiple reasons and I want to thank Eng. Samir, Magdy, Michael, Carmen, Donna, Katie and Eng. Gamal, as well as all church friends at the Hadaek El Koba Presbyterian Church in Cairo - Egypt, Bethesda Pentecostal Church in St. John's - Newfoundland, Cedarview Alliance Church and Altavista Baptist Church in Ottawa. In particular, I would like to thank Elders Nageeb Wahbah and Shoukry Amin (Cairo), Karen Regular and Elsie Wright (St.John's), and from Ottawa, Pastor Dorothy, Lorne & Vivian Anderson, Deaconess Ann Robertson, Deaconess Edith Sparrow, and Pastor Waleed & Rima Bitar whose unconditional love and prayers helped me recover from a tremendous spiritual warfare.

Above all, I praise my rock of salvation **Jesus Christ** who has been my **S**trenght in my weakness and my Shield in my spiritual warfare, my **H**ope when there seemed to be no hope, my **E**mannuel (God with us), my **P**ortion, my Joy in the midst of tribulations, my **H**oliness and my righteousness, my **E**verlasting king, my **R**ock, my **D**eliverer, my Strong Tower and my All in All. He is my good **SHEPHERD**. His faithful promises to me throughout my PhD journey are: *"Do not fear, for I am with you; do not be dismayed, for I am your God. I will strengthen you and help you; I will uphold you with my righteous right hand. All who rage against you will*

surely be ashamed and disgraced; those who oppose you will be as nothing and perish. Though you search for your enemies, you will not find them. Those who wage war against you will be as nothing at all. For I am the LORD, your God, who takes hold of your right hand and says to you, Do not fear; I will help you." Isaiah 41: 10-13

LORD JESUS, I adore You for who You are; victorious, almighty, redeemer and the lover of my soul. I thank You for winning the battle. If it were not for You and for Your goodness and mercy, this thesis would not have come to fruition. Therefore, I give you back all my life, work, worship and adoration. You are truly **WONDERFUL!**

Prelude to Thesis

The pathogenesis of salt-sensitive hypertension involves a complex combination of genetic and dietary factors that influence salt retention and excretion. The genetic and molecular bases of salt-sensitive hypertension are not fully understood. Therefore, understanding how specific genes contribute to salt-sensitive hypertension, as well as the complementary molecular mechanisms that impact how genes function is an exciting avenue for the study of genetics. The premise of the following genetic analysis study was to screen for sequence variability in genes related to salt-sensitive hypertension such as the epithelial sodium channel alpha, beta and gamma genes (ENaC α, β, & γ genes). Screening for sequence variability was performed in genetically-determined models of salt sensitive hypertension namely Dahl salt sensitive (S) rats and compared to their normotensive counterparts, Dahl salt resistant (R) rats. Following the above comparative sequence analysis study between hypertensive and normotensive rats, a study of the existence of α ENaC alternatively spliced forms as well as the putative mechanisms by which these spliced forms might influence salt retention and/or excretion was explored. Given that some people are resistant to salt-sensitive hypertension and some are not, identifying the underlying molecular mechanism to combat salt-sensitive hypertension is clinically relevant. In addition, from a treatment perspective, it would be valuable to know if subjects with salt-sensitive hypertension might be diagnosed earlier based on their unique genetic profile, and if salt-sensitive patients in whom alternative splicing is involved in their pathogenesis might be subjects for specific pharmacotherapeutic treatments.

This thesis outlines the relevance of salt-sensitive hypertension as a disease state, describes the selection of ENaC α, β, & γ as candidate genes in salt-sensitive hypertension and examines closely ENaC regulation via RNA alternative splicing, through 4 manuscripts looking

at the significance of ENaC in blood pressure regulation, the descriptive sequence analysis of ENaC α, β & γ genes in Dahl S *versus* R rat models, the existence of α ENaC alternatively spliced forms as well as the putative role of RNA alternative splicing in ENaC regulation in Dahl rats. Finally, a possible explanation of the mechanism of salt-sensitive hypertension in Dahl S rats and salt-resistance in Dahl R rats will be discussed.

1.0 Chapter 1: General Introduction

Adapted from a review published by the International Archives of Medicine by Shehata, M.F, 2009 and entitled "Regulation of the epithelial sodium channel [ENaC] in kidneys of salt-sensitive Dahl rats: Insights on alternative splicing"

Hypertension is defined as a consistent elevation in blood pressure above 140/90 [1], where the top number refers to the systolic blood pressure and the bottom one refers to the diastolic blood pressure. The definitions and classifications of hypertension are found in table 1.1. In Canada, over one-fifth of Canadian adults are diagnosed with hypertension [2]. Despite the fact that hypertension is the primary risk factor for stroke and heart disease, and has been labeled by the "silent killer disease", yet 42% of Canadians are still unaware of their increased blood pressures [3].

Hypertension can be classified as essential (primary) or secondary depending on the etiology of the disease. Essential hypertension describes the increase in blood pressure without a specific medical cause and it affects over 91% of hypertensive subjects [4]. On the other hand, secondary hypertension affects the remaining 10% of the population and refers to the rise in blood pressure as a result of a medical condition (such as kidney disease, hormonal disorders or the use of certain medications).

Of the 91% of patients diagnosed with essential hypertension, statistics have shown that over 50% are salt-sensitive [5], which makes salt-sensitive hypertension a highly common form of hypertension. These above statistics, combined with the realization that salt-sensitive hypertension exacerbates mortality rates [6], worsens manifestations of target organ damage [7], [8] and is a common finding in aging populations, emphasize the importance of identifying novel targets for prevention and treatment of salt-sensitive hypertension. The major contributor to the pathogenesis of salt-sensitive hypertension is dietary salt [9]. Dietary sodium, in turn, has sodium chloride (NaCl) as its major constituent. The sodium ion (Na^+) is transported into the cell primarily via the amiloride-sensitive Epithelial Sodium Channel (ENaC). Owing to the fact that inadequate Na^+ excretion is a risk factor for hypertension, ENaC represents an attractive

therapeutic target to study in salt-sensitive hypertension.

ENaC is critical for the maintenance of sodium balance, extracellular fluid volume and long term blood pressure control. Monogenic disorders causing ENaC hyperactivity have led to a severe form of hereditary hypertension in humans, known as Liddle's syndrome. Similarly in animal models, ENaC hyperactivity has been well documented in kidneys of salt-sensitive (S) Dahl rats (a genetic model of salt-sensitive hypertension) *versus* their normotensive control [Dahl salt-resistant (R) rats]. The purpose of the present literature review is to highlight the importance of ENaC in blood pressure regulation, the role of ENaC in salt-sensitive hypertension and the differential regulation of ENaC in kidneys of Dahl S *versus* R rats. A systematic overview of the putative role of alternative splicing of the principle α subunit of ENaC (α ENaC) in modulating ENaC expression in kidneys of Dahl rats will be discussed. Finally, a better understanding of the meaningful contribution of ENaC in the pathogenesis of salt-sensitive hypertension will be achieved upon completion of this review.

Table 1.1: Definitions and classification of blood pressure levels (mm Hg)

Category	Systolic	Diastolic
Optimal	<120	<80
Normal	<130	<85
High-normal	130-139	85-89
Grade 1 hypertension (mild)	140-159	90-99
Grade 2 hypertension (moderate)	160-179	100-109
Grade 3 hypertension (severe)	≥180	≥110

World Health Organization and International Society of Hypertension, 2003

I. ENaC as a candidate gene for blood pressure regulation

ENaC is highly selective for Na^+ and mediates Na^+- entry (down an electrochemical gradient) through the apical membrane of renal epithelial cells. ENaC also regulates sodium transport in other epithelia such as the alveolar epithelium, distal colon, brain, salivary duct and sweat glands [10-12]. Additionally, ENaC has proved essential for lung fluid clearance in newborn mice [12] and the entire salt taste perception in rodents.

Although the ENaC accounts for a small proportion of renal sodium reabsorption (<5%), nevertheless it still constitutes the rate-limiting step of sodium reabsorption in the distal nephron. The control of Na^+ movements in these epithelia is critical for the maintenance of extracellular fluid volume, electrolyte balance and long term blood pressure.

One of the major breakthroughs in understanding the central role played by ENaC in blood pressure was the demonstration of linkage between the ENaC and a rare form of hereditary severe salt-sensitive hypertension (Liddle's syndrome) [13]. Gain-of-function mutations and/or truncations in ENaC β and γ genes have been identified in patients with Liddle's syndrome. Later on, transgenic mouse models engineered with Liddle's mutations confirmed the critical role of ENaC in blood pressure regulation [14].

In contrast, loss-of-function mutations in the α and β subunits of ENaC have been identified in patients with pseudohypoaldosteronism, a salt-wasting nephropathy that results in defective sodium transport in many organs containing the ENaC, such as the kidney, lung, colon, sweat and salivary glands.

In summary, ENaC serves as an attractive candidate gene to study in salt-sensitive hypertension for the following reasons: i) ENaC serves as a key channel in controlling the rate of renal sodium reabsorption [11], ii) Genetic defects causing ENaC hyperactivity have led to a

monogenic form of hereditary hypertension in humans (Liddle's syndrome). This suggests that salt-sensitivity might arise from subtle defects in ENaC function and/or regulation [15]. iii) Moreover, ENaC activity is twice as high in renal collecting ducts of high salt-fed genetically predetermined salt-sensitive Dahl S rats *versus* their normotensive controls (Dahl R rats) that remain resistant to salt-sensitive hypertension on high salt diet [16], iv) Finally, ENaC blockade in the brain by benzamil rescued Dahl S rats from salt-induced hypertension [17]. Therefore, owing to the established importance of ENaC in blood pressure regulation, and in an attempt to understand the genetic differences in ENaC among Dahl S and R rats, the present review will highlight the putative mechanisms of ENaC regulation via alternative splicing. A comprehensive review of ENaC structure, function and differences in Dahl S *versus* R rats will be explained in details as a prelude to alternative splicing regulation of ENaC.

II. Structure of ENaC

The amiloride-sensitive epithelial sodium channel (ENaC) is composed of three homologous α, β and γ protein subunits of corresponding 698, 638 and 650 amino acids in length [18, 19]. ENaC α, β and γ subunits share approximately 30% homology at the amino acid level and each subunit correspond to a molecular mass of 70-85 kDa. The three ENaC subunits are inserted into the plasma membrane with a proposed stoichiometry of 2:1:1 [20] or 3:3:3 [21, 22]. The structure of the ENaC is found in figure 1.1.

Each ENaC protein subunit is formed up of four major domains: a cytoplasmic N-terminus, a large extracellular loop, two short hydrophobic segments known as the transmembrane domains 1 and 2 (TM1 and 2) and a cytoplasmic C-terminus. The N- and C-termini face the cytosolic side, while the extracellular loop faces the extracellular side [23]. The

channel domains are important for basic channel function such as the translocation of Na^+- ions across the membrane and for the modulation of ENaC activity at the cell surface. All three subunits cooperate to form the channel pore.

Among the channel domains the C-terminus has gained considerably high attention because almost all mutations discovered so far affecting the C-terminus cause a rare form of hereditary hypertension called the Liddle's syndrome. These mutations target the PY motif (PPPXY, where P=proline, X=any amino acid and Y=tyrosine) within the intracellular C-termini of the three subunits [24, 25]. The PY motif provides a mechanism for enhancing ENaC retrieval from the plasma membrane. Therefore, mutations of the PY motif prolong the half-life of the channel at the cell surface as a result of impaired internalization of ENaC [25].

At the genomic level, ENaC α, β, and γ protein subunits are encoded by three different genes located on separate chromosomes. The gene encoding the α ENaC subunit (Scnn1a) is located on chromosome 4q42, while the β and γ genes (Scnn1b and g) are located at a close proximity from each other on chromosome 1q36-q41. The genomic organization of ENaC genes is found in figure 1.2.

Figure 1.1: Structure of the Epithelial Sodium Channel (ENaC). The amiloride-sensitive epithelial sodium channel (ENaC) is composed of three homologous α, β and γ protein subunits of corresponding 698, 638 and 650 amino acids in length [18, 19]. ENaC α, β and γ subunits share approximately 30% homology at the amino acid level and each subunit correspond to a molecular mass of 70-80 kDa. The three ENaC subunits are inserted into the plasma membrane with a proposed stoichiometry of 2:1:1 [20] as shown in the figure or 3:3:3 [22]. Each ENaC protein subunit is formed up of four major domains: the cytoplasmic N terminus, the large extracellular loop, the two short hydrophobic segments known as the transmembrane domains 1 and 2 (TM1 and 2) and the cytoplasmic C-terminus. The N- and C-termini face the cytosolic side, while the extracellular loop faces the extracellular side [23]. All three subunits cooperate to form the channel pore via the transmembrane domains.

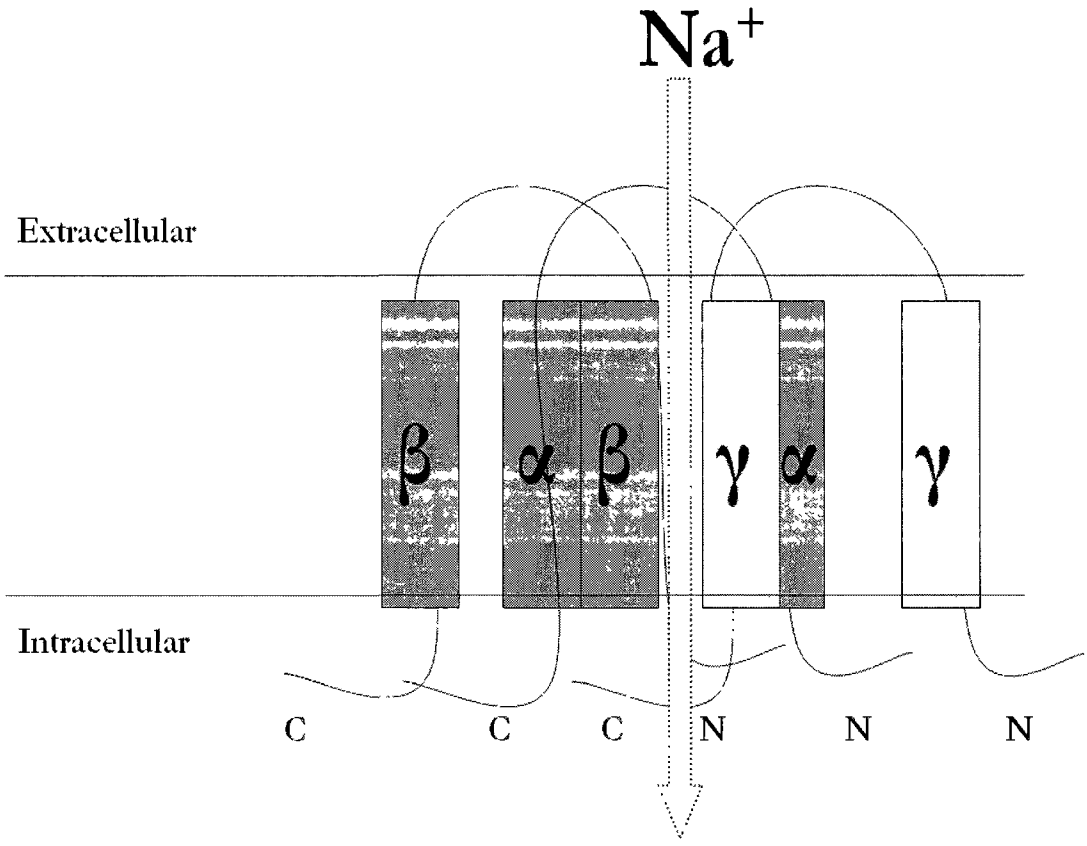


Figure 1.1

Figure 1.2: Genomic Organization of ENaC α, β, and γ. At the genomic level, ENaC α, β, and γ subunits are encoded by three different genes located on separate chromosomes. The gene encoding the α ENaC subunit (Scnn1a) is located on chromosome 4q42, while the β and γ genes (Scnn1b and g) are located at a close proximity from each other on chromosome 1q36-q41 (RGD: Rat Genome Database). α ENaC is composed of 12 exons, whereas each of the β and γ ENaC genes are composed of 13 exons. Translation starts in exon 1 for α ENaC-and starts in exon 2 for β and γ ENaC. Translation ends in exon 12 for α ENaC-and in exon 13 for β and γ ENaC. Therefore the 5'untranslated region (UTR) is included in exon 1 of α ENaC-and in exons 1 and 2 of β and γ ENaC genes, while the 3'UTR is included in exon 12 of α ENaC-and exon 13 in each of β and γ ENaC. Light shaded boxes represent the translated regions, while the black boxes represent the 3' and 5' UTR.

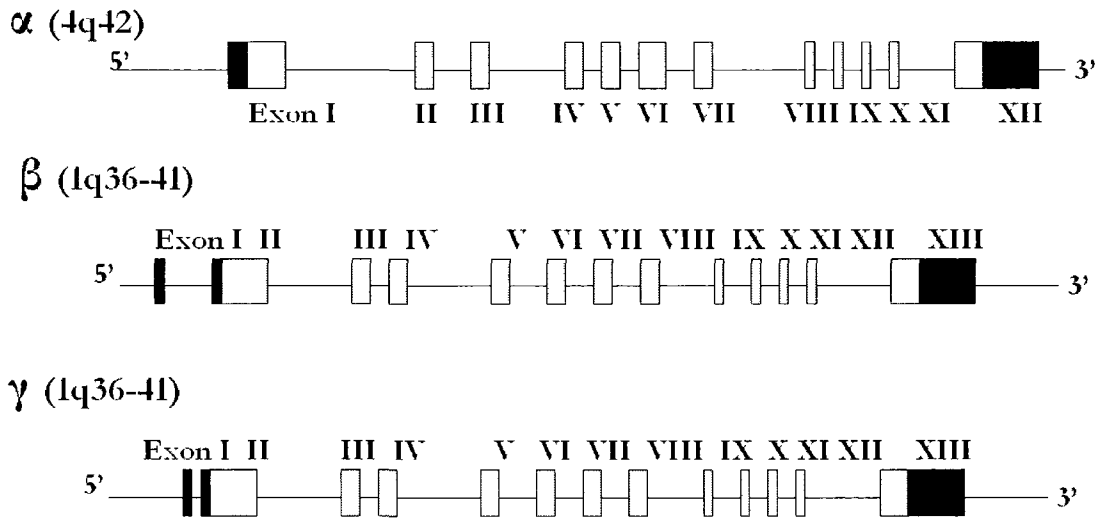

α (4q42)
5'
3'
Exon I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
β (1q36-41)
Exon I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
5'
3'
γ (1q36-41)
Exon I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
5'
3'

Figure 1.2

III. Significance of α ENaC *versus* β and γ ENaC

Of the three ENaC subunits, the α ENaC-alone is critical to the formation of a functional channel. This is because the expression of α ENaC-alone in Xenopus oocytes confers a low amiloride-sensitive sodium current, whereas neither the β nor the γ subunits can form conducting functional channels when expressed alone in Xenopus oocytes. β and γ ENaC only serve to maximize channel activity [19, 26].

The critical role of α ENaC is highlighted not only in expression studies in Xenopus oocytes, but also by knockout mice models. α ENaC knockout mice died within 40 hours of birth because of water-clogged lungs and failure of fluid clearance [27]. Moreover, decreased α ENaC expression (without necessarily knocking out α ENaC) predisposes animals to a respiratory distress syndrome [28]. The β and γ subunits have only minor effects on lung fluid clearance. Owing to the critical role of α ENaC in the functionality of the channel, and the fact that it is the only ENaC subunit with currently published alternatively spliced forms in Dahl rats, α ENaC regulation by alternative splicing will be discussed in the current review.

IV. Critical role of ENaC in kidneys of Dahl rats

Dahl rats serve as good candidates for studying ENaC. Dahl rats are separated into two strains; the salt-sensitive (S) and the salt-resistant (R) strain because of the inherent genetic propensities of Dahl S, but not R rats to develop hypertension on high salt intake [29, 30]. Renal cross-transplant studies demonstrated the decisive role of the kidneys in regulating blood pressure in Dahl S rats on regular salt diet. Indeed, Morgan et al. were able to demonstrate clearly that Dahl R rats when receiving an R kidney did not develop hypertension on high salt diet, but did with an S kidney [31]. This highlighted the critical role of the kidney in salt-sensitive hypertension

in Dahl S rats.

Additionally, in vitro studies do indicate that Dahl S rats exhibit enhanced Na^+ transport related to ENaC. This is because monolayers of inner medullary collecting duct cells when cultured in vitro and then examined electrophysiologically showed twice the rate of Na^+-transport when obtained from S *versus* R rats. This increase in sodium transport related to ENaC in Dahl S *versus* R rats is apparently due to a primary increase in the conductive permeability of the apical membrane to Na^+. The authors concluded that ENaC is intrinsically different or differently regulated in kidneys of S and R rats [16].

To date, there are few reports on the regulation of ENaC in these rat models. Aoi et al. just recently reported an abnormal increase in α ENaC mRNA (2.5-fold) in the kidneys of Dahl S rats on high *versus* regular salt intake for 4 weeks [32], while Dahl R rats showed a decrease in α ENaC mRNA [33]. Changes in ENaC protein abundance have not been reported, which is important since ENaC undergoes extensive post-translational regulation.

V. ENaC Differential Regulation in Dahl S *versus* R rats

It is essential to recognize that ENaC mutations might be the reason behind the enhanced α ENaC expression and overall ENaC activity in Dahl S *versus* R rats. A comprehensive ENaC α, β, and γ screening study is worthwhile to rule out or rule in the contribution of genetic mutations in the enhanced overall ENaC activity in Dahl S *versus* R rats. On the other hand, lack of mutations in ENaC genes in Dahl S and R rats will leave us with poorly understood mechanisms behind the enhanced ENaC activity in Dahl S *versus* R rats. An additional potential strategy for the differential ENaC regulation in Dahl rats - besides sequence variability of ENaC genes in Dahl S *versus* R rats - is via alternative splicing for the principle α ENaC subunit, which is the

focus of the present review. Although ENaC activity depends on the regulation of the channel transport to the plasma membrane, little is actually known about the check points and different factors involved in this regulation. In addition, interactions of ENaC with ENaC alternatively spliced forms and the outcome of such interactions on channel-subunits expression is important for channel assembly, localization to the luminal membrane and activity, and yet remains nebulous. Finally, in light of increased ENaC mRNA levels detected in Dahl S rats fed high-salt diet, increased ENaC activity in Dahl S rats can be attributed to increase in protein levels and corresponding increase in channel activity.

VI. Regulation of α ENaC-by alternative splicing: What is currently known?

Naturally occurring alternatively spliced forms have been reported for the α ENaC (not the β, or γ ENaC) in humans [34], mice [35], and chicken [36] suggesting that alternative RNA splicing is most likely a mechanism regulating α ENaC-activity. To date, there are two alternatively spliced forms (α ENaC-a and -b) of the α ENaC subunit that are currently published in rats [37, 38]. α ENaC-a and -b are identified in the rat kidney and tongue taste tissues [37]; [38]. The exon-intron organization of these two alternatively spliced forms are found in figures 1.3 and 1.4. The potential biological role of these alternatively spliced forms in ENaC regulation prior to and after salt loading in Dahl S rats is yet to be examined. Interestingly the 5' donor splice site (CCTGGG) used to create the α ENaC-a and -b was also utilized to create the α ENaC +22 splice variant in humans [34] and the 3399 bp variant in chicken [36]. This conservation for the 5' splice site across species underscores the significance of α ENaC-a and -b spliced forms in ENaC regulation.

Figure 1.3: α ENaC-alternatively spliced forms. An illustration of protein, genomic and structural organization of the 2 alternatively spliced forms of α ENaC (α ENaC-a & -b) that have been published in rats [37] in comparison to α ENaC wildtype major transcript. The protein sequence of α ENaC wildtype along with α ENaC-alternatively spliced forms are shown above. α ENaC wildtype is 698 amino acids in length (2100 bp). Amino acid residues from 1 to 110 reside in the cytoplasm, amino acid residues from 111 to 131 constitutes the first transmembrane domain, residues 132 to 589 constitute the extracellular loop, residues 590 to 610 constitute the second transmembrane domain, and residues 611-698 are cytoplasmic. α ENaC-a alternatively spliced form is formed by the deletion of 23 nucleotides from exon 8, whereas α ENaC-b is formed by the deletion of 79 nucleotides that involved exon 8 skipping. These deletions introduced a premature stop codon and resulted in shorter proteins at the carboxy terminus by 199 in α ENaC-a and 216 amino acids in α ENaC-b, making α ENaC-a 499 amino acids (2077 bp) and α ENaC-b 482 amino acids (2021 bp) in length . These resultant shorter proteins lacked the second transmembrane domain (TDM2) which is important in channel pore formation. α ENaC-a alternatively spliced form is a low abundance transcript that is expressed in the rat kidney, tongue epithelia and tongue taste tissues. α ENaC-a binding with the channel blocker (phenamil) was greatly enhanced. This demonstrates that the amiloride-binding site (i.e ENaC blocker site) resides in the extracellular loop of the channel and not the second transmembrane domain that is presently missing in α ENaC-a (CD: cytoplasmic domain, TDM1: transmembrane domain M1, EC: extracellular loop, TDM2: transmembrane domain M2).

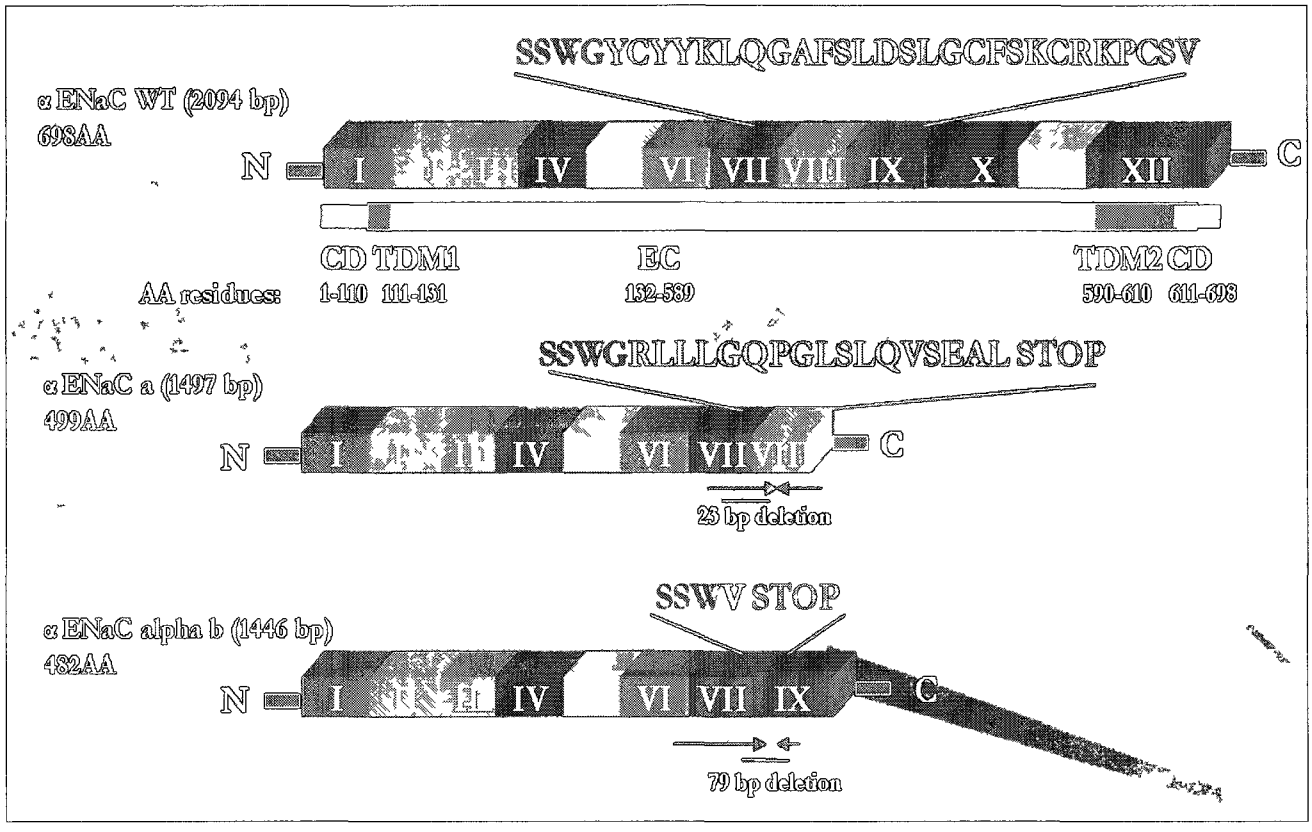
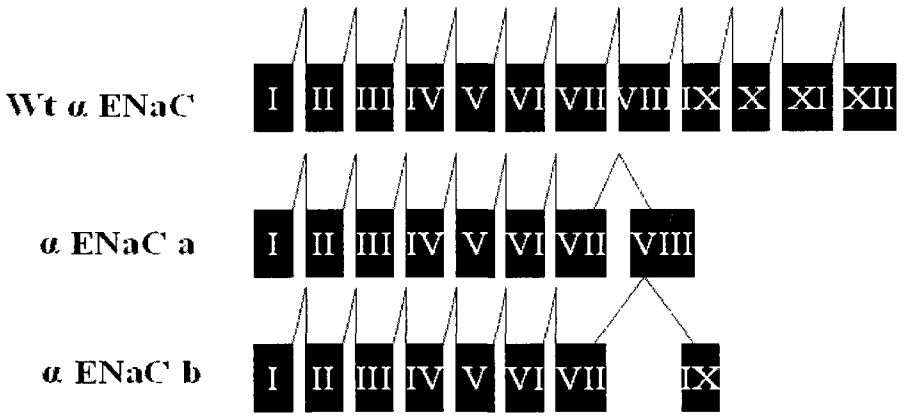

SSWGYCYYKLQGAFSLDSLGCFSKCRKPCSV
α ENaC WT (2094 bp)
698AA
N
I
IV
VI
VII
VIII
IX
X
XII
C
CD TDM1
EC
TDM2 CD
AA residues:
1-110 111-131
132-589
590-610 611-698
SSWGRLLLLGQPGLSLQVSEAL STOP
α ENaC a (1497 bp)
499AA
N
I
IV
VI
VII
C
23 bp deletion
SSWV STOP
α ENaC alpha b (1446 bp)
482AA
N
I
IV
VI
VII
IX
C
79 bp deletion

Figure 1.3

Figure 1.4: Schematic representation of wildtype and the alternatively spliced forms α ENaC-a & -b A. A schematic illustration of the mRNA sequences of α ENaC wildtype, -a and -b forms. α ENaC wildtype is made of 12 exons, while α ENaC-a is formed of exons I to VIII, with a 23 bases deleted from exon VIII. On the other hand, α ENaC-b is formed of exons I to IX with a skipping of exon VIII (79 bases). **B. The genomic sequence of α ENaC wildtype, -a and -b forms**. The alternatively spliced forms α ENaC-a & -b share the same splicing site (CCTGGG) which is located within exon VII. α ENaC-a & -b had 23 and 79 bases deleted respectively resulting in the iformation of a premature stop codon. **C. The protein sequence of α ENaC wildtype, -a and -b forms.** The deletions of 23 and 79 bases respectively in α ENaC-a & -b introduced a premature stop codon and resulted in shorter proteins at the carboxy terminus by 199 in α ENaC-a and 216 amino acids in α ENaC-b, making α ENaC-a 499 amino acids (1497 bp) and α ENaC-b 482 amino acids (1446 bp) in length . The α ENaC-a and -b truncated proteins of approximately 55 and 53 kDa respectively, are identical to wildtype α ENaC up to amino acids 481 and 480 respectively, followed by 17 and 1 novel amino acids unique to the spliced form after which the stop codon terminates translation.

A.



B.

Wt α ENaC

CCTGGGGCTATTGCTATTATAAACTGCAGGGCGCCTTCTCCTTGGACAGCCTGGGCTGTTTCTCCAAGTGTCGGAAGCCTTGTAGTGTGATCAACT
AC

α ENaC-a

CCTGGG----------GGCGCCTTCTCCTTGGACAGCCTGGGCTGTTTCTCCAAGTGTCGGAAGCCTTGTAGTGTGATCAACTAC

α ENaC-b

CCTGGG----------TGTGACCAACTAC

C.

Wt α ENaC EFCDYRKQSSWGYCYYKLQGAFSLDSLGCFSKCFSKCRKPCSV

α ENaC-a EFCDYRKQSSWGRLLLGQPGLSLQVSEALSTOP

α ENaC-b EFCDYRKQSSWVSTOP

Figure 1.4

Prior to discussing in depth the α ENaC-alternatively spliced forms, we need to discuss the α ENaC subunit in depth. α ENaC is composed of 698 amino acid with a molecular weight of 78.8 kDa. Amino acid residues from 1 to 110 reside in the cytoplasm, amino acid residues from 111 to 131 constitutes the first transmembrane domain, 132-589 constitute the extracellular loop, 590-610 constitute the second transmembrane domain, and 611-698 are cytoplasmic. The exon-intron structure of α ENaC is found in figure 1.2.

α ENaC-a transcript is a low abundance transcript compared to full length α ENaC- and has been studied in terms of expression, functionality and binding to ENaC blocker [37]. On the other hand, α ENaC-b is yet to be characterized. α ENaC-a alternatively spliced form is formed by the deletion of 23 nucleotides from exons 7 and 8 [37, 38]. This deletion introduced a premature stop codon and resulted in a shorter protein at the carboxy terminus by 199 in α ENaC -a. This resultant shorter protein lacked the second transmembrane domain which is important in channel pore formation. The α ENaC-a form alone has been studied in depth in terms of expression, tissue distribution, functionality in vivo and binding with the phenamil compound (phenamil is a derivative of amiloride and acts as a channel blocker) [37, 38]. α ENaC-a alternatively spliced form is expressed in the rat kidney, tongue epithelia and tongue taste tissues enriched in circumvallate papillae. Regarding functionality, α ENaC-a failed to generate amiloride sensitive Na^+ current when expressed in Xenopus oocytes, but still retained binding with the channel blocker (phenamil) that was greatly enhanced. This demonstrates that the amiloride-binding site resides in the extracellular loop of the channel and not the second transmembrane domain.

On the other hand, α ENaC-b formation involves exon 8 skipping (79 nucleotides). α ENaC-b is a truncated protein of 53 kDa that is identical to full length α ENaC up to amino acid 480, followed by one novel amino acid unique to α ENaC-b after which the stop codon

terminates translation. α ENaC-b lacks the second transmembrane domain which is critical in channel pore formation (figure 1.1). The significance of the second transmembrane domain is highlighted by the presence of the ENaC selectivity filter (that allows for a high selectivity for Na^+) in a region localized to a three-residue (G/SxS) track immediately preceding the second transmembrane domain of the ENaC subunits. This track resides in the narrowest part of the pore to exclude all, but the smallest cations. The three-residue track is located at amino acid 587, 529, 534 for ENaC α, β, and γ respectively [39]. Not only does TM2 control ion selectivity, but also contribute to ion permeation. Point mutations of selected residues within TM2 particularly amino acids 595 and 602 reduced Na^+ currents significantly and allowed for K^+ permeation over Na^+ permeation [40]. Owing to the critical role of the second transmembrane domain, α ENaC-b is expected to be a non functional transcript similar to α ENaC-a that previously failed to generate amiloride sensitive Na^+ current when expressed in Xenopus oocytes [37]. Because of the non functionality of all α ENaC-alternatively spliced forms in all species, it has been proposed to act as dominant negatives on the wildtype α ENaC. The genotoxic effects of alternatively spliced forms have been widely reported in several proteins, channels and membrane receptors and will be highlighted in section VI.

In agreement with the potential significance of alternative splicing in regulating ENaC, Xu et al., demonstrated a suppression in α ENaC spliced forms by oxidative stress in the lung epithelial cells in humans [41]. This finding is critical for ENaC regulation in Dahl rats because of the remarkable oxidative stress levels reported in most of the tissues of high-salt-fed Dahl S rats [42-44].

Our current review examines two potential mechanisms by which α ENaC spliced forms regulate the renal full length α ENaC possibly by a dominant negative effect. The first mechanism is through the enhanced binding of α ENaC spliced forms protein to the full

length α ENaC. Enhanced binding of α ENaC spliced forms to the full length α ENaC might in itself hinder proper channel assembly and interfere with proper channel activity. It might as well facilitate ENaC degradation in the cytoplasm and/or inhibit proper ENaC insertion into the plasma membrane, and therefore, contribute to the formation of non functional channels. The second mechanism is through an enhanced degradation of full length α ENaC- as a result of direct binding to α ENaC spliced forms, a phenomenon that has been reported previously in several other channels and membrane proteins [45-50].

VII. Implications of alternative splicing of ion-channels: Possible confounders in the "Genotoxicity" of α ENaC-alternatively spliced forms

Alternative splicing is a regulated process that takes place when the introns of a certain pre-mRNA are spliced in more than one way to yield several possible mature mRNAs from a single gene. 59% of human genes have more than one splice form [51], and 80% of alternative splicing changes the encoded protein [52]. The functional significance of ion channel-alternatively spliced forms vary considerably from altering the channel activation and inactivation rates for K^+ channels [53, 54], to altering gating properties for Ca^{++} channels [55], unit conductance , ion selectivity or sensitivity [56], or fine physiological tuning for optimal tissue performance [45, 57].

Aside from the fact that alternative splicing is a major contributor to the structural and functional diversity of ion channels such as the Na^+, K^+, and Ca^{++} channels, a major surge of interest has been recently witnessed in the genotoxic potential of alternatively spliced forms on full length forms [45-49]. Often, dominant negative alternatively spliced forms sequester the full length form in the cytoplasm and subsequently enhance its proteolytic degradation, such an intriguing phenomenon that greatly emphasize the importance of

alternative splicing in physiology, development and disease [58-61].

Moreover, the biological impact of alternatively spliced forms, particularly those lacking functional domains such as the second transmembrane domain in α ENaC-a and -b, may go as far as a switch-off effect [62]. However, one might wonder if the "genotoxicity" of a given spliced form is exerted mainly at the expense of full-length transcription and/or translation (potentially by accelerating full-length proteolytic degradation); or if it is primarily impacting channel assembly and/or translocation to the plasma membrane; or if it solely hinders channel cell surface expression and/or activity? (Figure 1.5).

Alternatively spliced forms have been shown to impair any of the above mentioned cellular processes, either independently or in synergy. For example, a spliced form of the K^+ channel (SV1) was shown to impair full-length translation, subunit assembly, translocation to the plasma membrane, cell surface expression and activity. SV1 specifically inhibited cell surface expression of the full-length K^+ channel α or β subunits by ~80%, by trapping them in the endoplasmic reticulum (ER) [45]. SV1, in turn, prevented subunit trafficking to the plasma membrane because of retention in the endoplasmic reticulum. Moreover, SV1 diminished protein expression of the K^+ channel subunits and cells that express it failed to generate a current.

Similarly, splice variants of the cation-channel (TRPV4) impaired subunit oligomerization, enhanced subunit accumulation in the endoplasmic reticulum and hindered trafficking [63]. Additionally, a spliced form of the inhibitor of apoptosis protein family was shown to be expressed at mRNA levels that were 2-3% of the levels of the full-length transcript, yet it encodes a protein that accumulates 50-fold higher levels than full-length and this accumulated protein competes with full-length form for activity [64]. Likewise, co-expression of the calcium sensing receptor splice variant with its full-length form reduced the

expression and activity of the full-length form in a dose-dependent fashion [65]. Consistent with the notion of a so-called "dominant negative" effect of alternatively spliced forms on full-length forms, a spliced form of the GTP cyclohydrolase enzyme (GCH) suppressed full-length GCH form expression levels in a dose-dependent manner, possibly by heteromeric interactions that ultimately decreased the stability and activity of the full-length form [49].

As such, common findings that support the role of a spliced form as a dominant negative expression regulator can be summarized as follows: a) the spliced form is non-functional [46], b) the frequency of the spliced form is higher relative to the full-length form; c) the spliced form heterodimerizes with the full length form [49]; and finally d) the spliced form accelerates full-length form degradation by trapping the latter in the endoplasmic reticulum [46].

Figure 1.5: Control points impinging on ENaC cell surface expression and activity. Schematic representation of the steps involved in regulating α ENaC-cell surface expression and activity. These steps include α ENaC subunit synthesis, assembly with the β and γ subunits, trafficking, insertion into the plasma membrane and activity. α ENaC-b may hinder any of these steps causing a suppressed channel cell surface expression and/or activity in Dahl R *versus* S rats that is augmented on high salt diet.

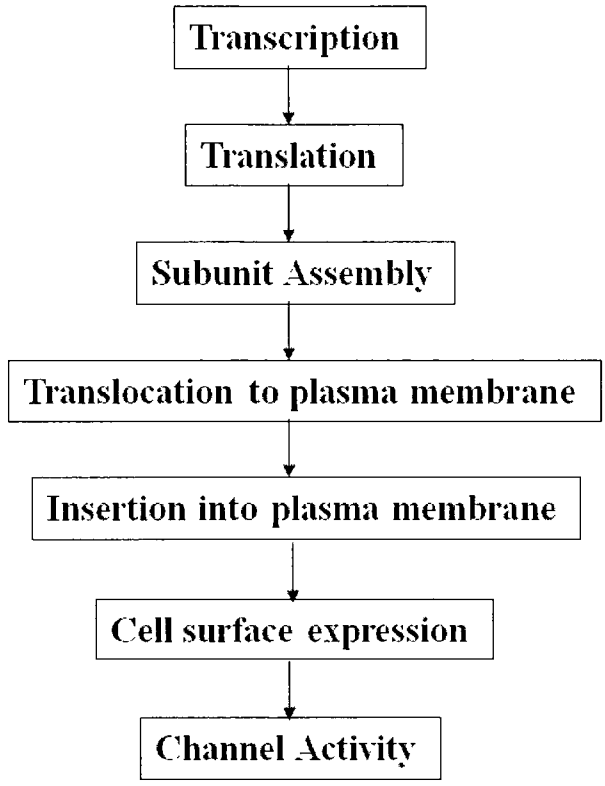

Transcription
Translation
Subunit Assembly
Translocation to plasma membrane
Insertion into plasma membrane
Cell surface expression
Channel Activity

Figure 1.5

VIII. Conclusion and future perspectives

Owing to the fact that alternative splicing is a strictly regulated process, and that alternatively spliced forms either serve as important regulators for the parent gene, possibly by a dominant negative effect, or as diagnostic markers for several pathological states particularly human genetic diseases, therefore, this review was meant to highlight recent findings with regards to the putative mechanism by which α ENaC-alternatively spliced form(s) modulate ENaC activity in response to high salt diet in Dahl-S *versus* R rats. Understanding the significance of α ENaC-alternative splicing in modulating ENaC in kidneys of Dahl rats is worthwhile because of the enhanced ENaC activity in Dahl S *versus* R rats.

Knowledge of the mechanism by which α ENaC spliced forms regulate full length α ENaC-and possibly prevent the hyperactivity of ENaC in Dahl S rats and the subsequent genesis of salt-dependent hypertension [a disease that comprises a large subgroup (over 50%) of Canadian adults] would certainly enhance the understanding of the basic regulation of ENaC and the pathophysiology of ENaC-associated disorders such as salt-sensitive hypertension. It may also create one or more specific targets for the development of novel anti-hypertensive drug or gene therapy.

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Prelude to Manuscript II

Statement of the rationale, hypothesis and objectives

Rationale

Salt-sensitivity denotes an enhanced response to dietary sodium, which is proposed to be caused by genetic variations leading to inter-individual variability in the responsiveness to salt loading. Findings from candidate gene studies will be valuable in the identification of novel pathways involved in the regulation of blood pressure.

Hence, the following studies were undertaken to address possible ENaC α, β, γ genes polymorphisms in Dahl salt-sensitive (S) *versus* salt-resistant (R) rats that might explain the differential response to salt loading in Dahl S rat models of salt-sensitive hypertension.

We chose to pursue this genetic anaylsis study in an attempt to understand the enhanced activity of ENaC in Dahl S *versus* R rats. Finding genetic differences in Dahl S *versus* R rats might provide targets for diagnosis and later prevention and/or treatment of salt-sensitive hypertension in affected subjects.

Hypothesis

Dahl S rats harbor genetic polymorphisms (variants) in ENaC α, β, and/or γ genes that enhance ENaC activity and/function and contribute to their salt-sensitive hypertension.

Objectives

Objective 1. *Identify genetic polymorphisms in the complete coding regions of ENaC α, β, and/or γ genes in Dahl S versus R rats.* We aim to use two methods of genetic screening namely the denaturing high performance liquid chromatography (DHPLC) and automatic

sequencing for screening the complete coding regions and exon-intron boundaries of ENaC α, β, and γ genes genes in Dahl S *versus* R rats.

Objective 2. *Identify genetic polymorphisms in the 5' and 3' flanking regions of ENaC α, β, and/or γ genes in Dahl S versus R rats.* We will screen the 5' and 3' flanking regions for each of the ENaC α, β, and γ genes in Dahl S *versus* R rats using DHPLC and automatic sequencing.

Objective 3. *Locate any identified genetic polymorphsisms in the 5' flanking regions of ENaC α, β, and γ to important consensus sequences and/or boxes.* We aim to use the TRANSFAC® and TFSEARCH® webtools to locate any identified polymorphism to important consensus sequences in the 5' flanking regions of ENaC α, β, and γ genes.

2.0 Chapter 2: Manuscript II

Sequence Analysis of Coding and 3' and 5' Flanking Regions of the Epithelial Sodium Channel α, β, & γ Genes in Dahl S *versus* R rats

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Published Manuscript

BMC Genetics 2007 Jun 25; 8:35

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Abstract

Background: To test whether epithelial sodium channel (ENaC) genes' variants contribute to salt sensitive hypertension in Dahl rats, we screened ENaC α, β, and γ genes entire coding regions, intron-exon junctions, and the 3' and 5' flanking regions in Dahl S, R and Wistar rats using both Denaturing High Performance Liquid Chromatography (DHPLC) and sequencing. **Results:** Our analysis revealed no sequence variability in the three genes encoding ENaC in Dahl S *versus* R rats. One homozygous sequence variation predicted to result in a D75E substitution was identified in Dahl and Wistar rat ENaC α compared to Brown Norway. Six and two previously reported polymorphic sites in Brown Norway sequences were lost in Dahl and Wistar rats, respectively. In the 5' flanking regions, we found a deletion of 5GCTs in Dahl and Wistar rat ENaC α gene, five new polymorphic sites in ENaC β and γ genes, one homozygous sequence variation in Dahl and Wistar rat ENaC γ gene, as well as one Dahl rat specific homozygous insertion of -1118CCCCCA in ENaC γ gene. This insertion created additional binding sites for Sp1 and Oct-1. Five and three Brown Norway polymorphic sites were lost in Dahl and Wistar rats, respectively. No sequence variability in ENaC 3' flanking regions was identified in Dahl compared to Brown Norway rats. **Conclusions:** The first comprehensive sequence analysis of ENaC genes did not reveal any differences between Dahl S and R rats that were isogenic in the regions screened. Mutations in ENaC genes intronic sequence or in ENaC-regulatory genes might possibly account for increased ENaC activity in Dahl S *versus* R rats.

Keywords: Salt-sensitive hypertension, Dahl rat, ENaC genes analysis

Introduction

The epithelial sodium channel (ENaC) is made up of three homologous subunits named α, β, and γ that assemble together to form a highly Na^+- selective channel [1]. The structure of these subunits is characterized by the presence of two transmembrane domains separated by a large extracellular loop. Identification of mutations in ENaC subunits causing salt-sensitive hypertension and hypotension in humans (Liddle's syndrome and pseudohypoaldosteronism type 1) highlighted the impact of these genes on salt homeostasis and control of blood pressure (BP) [2, 3].

In rats, the three α, β and γ ENaC (rENaC) subunits are encoded by three distinct genes *Scnn1a, Scnn1b*, and *Scnn1g* respectively, located on chromosomes 4q42, 1q36-41 and 1q36-41. The three subunits share similar structures and show 33-37% amino acid sequence homology in human [4]. rENaC α gene is composed of 12 coding exons [5]. rENaC β and γ genes are composed of 13 exons, the translation initiation codon is present within the second exon for both genes [6,7].

Dahl rats represent a robust animal model of genetically determined salt-sensitive hypertension. High salt intake increases BP in Dahl salt-sensitive (Dahl S), but not in Dahl salt-resistant (Dahl R) rats. Blockade of ENaC in the brain by benzamil prevents the increase in BP in Dahl S rats on high salt diet [8,9].

So far, only the coding sequences of genes encoding the three subunits have been partially screened in Dahl S and R rats. Analysis of near full length (base 22 till the end, all numbering starts at the A nucleotide of the primary initiation codon) of the *Scnn1b* cDNAs derived from kidneys of Dahl S and R rats failed to reveal any coding sequence mutations that could affect the predicted peptide sequence of *Scnn1b* [10]. Sequencing of nucleotides 21667 to 22054, 31172 to 31492, and 29142 to 29522 in the carboxy termini of ENaC α, β,

and γ genes respectively revealed no differences in Dahl S *versus* R rats [11]. The sequence of the entire coding regions, intron-exon junctions, as well as the 3' and 5' flanking regions of ENaC three genes has not yet been reported in Dahl rats. In order to identify any variation, each sequence was analyzed using a combination of two screening methods, Denaturing High Performance Liquid Chromatography (DHPLC), offering 95-100% sensitivity and 100% specificity and automatic sequencing, offering 99.7-100% sensitivity and 100% specificity [12,13]. Dahl S and R rats, as well as Wistar rats, used as a control, were screened and the obtained sequences were compared to Brown Norway sequences retrieved from the rat genome database.

Results

Screening of ENaC α, β, and γ Genes Coding Regions for Variations between Dahl R and S rats

No sequence variability was identified in the entire coding regions, as well as in exon-intron junctions of ENaC α, β, and γ genes in Dahl S *versus* R rats (Table 2.1). One homozygous sequence variation G225T in exon 1 of ENaC α, predicted to result in a D75E substitution, was identified in Dahl S and R and Wistar rats compared to the published Brown Norway sequence (Table 2.1). Previously published Wistar rat sequences did not identify this variation [5]. It is therefore possible that Wistar rats are heterozygous at this position. One reported Brown Norway polymorphic site of ENaC α was lost in both Dahl and Wistar rats (Table 2.1). No sequence variability was identified in Dahl and Wistar rat ENaC β gene compared to the Brown Norway sequence available in the public domain. No sequence variation in ENaC γ gene was identified in Dahl and Wistar rats compared to the published Brown Norway sequence. However, five and one previously reported polymorphic sites in Brown Norway ENaC γ sequence were lost in Dahl and Wistar rats, respectively (Table 2.1). The Wistar rat alleles studied followed a Mendelian independent assortment.

Table 2.1: Allelic variants in the coding sequence of ENaC subunits in Dahl S, R, Wistar, and Brown Norway rats.

Subunit	Nucleotide position/exon	Dahl S and R	Wistar	Brown Norway	AA change
ENaC α	+225/1	TT	TT*	GG	D75E
	+10716/3	AA	AA	GG, GA, AA	R290R
ENaC γ	+4008/4	GG	GG	AA, AG, GG	E255K
	+22549/7	CC	TT, TC, CC	TT, TC, CC	D376D
	+24672/8	CC	TT, TC, CC	TT, TC, CC	C410C
	+29195/13	TT	CC, CT, TT	CC, CT, TT	C542C
	+29291/13	TT	GG, GT, TT	GG, GT, TT	C573W

Numbering starts at the A nucleotide of the primary initiation codon.

(www.genome.ucsc.edu).

AA: amino acid

* GG genotype was previously reported [5].

Screening of ENaC α, β, and γ Genes 3' and 5' Flanking Regions for variations between Dahl R and S rats.

We first defined the 5' flanking regions to be screened for each ENaC subunit. The predicted putative binding sites on Brown Norway rat ENaC sequences were identical using TRANSFAC® and TFSEARCH®. Because of the presence of potential kidney and brain transcription factor binding sites, we screened 1.8 kbp, 1.5 kbp, and 4.4 kbp of the 5' flanking regions of ENaC α, β, and γ respectively from the transcription start site (Figure 2.1). Compared to previously reported transcription factors binding sites [5-7, 14], the present analysis determined five, one, and ten new potential transcription factor binding-sites sequences on ENaC α, β, and γ, respectively (Figure 2.1).

No sequence variability was identified in both the 3' and 5' flanking regions of ENaC α, β, and γ genes in Dahl S *versus* R rats (Table 2.2).

Four homozygous sequence differences were found in both Dahl and Wistar rat ENaC α gene compared to Brown Norway: one homozygous 15 bp deletion (-1788 → -1803 bp), and the loss of three Brown Norway polymorphic sites (Table 2.2). In addition to previously described transcription factor-binding sites [5, 14], computer analyses suggest that the regions from –1248 to 1239, from –1052 to –1047, from -780 to -790, and from –736 to –746 represent putative binding sites for GATA-1, -2, and -3, GATA-1 and 2 and YY1, GATA-2, and Sp1 respectively (Figure 2.1A). The putative GATA-1, -2, and -3 binding site (–1248 to -1239) found in presence of the -1247A allele loses the binding site for GATA-2 in the presence of the T allele. The putative binding site for YY1 (–1052 to –1047) found in presence of the -1050G allele and overlapping GATA-1 and 2 sites, is lost in the presence of the A allele; while GATA-1 and 2 transcription binding sites remained unaltered. Both Dahl

and Wistar rats were homozygous TT and AA for respectively the T-1247A and the G-1050A polymorphisms.

The G-34648A ENaC β gene variant, not reported in Brown Norway rats, was present at the homozygous state in Dahl rats and at the heterozygous state in Wistar rats (Table 2.2). One Brown Norway ENaC β gene polymorphic site was lost in Dahl rats, but not Wistar rats (Table 2.2). In addition to previously described transcription factor-binding sites [6] the regions from –34650 to –34635 represent a putative binding site for STAT proteins respectively (Figure 2.1B). However, the presence of the A-34648G polymorphism within this STATX potential site is not predicted to alter STAT protein binding. Dahl rats were homozygous AA for the G-34648A polymorphisms, while Wistar rats were heterozygous.

Four ENaC γ gene sequence variations (A-1588C, G-2525A, A-2561G and T-3313C) were identified in both Dahl and Wistar rats compared to Brown Norway rats, these variations were all homozygous in Dahl rats; in Wistar rats T-3313C was found at the homozygous state, while A-1588C, G-2525A, A-2561G were heterozygous (Table 2.2). A 6 bp deletion at -1118 was only found in Dahl rat ENaC γ gene (Table 2.2). The A-2386G ENaC γ gene variant, not reported in Brown Norway rats, was present at the homozygous state in Dahl rats. Our screening of the Wistar ENaC γ gene only found the homozygous AA allele, however, the presence of the G allele was previously reported [7] (Table 2.2). One previously reported Brown Norway polymorphic site ENaC γ was lost in Dahl rats, but not Wistar rats (Table 2.2). In addition to previously described transcription factor-binding sites [7], the regions from –4102 to –4120, from –4010 to –4022, from –2483 to –2497, from –696 to –706, from –607 to –619, from –442 to –463, and from –400 to –413 represent putative binding sites for C/EBP a & b and CRE, Oct-1, C/EBP, CRE and C/EBP b, Sp1, c-Myc and C/EBP b, and USF respectively (Figure 2.1C). One polymorphism, the G-2525A

polymorphism, out of the seven found in the 5' flanking region of ENaC γ gene, is located within a potential consensus binding sites for C/EBP (Figure 2.1C). The C/EBP binding site is present when the -2525G allele is present and the binding site is lost in the presence of the A allele. Dahl rats were homozygous AA for the G-2525A polymorphism, while Wistar rats were heterozygous and Brown Norway rats homozygous for the G allele. Finally, the 6 bp homozygous insertion at position -1118, which is only present in Dahl rats, is predicted to create Sp1 and Oct-1 sites (CCCCA*CCCCA*TT) (TFSEARCH® scores 87.7 and 85.8, respectively).

Figure Legend

Figure 2.1: A. Location of the variants identified in the 5' flanking region of Dahl S, R, and Wistar rats ENaC α (A), β (B), and γ (C) genes on the Brown Norway rat genomic sequence (www.genome.ucsc.edu). Position of the variants identified in the current study is highlighted in bold. Boxes represent the putative transcription factor-binding sequences; the putative binding sequences found during the present sequence analysis are labeled in bold; the factor names are written above the boxes. The first three bases for the major kidney and brain transcription start sites are italicized and bold. A. The translation initiation codon (+1) is underlined. TFSEARCH® scores for the newly assigned putative binding sequences are 93.1, 89.7, and 89.7 for GATA 1, 2, 3 respectively; 89.0 and 88.5 for GATA 1, 2 respectively and 85.8 for YY1; 88.5 for GATA 2, and 87.7 for Sp1. **B. TFSEARCH® score for the newly assigned putative binding sequences for STATX is 92.3. C. The translation initiation codon (+1) is underlined.** TFSEARCH® scores for the newly assigned putative binding sequences are 89.2, 87.4, and 89.0 for C/EBP a & b and CRE, respectively; 85.8 for Oct-1; 89.3 for C/EBP, 87.9 and 85.5 for CRE and C/EBPb respectively; 87.7 for Sp1, 91.0 and 87.4 for c-Myc and C/EBPb respectively; and 85.9 for USF.

Figure 2.1A

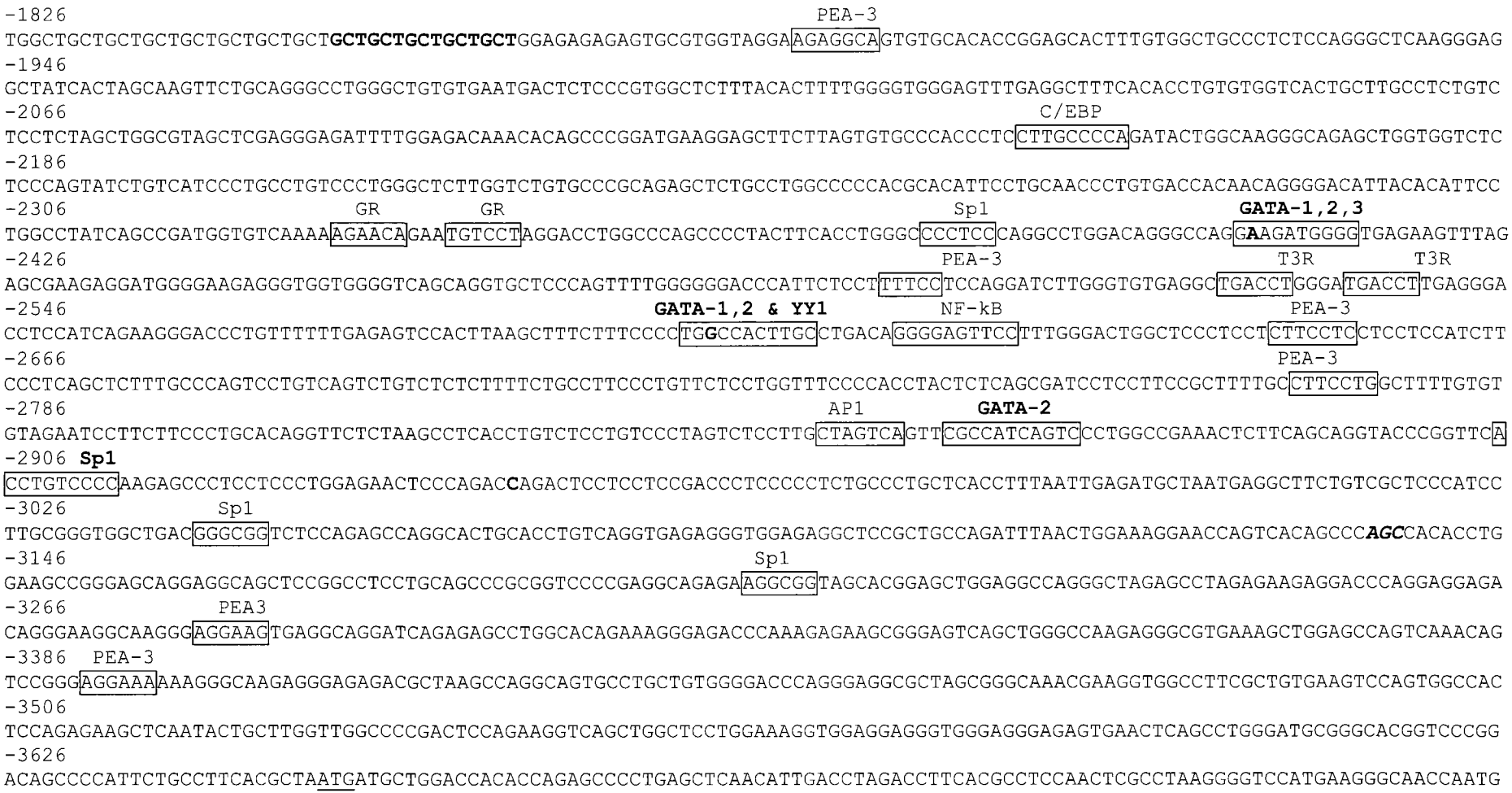
-1826
PEA-3
TGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGGAGAGAGAGTGCGTGGTAGGAAGAGGCAGTGTGCACACCGGAGCACTTTGTGGCTGCCCTCTCCAGGGCTCAAGGGAG
-1946
GCTATCACTAGCAAGTTCTGCAGGGCCTGGGCTGTGTGAATGACTCTCCCGTGGCTCTTTACACTTTTGGGGTGGGAGTTTGAGGCTTTCACACCTGTGTGGTCACTGCTTGCCTCTGTC
-2066
C/EBP
TCCTCTAGCTGGCGTAGCTCGAGGGAGATTTTGGAGACAAACACAGCCCGGATGAAGGAGCTTCTTAGTGTGCCCACCCTCCTTGCCCCAGATACTGGCAAGGGCAGAGCTGGTGGTCTC
-2186
TCCCAGTATCTGTCATCCCTGCCTGTCCCTGGGCTCTTGGTCTGTGCCCGCAGAGCTCTGCCTGGCCCCCACGCACATTCCTGCAACCCTGTGACCACAACAGGGGACATTACACATTCC
-2306
GR
GR
Sp1
GATA-1,2,3
TGGCCTATCAGCCGATGGTGTCAAAAAGAACAGAATGTCCTAGGACCTGGCCCAGCCCCTACTTCACCTGGGCCCCTCCCAGGCCTGGACAGGGCCAGGAAGATGGGGTGAGAAGTTTAG
-2426
PEA-3
T3R
T3R
AGCGAAGAGGATGGGGAAGAGGGTGGTGGGGTCAGCAGGTGCTCCCAGTTTTGGGGGGACCCATTCTCCTTTTCCTCCAGGATCTTGGGTGTGAGGCTGACCTGGGATGACCTTGAGGGA
-2546
GATA-1,2 & YY1
NF-kB
PEA-3
CCTCCATCAGAAGGGACCCTGTTTTTTGAGAGTCCACTTAAGCTTTCTTTCCCCTGGCCACTTGCCTGACAGGGGAGTTCCTTTGGGACTGGCTCCCTCCTCTTCCTCCTCCTCCATCTT
-2666
PEA-3
CCCTCAGCTCTTTGCCCAGTCCTGTCAGTCTGTCTCTCTTTTCTGCCTTCCCTGTTCTCCTGGTTTCCCCACCTACTCTCAGCGATCCTCCTTCCGCTTTTGCCTTCCTGGCTTTTGTGT
-2786
AP1
GATA-2
GTAGAATCCTTCTTCCCTGCACAGGTTCTCTAAGCCTCACCTGTCTCCTGTCCCTAGTCTCCTTGCTAGTCAGTTCGCCATCAGTCCCTGGCCGAAACTCTTCAGCAGGTACCCGGTTCA
-2906 Sp1
CCTGTCCCCAAGAGCCCTCCTCCCTGGAGAACTCCCAGACCAGACTCCTCCTCCGACCCTCCCCCTCTGCCCTGCTCACCTTTAATTGAGATGCTAATGAGGCTTCTGTCGCTCCCATCC
-3026
Sp1
TTGCGGGTGGCTGACGGGCGGTCTCCAGAGCCAGGCACTGCACCTGTCAGGTGAGAGGGTGGAGAGGCTCCGCTGCCAGATTTAACTGGAAAGGAACCAGTCACAGCCCAGCCACACCTG
-3146
Sp1
GAAGCCGGGAGCAGGAGGCAGCTCCGGCCTCCTGCAGCCCGCGGTCCCCGAGGCAGAGAAGGCGGTAGCACGGAGCTGGAGGCCAGGGCTAGAGCCTAGAGAAGAGGACCCAGGAGGAGA
-3266
PEA3
CAGGGAAGGCAAGGGAGGAAGTGAGGCAGGATCAGAGAGCCTGGCACAGAAAGGGAGACCCAAAGAGAAGCGGGAGTCAGCTGGGCCAAGAGGGCGTGAAAGCTGGAGCCAGTCAAACAG
-3386
PEA-3
TCCGGGAGGAAAAAAGGGCAAGAGGGAGAGACGCTAAGCCAGGCAGTGCCTGCTGTGGGGACCCAGGGAGGCGCTAGCGGGCAAACGAAGGTGGCCTTCGCTGTGAAGTCCAGTGGCCAC
-3506
TCCAGAGAAGCTCAATACTGCTTGGTTGGCCCCGACTCCAGAAGGTCAGCTGGCTCCTGGAAAGGTGGAGGAGGGTGGGAGGGAGAGTGAACTCAGCCTGGGATGCGGGCACGGTCCCGG
-3626
ACAGCCCCATTCTGCCTTCACGCTAATGATGCTGGACCACACCAGAGCCCCTGAGCTCAACATTGACCTAGACCTTCACGCCTCCAACTCGCCTAAGGGGTCCATGAAGGGCAACCAATG

Figure 2.1B


-34803
NF-kappaB
GGGAAGAGCAAAACTCAATTGAGAAAACGTCTCCATCAAATTGGCCTGTCAGCAAATTTTGTGGGCATTCTTGATCAATTATTGATGTGGGAGGACCCAGCCCACTGTGGGAGCTGCCAC
-34923
CCCCTGGCAGGTGGTCCTGGGTTGTATAAGAAAGCGGGCTGAGCAAATCATGGGGCACAAGCCAGTAAGCACTAGTGTACCACGGCCTCTGCTTCAGCTCTGACTTCCAGAGCTGCTTAT
-35043
GTTCCTACCTTGACACCCTTTCTGGTATTTATCACAGCATAGAAAACAAACTAGGACAGCCATGCACATGAAGACTCTGGAGTCTTCAAAGATTTTTAAAAGTATTATAGGTACCTGTGA
-35163
Oct-1
PEA-3
STATX
PEA-3
TGCAGTCTTCAAGAACTCATATATTAGAAGCATCTGTGGAAAGATACACAGGAAATGGAACCGGTGGTTACAGGAAAGAAAGCTGAATGGACAGAGAGTAGCTTTGATCTGAGGAGAGGAG
-35283
Sp1
ACAGCAGAGGGGAGGGGAGGGAGTGACGAGTACAGGTGCCCTTAATCTATTCTGTGGTAAATACCACGATATGGATACGTTCAAAAGAAAAGTTTGCTTGGGCTCCCAGCTCTGGACCAA
-35403
AP1
GGTCACTAGGTCCTGCACCTGGTGATACCCTTCTTGTTGGCAGAGCCCCAGAGTCAAGTTGGGTGCTCCTGGGAGATCTAGTCAAGTTGGCTGCAAAGACGAGATTACCATTGATCCATT
-35523
AP1
ACCCAAATAGATGAGTTAATCTGTTAACTGAGTAGATGAGCTATCCCACTCCTAAGGGGTCTCCTAATCACCCCTTCAAGTGCCACCTCTTCTCCTCTTAAAACGGAGGCTCGGGGTTGG
-35643
Ap-1
GGATTTAGCTCAGTGGTAGAGCGCTTGCCTAGCAAGCGCAAGGCCCTGGGTTCGGTTCCCAGCTCCGAAAAAAAGAAAAAAAAATAGAACGGGGCTGTGAGGTGATGTGTCACCGTGTAT
-35763
Oct1
PEA-3
Sp-1
GTGATGCGCTGAAGCATGCGTGTCTTATGCTGGTTATGGAGTGAGGATACACGGAGGAAGGCTTTTCTGCACCAATGTAGGGCATGGAGTCCCCTGTCGTTGCTTTCCCCCAACCCCCAC
-35883
Sp-1
Sp-1
AP-1
CCCCACCCTGTGTATAGAGAATGGGGCTGGGAGGTGAGAAGTGGCCTGTGGGGACTACAGAGGATACTGACCTGGAGCCTGTCAACTGGAACAAAGTGGCCTGGGAGCCTGACAGGTAGC
-36003
Sp-1
Sp-1
Sp-1
Sp-1
CCGACTAACCCAGGAAGGCAGGCTCCTCCCGCAGGCCCTCCCGGTTGGTCCCCGCCCCCCGCGCTCCCTCCGCCTACAGCGTCCCTGGCTCTACAGGTGACCCAGCTCCAGCCACTCGGA
-36123
CGGACGCCACCACCTTAGCTGCCATCACTGCACATTGGAGCAGCTTTCTAAACAGGTATGACAGCGACCTCCGCGCGCGCGCTCGCACCCGGGCAATGACCGCAGTTCCGTCCCTGACAG

Figure 2.1C


-4399
GATTTTAGGGGATTTTTCTTTACATTGTTCTTCAATAATGCTTGACATGTTTCTACCCACCAAAAGTCTGATCTTTGGCTGGGCAAAAGAAGCATCTTGAGAGGTTC. . . . . .
-4639
IL-6 C/EBPa&b & CRE
AP-2
GRE
TTCGGTTTATGTAATAGTGGGGATGAGCCCCGGGGCTTCAGTTATATTAAACAAGCTCTCTACCAACTGATCTACATCCTCAACTCAAGAATCTGCATTTTAACAAGGTCTGTAGGTGGA
-4759
Oct-1
TCTAAACACATTTGAGTCTAAGATGTTTTAATACTTATTAACACCCTTTCTTTTGTGGGGCCATGTTTCTTCCCAGATCAAAGGGATTGTAACTTGGACACTTGTCTT. . . . . .
-5119
Ap-1
E-box
Sp1
AP-2
CAAGCACGCACGGTGCCACCGCAAGACTGCGCAGCCCGGGTTGAGGCGCGGAGCTGACTCAGGACGGCAGGTGGCACTGCGGGGTCGCGGCTTACTCGGGGCTGAGACGAATCGGGGGTG-
5239
GC box
PEA3
GC box
AP-2
GCCTGGGGAAAACACCTGGGCAGGTGTGGGCGGAGTCTGAGTGAGGAGGAAACTCTAGGGGCGGGGCTTGAGCCTGACCCACGGGCGCTTCTGGCCGTGCCACTTGG. . . . . .
-5599
GRE
GAGCCGGTGTGAGTGGGAGGAGCGCATCTCGGATCTTCTCTGCCCAGCTCAACCGGCTTCCCAGCCTTGAAGAGCATGCATTGACCGCGACTGTTCTTTCCGCCGCGGGAAAGCTTTTCT
-5719
IL-6
Sp1
TTAGAGGTGCCTGCTGCAGAAAGAAACAAGCACTTGAAGTGGGCTAGTTGAAAGAGGAGCCCGAGACTGCAGCGGCGCGAAAGCTGGCGCGAAAGCTGGCGCGAAGCAGATGGCTTTTGC
-5839
AP-2
CGCAAAACTGCGCCAGGTCGCTTCCCAAAATGCAGCGAGTCAGTGGGTAGCTCTGTCCCAGGGCTTGAGGTTCTGAGGCTCAAGTTATTTCTGAATAGCACCGTGGCATGGCGGGAATCA
-5959
CRE
Sp1
AAACATTGCCTGATCCCAGGCTTTTGTGCCCAAACTCTAGAATGAACAGAGCCTCGCAAACATGTCACCCCTCGTCAAGCTTCAGTTTCCTGACGGGGACAGAGGCAAGGATGGGGGGGGC
-6079
IL-6 PEA3
GGGTGAGAGGGAAGGGAGCAAGTGTGGTAAGGAAAATGGAGACTTGGAATTTCAAAGAATTCTTTCGTTCTTTCATACTGTGTGGCCTTGGGCAGATTACCTAACCTTTTTACACCTGTG
-6199
C/EBP
TTTGCATCTCTGTGGGATGGGGATAGAAATGACACATCATCTTGTCTGTAGTGAGAGTTAAATTAGGTGGAAACGTTGGAATAGGAATCCTGGGCACGGCTGTATCTGTAACTGATATCT
-6319
IL-6
GCCATTATTGCTGCTGCAGCAGTTGTCACCTGCGACCACTATCATTAAGCAATGACATAACTAGTATGTGGTCTTGGGTTCAAATCCTAGCCCAGACACTTTCTATTATGACCTTGGGCA
-6439
CACTACTTAGTCTATCTGTGTGCCTCCATTTCATTTGTGTAGTGGGGAAATTACGATATCTACCGGAGAAGACCATTGAGAGTATTCTGTGAGTTAACTCTTGAGATGAG. . . . . .
-7639
↓
TGTTACCCCCTTTCCCCTACCCACACACCCACCCCACCCCCATTATTTTATGCACCATCTTGAAAAAGACAAAGAAAAGAACATGATGGGGCCATCAAAGTCCCCAACTT. . . . . .
-7999
CRE, C/EBP b
CTGCCGCTGAGATGCAACATACACATGTGAATGTGTTCATTCGTTTCCCAATACTTACCCCAGAGGCTGTCTCTAAATTAGGTAAAATCAGGTCAAATGACCTGGGGCTCGTAAATATGG
-8119
Sp1
AGAGGCCAGCAGTCATGGGGCTGATGTGACACCAGCTGCTATTGACCTCGGTAAGGAGAAGAAATGAAACAGAAATCATTTCACTTAGAAGAAAGCAACATGGAAGAGGGGCAGGGGAAA
-8239
c-Myc & C/EBP b
CATGGTCAGGACAGAATCTACAAGCTGCTGGGAAATTGTGGGAGATTTCATCAGACAGTGAACACATTGCCTGACATGTGCTCTGTGCAGGAGGGACATTTCACTAGGCACCAGGAGACA
-8359
USF
TTGTAAAACAGGAGGGCTGCTCTCAACAAGTGGCCAATGGAAGCGATAAGGCACGGGTTATAGTGCCTCTCTGCCTGATAAGAGAAGTCTGGCTGGGTTGCTTT. . . . . .
-8719
CTGACAGGTCTGTGCTTGCAAACTCACTAACCCCTCTGTCCCTTCAGAACTCTGCAGGCAGCAAAGTCCTGTCCTTACCATGGCGCCTGGAGAGAAGATCAAAGCCAAAATCAAAAAGAA

Table 2.2: Allelic variants in the 5' flanking regions of ENaC subunits in Dahl S, R, Wistar, and Brown Norway rats.

Subunit	Nucleotide Position	Dahl S and R	Wistar	Brown Norway
ENaC α	-705	TT	TT	CC, CT, TT
	-1050	AA	AA	GG, GA, AA
	-1247	TT	TT	AA, AT, TT
	-1788	Deletion of 5GCTs	Deletion of 5GCTs	
ENaC β	-34381	AA	GG,GA, AA	GG, GA, AA
	-34648	AA	GG, GA, AA	GG
ENaC γ	-1118	Insertion of CCCCCA		
	-1588	CC	AA, AC, CC	AA
	-2054	TT	TT, CT, CC	CC, CT, TT
	-2386	GG	AA*	AA
	-2525	AA	GG, GA, AA	GG
	-2561	GG	AA, AG, GG	AA
	-3313	CC	CC	TT

Numbering starts at the A nucleotide of the primary initiation codon (www.genome.ucsc.edu).

* GG genotype was previously reported [7].

Discussion

The results of the current study show that Dahl S and R inbred rats are isogenic in the entire coding regions, exon-intron junctions, 3' and 5' flanking studied regions of ENaC α, β, and γ genes. These results are in agreement with the initial partial screenings of ENaC subunits in Dahl rats [10, 11]. Nine homozygous sequence variations were identified in ENaC genes in Dahl rats compared to the Brown Norway sequence available in the public domain and eight of these nine sequence variations (5 polymorphic and 3 homozygous) were also identified in Wistar rats. However, the 6 bp deletion at -1118 which in the γ ENaC 5' flanking region, was specific for Dahl rats and not found in Wistar or Brown Norway rats. Eleven and five Brown Norway polymorphic sites were lost in Dahl and Wistar rats respectively. Among the identified variations in Dahl and Wistar rats, three are non synonymous variations. Four variants in the 5' flanking regions of ENaC α, β, and γ genes are present within putative DNA consensus regulatory elements and two putative DNA consensus sites were introduced with the Dahl rat specific 6 bp insertion at -1118 in ENaC γ.

Of the three non synonymous variants identified in the present study (αD75E, γE255K, and γC573W), only γC573W, located in the transmembrane domain M2, was previously functionally assessed and was found not to modify ENaC activity in *Xenopus* oocytes [11]. The αD75E substitution is present in the N terminus of the channel, prior to the transmembrane domain M1 and close to the channel pore, a critical region for kinetics properties of the channel predicted to participate in channel gating [11, 15]. γE255K located in the extracellular loop might alter the amiloride sensitivity since residues essential for

the formation of the high affinity amiloride-binding sites reside within this domain [1]. The silent polymorphism C542C was previously documented in ENaC γ carboxy terminus within and between rat strains [11].

Previous studies analyzed 1.5 kbp of ENaC α, 1.3 kbp of ENaC β, and 4.2 kbp of ENaC γ from the transcription start site in Wistar, Sprague Dawley and Wistar rats respectively as well as the first intron in Wistar rat ENaC γ [5-7, 14]. In the present analysis, we determined five, one, and ten new potential transcription factor binding-sites sequences on ENaC α, β, and γ, respectively. Among all variants identified within putative transcription factors binding sites, one of them, the insertion of -1118CCCCCA, located on ENaC γ gene was only present in Dahl rats. To our knowledge, this variant has not been previously reported on Dahl rats or any other rat strains [7]; www.genome.ucsc.edu; http://rgd.mcw.edu/VCMAP/). It creates binding sites for Sp1 and Oct-1. Sp1 is a DNA-binding protein which interacts with a variety of gene promoters containing GC-box elements. Moreover, the activity of TATA-less promoters is frequently dependent on Sp1 sites in the proximal promoter region. Deletion of one of the two clusters of Sp1 consensus binding sites within the 5' flanking region of the ENaC β gene indicated that the proximal cluster was essential to basal promoter activity in transfected cell lines [6]. Studies of both the human and rat γ -subunits [7, 16] also reported the absence of a TATA box in their promoters and the presence of GC boxes and Sp1 consensus sites. In human ENac γ, Sp1 may be part of the transcription complex that binds at the core promoter [16]. Sp1 protein is part of a much larger family of mammalian transcription factors, the Sp/XKLF family [17], it is ubiquitously expressed, and contains

three highly conserved C2H2-type zinc fingers in the C-terminal region and have a glutamine-rich activation domain. Sp1 can bind GC boxes, and acts as a transcriptional activator. Therefore the presence of a potential additional Sp1 consensus site in Dahl rats may enhance the transcription of the ENaC γ gene in Dahl rats compared to other rat strains. Similarly, Oct-1 sites have previously been reported to be able to function as repressors or activators of transcription depending on context [18-20]. Overexpression of ENaC γ in collecting duct cells has been shown to enhance Na^+ transport [21]. Remarkably, overexpression of marginally detectable amount of ENaC γ was sufficient to produce a full increase in Na^+ transport [21]. However, determination of the precise mechanism of all above variants in influencing promoter activity awaits further investigation.

It remains possible that mutations in the intronic regions of the ENaC are involved in the generation of hypertension in Dahl S rats on high salt intake. Commonly occurring ENaC variants, including intronic substitution (i12-17CT) were found associated with an increased urinary potassium excretion rate in relation to the renin levels as well as with hypertension in humans [22]. However, when expressed in *Xenopus* oocytes, the variants did not show a significant difference in activity compared with ENaC wild-type [22].

One can speculate that mutations in genes encoding a protein interacting with ENaC to regulate its activity might increase ENaC activity leading to hypertension. SGK1, which activates ENaC in tubules, maps to a known BP QTL [23]. Abnormal regulation of SGK1 mRNA and protein level by aldosterone in Dahl S compared to Dahl

R rat was observed suggesting that regulation of ENaC *via* SGK1 signaling pathway may be disturbed in Dahl S rat [24].

Conclusion

To our knowledge, this report presents the first comprehensive screening for variations in the entire coding sequences including intron-exon junctions, and in the 3' and 5' flanking regions of ENaC three genes in the hypertensive Dahl S rats and their normotensive Dahl R control rats together with an additional control group of Wistar rats. We could not link salt induced hypertension in Dahl S to differences in ENaC sequences in Dahl S *versus* R rats. Further characterization of SNPs across candidate genes contributing to the salt-sensitive hypertension phenotype will be useful in designing genetic mapping panels for association studies. If disordered activity of the epithelial cell sodium channel contributes to the pathogenesis of hypertension in Dahl S rats, it appears to stem from genetic variations in genes encoding proteins that regulate ENaC or in intronic sequences important for the structure or function of the sodium channel.

Materials and Methods

Animals: Male Dahl S and R rats (4 rats/group), 4–5 wks of age, were obtained from Harlan Sprague Dawley (Indianapolis, IN) and handled as previously described [8]. To assess the salt sensitivity of Dahl S rats, at 5 wks of age, Dahl S and R rats were placed on a high-salt (1,370 μmol Na/g, Teklad; Madison, WI) diet for 4 wks. After 4 wks, BP was measured invasively by intraarterial catheter and the average mean arterial pressure was estimated to be 156 ± 11 for S, and 131 ± 1 for R rats ($P<0.05$). Wistar rats (Charles River Breeding Laboratories Montreal, QC, Canada) were used as control, and were not subjected to high salt diet. The animals were then killed by decapitation and whole blood was collected for DNA isolation. All experiments were carried out in accordance with the guidelines of the University of Ottawa Animal Care Committee for the care and use of laboratory animals.

Genomic DNA isolation and amplification: Genomic DNA was isolated from white blood cells (Qiagen FlexiGene, Qiagen Canada, Mississauga, ON, Canada). See appendix E on page 162). Using PRIMER3-based Web application (http://primers.niob.knaw.nl), 73 sets of specific oligonucleotide primers (appendix D page 154) were designed based on the sequences retrieved from www.genome.ucsc.edu (November 2004 rat (*Rattus norvegicus*) genome assembly) in order to screen the coding sequence of ENaC three genes, including the exon-intron boundaries, as well as the 5' and 3' flanking regions [GenBank: NM_031548; NM_012648; NM_017046] [GeneID: 25122; 24767;24768]. For large exons (>350 bp) overlapping primer sets were employed.

The nucleotides representing the entire coding sequences and the 3'UTR and flanking regions that were screened in ENaC genes are as follows (numbering starts at the A

nucleotide of the primary initiation codon): for ENaC α, nt. 1 to 590, 9199 to 9652, 10553 to 10887, 15934 to 16281, 16442 to 16777, 17027 to 17271, 17246 to 17536, 20442 to 20730, 20663 to 20874, 20927 to 21134, 21117 to 21372, and 21582 to 23041; for ENaC β, nt. 1 to 413, 3287 to 3686, 5622 to 6012, 23724 to 23969, 25539 to 25839, 25974 to 26213, 27914 to 28154, 28987 to 29221, 29116 to 29351, 29910 to 30157, 30857 to 31100, and 31078 to 32049; for ENaC-γ nt, 1 to 378, 2108 to 2552, 3781 to 4141, 5153 to 5403, 8093 to 8385, 22437 to 22674, 24560 to 24797, 25439 to 25676, 25616 to 25849, 25842 to 26091, 28671 to 28918, 29065 to 30547. As for the 5' flanking regions of ENaC genes, -2078 to +1 bp, -34812 to -33582 bp, -4359 to +1 bp of ENaC α, β, and γ genes respectively were amplified.

Sequence Analysis: a) DHPLC (Helix, Varian, Palo Alto, CA) analysis. DHPLC runs were performed as recommended by Varian using buffer A and buffer B (Varian) and a flow rate of 0.45ml/min. Freshly prepared PCR products were denatured at 95°C for 3 min and re-annealed by decreasing the temperature from 95°C to 64°C at a rate of 1°C /min (appendices F to K). Optimal melting temperatures for the PCR products were determined using the Stanford University website (http://insertion.stanford.edu/melt.html). The chosen temperatures correspond to the point at which the retention time was 75% of (t_{rmax} - t_{rmin}). b) Automatic sequencing (ABI 310, PE Applied Biosystems, Foster City, CA). Sequencing was performed using the DYEnamic ET Terminator kit according to the instructions provided by the manufacturer (PE Applied Biosystems, Foster City, CA). Sequencing products were purified (DyeEx 2.0 spin kit columns; Qiagen Canada, Mississauga, ON, Canada) and analysed on 3100 DNA analyser (Applied Biosystems, Foster City, CA). Resulting sequences were

compared to the Brown Norway sequence retrieved from www.genome.ucsc.edu. c) ENaC 5' flanking regions analysis. A comprehensive analysis of putative kidney or brain transcription factor binding sites was performed using both literature reports [6, 7, 14] and two well-known and large-scale databases, TRANSFAC® [25] and TFSEARCH® [26], Using the cell selectivity track, the database searches were refined to transcription factors active in the rat kidney and brain where ENaC contributes to BP control.

Authors' Contributions

MS performed the all experiments and sequences analysis, participated in experimental design, drafted the manuscript.

FL conceived the project, finalized the manuscript.

FT designed the experimental strategy, supervised the study, finalized the manuscript.

All authors read and approved the final manuscript.

Acknowledgments

We are grateful for the generous advice and assistance of Drs. M. Ahmad, N. Sylvius and K. Adamo and Mrs. P Bolongo.

This study was supported by an operating grant MOP-74432 from the Canadian Institutes of Health Research, and by Program Grant PRG5275 from the Heart and Stroke Foundation of Ontario. F. H. H. Leenen holds the Pfizer Chair in Hypertension Research, an endowed chair supported by Pfizer Canada, the University of Ottawa Heart Institute Foundation, and the Canadian Institutes of Health Research. M Shehata was supported by an Ontario Graduate Scholarship and an Ontario Graduate Scholarship for Science and Technology.

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Prelude to Manuscript III

Statement of the rationale, hypothesis and objectives

Rationale

Because ENaC α, β, and γ genes were isogenic in the complete coding regions, exon-intron junctions and 3' and 5' flanking regions in Dahl S and R rats, we chose to direct our attention to the alternative splicing regulation of ENaC for the following reasons. Alternative splicing contributes significantly to the structural and functional diversity of ion channels such as the K^+, and Ca^{++} channels. Recently, a great emphasis has been placed on the dominant negative effect of some alternatively spliced forms on full-length forms. Dominant negative alternatively spliced forms sequester full-length forms in the endoplasmic reticulum (where channel subunits are assembled) and subsequently enhance their proteolytic degradation. The above mechanism makes alternative splicing an important process to study, because of its putative role in physiology, development and disease. ENaC splicing in Dahl rats has not yet been examined. Therefore, we chose to study the principle α subunit of ENaC in terms of RNA alternative splicing because of its critical role in channel functionality.

Hypothesis

α ENaC is regulated by RNA alternative splicing via the formation of alternatively spliced forms. α ENaC-alternatively spliced forms are overexpressed in kidneys of Dahl R *versus* S rats and on high *versus* normal salt diet.

Objectives

Objective 1. *To test whether α ENaC subunit is associated with alternatively spliced forms in the kidney cortex of Dahl rats.* We will search for previously reported alternatively spliced forms in kidney tissues of Dahl rats.

Objective 2. *To examine the mRNA levels of alternatively spliced forms of α ENaC in Dahl S versus R rats.* Using real-time PCR, we will quantitate α ENaC-alternatively spliced forms in kidney tissues of Dahl S *versus* R rats.

Objective 3. *To examine the dietary sodium influence on the mRNA levels of expression of alternatively spliced forms of α ENaC.* Using real-time PCR, we will quantitate α ENaC-alternatively spliced forms in kidney tissues of Dahl S *versus* R rats on high and normal salt diet.

3.0 Chapter 3: Manuscript III

Characterization of Epithelial Sodium Channel Alpha Subunit Transcripts and Their Corresponding mRNA Expression Levels in Dahl S *versus* R rats' Kidney Cortex On Normal and High Salt Diet.

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Published Manuscript

International Archives of Medicine 2009 Mar 13; 2(1):5

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Abstract

AIMS/HYPOTHESIS: The α-subunit of the amiloride-sensitive epithelial sodium channel (α ENaC) is critical for the expression of functional channels. In humans and rats, non functional alternatively spliced forms of α ENaC have been proposed to act as negative regulatory components for ENaC. The purpose of this study was to examine the presence and consequently investigate the mRNA expression levels of alternatively spliced forms of α ENaC in kidney cortex of Dahl salt-resistant rats (R) *versus* Dahl salt-sensitive rats (S) on high salt and normal diets. METHODS: Using quantitative RT-PCR strategy, we examined the mRNA expression levels of previously reported α ENaC-a and -b alternatively spliced forms in kidney cortex of Dahl S and R rats on normal and four-week high salt diet and compared their corresponding abundance to the wildtype α ENaC mRNA levels. We identified 2 novel non coding C-terminus spliced forms and examined their mRNA expression in Dahl S *versus* R rat kidney cortex. We also tested the presence of five previously reported lung-specific α ENaC spliced forms in Dahl rat kidney cortex (CK479583, CK475461, CK364785, CK475819, and CB690980). RESULTS: Previously reported α ENaC-a and -b alternatively spliced forms are present in Dahl rat kidney cortex and are significantly higher in Dahl R *versus* S rats ($P<0.05$). Four-week high salt diet significantly increased α ENaC-b ($P<0.05$), but not α ENaC-a transcript abundance in Dahl R, but not S rats. Two non coding α ENaC spliced forms -c and -d are newly identified in the present study, whose levels were comparable in Dahl S *versus* R rats. Compared to α ENaC-wt, α ENaC-a, -c and -d were low abundance transcripts (4 +/- 2, 110 +/- 20, and 10 +/- 2 fold less respectively), in contrast to α ENaC-b transcript abundance that was 32 +/- 3 fold higher than α ENaC-wt. We could not identify any of

the five previously reported lung-specific α ENaC spliced forms (CK479583, CK475461, CK364785, CK475819, and CB690980) in Dahl rat kidney cortex.

CONCLUSIONS/INTERPRETATION: α ENaC-alternative splicing might regulate α ENaC-by the formation of coding RNA species (α ENaC-a and -b) and non coding RNA species (α ENaC-c and -d). α ENaC-a and -b mRNA levels were significantly higher in Dahl R *versus* S rats and α ENaC-b transcript was significantly higher after 4-weeks of high salt diet compared to normal salt diet in Dahl R rats. Among the four α ENaC transcripts (-a, -b, -c and -d), only α ENaC-b was a high abundance transcript that exceeded α ENaC-wt abundance by 32 fold. α ENaC-a and -b spliced forms, particularly, α ENaC-b, might act as dominant negative proteins for ENaC activity, thereby rescuing Dahl R rats from developing salt-sensitive hypertension on high salt diet. Non coding α ENaC-c and -d might assist alternative splicing, facilitate RNA processing, or regulate α ENaC-as well as each other.

Keywords: Salt-sensitive hypertension, Dahl rat, mRNA expression levels, α ENaC transcripts, alternatively spliced forms

Introduction

The amiloride-sensitive epithelial sodium channels (ENaC) plays a key role in active sodium reabsorption by epithelia throughout the body, including kidney tubules, the lung, the distal colon, sweat and salivary glands, and the brain [1]. ENaC has been purified from the kidney as a large protein complex [2-4]. Molecular cloning studies indicate that ENaC consists of at least three subunits denoted by α, β, & γ, each of which possesses two transmembrane domains, a large extracellular loop, a cytoplasmic C-terminus and N-terminus domains [5, 6, 6]. α ENaC subunit is critical to the formation of ion conductive membrane pore, whereas β & γ ENaC subunits are required for maximal channel activity. α ENaC knockout in mice was lethal within 40 h of birth due to failure of pulmonary fluid clearance, clearly demonstrating the pivotal role of α ENaC in forming a functional Na^+ channel complex in vivo [7]. Moreover, decreased α ENaC expression (without necessarily knocking out α ENaC) predisposes animals to a respiratory distress syndrome [8], unlike the β and γ subunits that have only minor effects on lung fluid clearance.

ENaC activity is twice in kidneys of Dahl S *versus* R rats, possibly explaining the salt-sensitivity seen in Dahl S on high salt diet [9, 10]. The reasons for this variability in ENaC activity in Dahl S *versus* R rats are poorly understood at present. We have screened ENaC α, β, and γ complete coding sequences, exon-intron junctions, 5' and 3'flanking regions in Dahl S *versus* R rats and indeed there were no genetic differences between both strains in the above sequenced regions [11]. Alternative splicing of pre-mRNA represents a widespread mechanism for increasing variability of eukaryotic gene

expression and has been associated with human pathologies, such as cancer, Alzheimer's, amyotrophic lateral sclerosis, ataxia telangiectasia, cystic fibrosis, and senescence [12, 13]. Alternatively spliced forms of α ENaC have been described in rats [14-17], humans [14-17], mice [14-17] and chicken [14-17]. Non functional α ENaC-alternatively spliced forms have been proposed to serve as negative regulatory components for ENaC activity in humans [16]. Additionally, other spliced forms such as that of the inhibitor of apoptosis protein family was shown to be expressed at mRNA levels that were 2-3% of the levels of the full-length transcript, yet it encodes a protein that accumulates 50-fold higher levels than full-length and this accumulated protein competes with full-length form for activity [18]. The above facts have prompted us to examine the existence and consequently the mRNA expression levels of α ENaC-alternatively spliced forms -a and -b in Dahl S *versus* R rats on normal and 4 week-high salt diet, and to test the presence of five previously reported lung-specific α ENaC spliced forms in Dahl rat kidney cortex. Additionally, we identified and characterized two novel non coding C-terminus spliced forms in Dahl rat kidney cortex, and consequently determined their corresponding abundance in Dahl rats on normal and four-week high salt diet.

Materials and Methods

Animals: Male Dahl S and R rats (n=24), 3-4 wks of age, were obtained from Harlan Sprague Dawley (Indianapolis, IN) and handled as previously described [19]. The rats were divided into 4 groups (6 rats/group) by placing them on either regular (120μmol Na+/g) or high-salt (1,370 μmol Na+/g, Teklad; Madison, WI) diet for four wks. After 4 wks, BP was measured invasively by intraarterial catheter and the average mean arterial pressure was estimated to be 192 ± 10 for S, and 125 ± 5 for R rats ($P<0.05$). The animals were then killed by decapitation and kidney tissues were removed and placed in cold methylbutane and then on dry ice. Tissues were preserved at -80 ° C for later RNA isolation. All experiments were carried out in accordance with the guidelines of the University of Ottawa Animal Care Committee for the care and use of laboratory animals.

RNA isolation: Approximately 100 mg of kidney cortex was cut out microscopically and homogenized with polytron. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Isolated RNA was subjected to DNAse treatment for removing potential genomic DNA contamination using DNase treatment kit (Ambion). Total RNA was quantified by UV light absorbance at 260 nm by a spectrophotometer. The OD ratio of 260/ 280 nm was within the range of 2.0 ± 0.1 in all samples.

Reverse transcription (RT): 5 μg of RNA were transcribed to cDNA in a total of 20 μl reaction volume. Briefly, a mixture of 1 μl (500 ng) oligo $(dT)_{12-18}$ primer), 1 μl 10 mM deoxyribonucleotides (dNTP) mix (final conc. 0.5 mM) and water were added to the calculated volume of RNA to make 12 μl. The mixture was heated to 65° C for 5 minutes, then quickly chilled on ice, and briefly centrifuged. 4 μl 5 X first strand buffer,

2 μl 1 M DTT, 1 μl RNAse OUT Recombinant Ribonuclease Inhibitor (40 units) and 1 μl of Superscript II RNase H- Reverse Transcriptase (200 units) (Invitrogen, Burlington, ON) were added to the tube. The final mixture (20 μl) was incubated in a water bath at 42° C for 50 minutes. The reaction was inactivated at 70° C for 15 min. First strand cDNA thus obtained was cooled in ice and then stored at -20° C until PCR was performed.

Primer Design: Primers were designed specifically to amplify the α ENaC-a and -b alternatively spliced forms by including the nucleotide deletions (23 bp in α ENaC-a sense primer, and 79 bp in α ENaC-b reverse primer). The primers used to amplify α ENaC-a, -b, -c, -d and the previously reported lung spliced forms (CK479583, CK475461, CK364785, CK475819, and CB690980) are mentioned in Table 3.1. For α ENaC-a and b, we used the sequences previously reported [15]; whereas for the lung spliced forms we used the sequences retrieved from the www.genome.ucsc.edu. The accession numbers for the five previously reported lung spliced forms are CK479583, CK475461, CK364785, CK475819, and CB690980. For α ENaC-c and -d, 2 different primer pairs were used to confirm the newly identified spliced forms.

Table 3.1. Primers used for reverse transcription polymerase chain reaction and expected sizes of cDNA fragments.

Accession ID	Amplic on Size	Primers used
α ENaC-wt	325 bp	Sense: 5'-TCATGCTGCTACGCCGGTTCC-3' Antisense:5'-TCCATCAGTTTACAAGGGAG-3'
α ENaC-a	266 bp	Sense: 5′-CAGAGCTCCTGGGGGCGCCTT-3′ Antisense: 5′- TCCTTCTGTCACGATGGTCA-3′
α ENaC-b	278 bp	Sense: 5′-GGATGATGGTGGCTTCAACT-3′ Antisense: 5′-ACACCCAGGAGCTCTGCTTT-3′
α ENaC-c	59 bp 59 bp	Sense: 5′-GCTCCTGGGTGTGATCAACTA-3′ Antisense: 5′-AAAGAAGGTGGTGGGGATGT-3′ Sense: 5′-GGATGATGGTGGCTTCAACT-3′ Antisense: 5′-AAAGAAGGTGGTGGGGATGT-3′
α ENaC-d	99 bp 99 bp	Sense: 5′-CCACGCGTCCGAAGAAAC-3′ Antisense:5′-AAAGAAGGTGGTGGGGATGT-3′ Sense: 5'-CCACGCGTCCGAAGAAACTG-3' Antisense:5'-TAAAGAAGGTGGTGGGGATG-5'
CK479583	767 bp	Sense: 5′-GCCCCAGCAGGGCATGACCC-3′ Antisense: 5′-CTAGAGTAGCATAGGCAGGT-3′
CK475461	814 bp	Sense: 5′-TCCAAGTATACACAGCAGGT-3′ Antisense: 5′-TACAGGAGTCAGGCTGTGAG-3′
CK364785	764 bp	Sense: 5′-GGGGCTATTGCTATTATAAA-3′ Antisense:5′-CCACACAGAGGGCTCGGCGGG-3′
CK475819	748 bp	Sense: 5′-GATTCCCGGGATCTTGGA-3′ Antisense: 5′- AGGCTGACCATCGTGACAG-3′
CB690980	376 bp	Sense: 5′-CCACGCGTCCGAAGAAAC-3′ Antisense: 5′- AAAGAAGGTGGTGGGGATGT-3′
Pgk	263 bp	Sense: 5′-GCTGCAGAACTCAAATCTCT-3' Antisense:5′-TGTGTGCAGTCCCAAAAGCA-3′

Cloning: α ENaC-a, -b, -c and -d were cloned using DH5 α competent cells, and TOPO TA cloning kit ® (Invitrogen, ON, Canada). The resulting clones were sequenced using ABI prism 3100 (PE Applied Biosystems, Foster City, CA). Sequencing was performed using the DYEnamic ET Terminator kit according to the instructions provided by the manufacturer (PE Applied Biosystems, Foster City, CA). Sequencing products were purified (DyeEx 2.0 spin kit columns; Qiagen Canada, Mississauga, ON, Canada). The newly identified α ENaC-c and -d spliced forms were examined using TRANSEQ® computer program for translation in silico (http://www.ebi.ac.uk/emboss/transeq/index.html?).

Quantitative Real-time PCR: Real-time PCR amplifications were performed with a Roche Light Cycler using Fast Start DNA Master SYBR Green I (Roche Diagnostics, Montreal, QC). 2 μL of 1:10 diluted RT product from kidney cortex were used for the PCR reaction in a 20 μl reaction using the previously described primers for each splice variant. The real-time PCR conditions were as follows (appendix M): first, 95°C for 10 min to activate Taq polymerase, followed by 45 cycles of denaturation at 95°C for 5 sec, annealing for 10 sec at 62° for PGK and α ENaC-wt; 65°C for α ENaC-a, -c and -d and 67°C for α ENaC-b. An extension step was performed at 72° C and the extension time was determined by the formula of amplicon length/25 sec. The specificity of real-time PCR products was documented with a melting curve analysis. In addition, a high resolution gel electrophoresis was performed which resulted in the amplification of a single product of the appropriate size (Table 3.1). Real-time RT-PCR analysis was performed in triplicate.

Plasmids containing the cDNAs of α ENaC-wt, -a, -b, -c and -d transcripts were used to generate external standard curves. α ENaC subunit full length cDNA plasmids was a gift from Dr. Bernard C. Rossier, Univ. of Lausanne, Switzerland. All of the construct concentrations were quantified by absorbance at 260 nm. Serial 10-fold dilution (e.g. 100 pg, 10 pg, 1 pg, 0.1 pg, 0.01 pg, 0.001 pg etc.) of each plasmid clones were used to generate an external standard individually by using same condition of real-time PCR described above for each gene. Expression was normalized to PGK levels as an endogenous reference. Normalization was achieved by dividing the amount of cDNA of each ENaC transcript by the PGK quantity. Real-time PCR efficiencies were calculated according to the equation $E=10^{[-1/slope]}$: for α ENaC-wt-1.97; a-2.00, b-1.99, c-1.98, d-1.95 and PGK-1.94.

Examining in silico translation for α ENaC-b, -c and -d

Using Transeq ® computer software, α ENaC-b, -c and -d were examined for translation in silico. Transeq ® free web tool can translate any nucleic acid sequence to the corresponding peptide sequence in any of the three forward and three reverse sense frames.

Statistical Analysis: Differences between the expression levels of α ENaC-wt and alternatively spliced forms were analysed in Dahl S *versus* R rats on normal and high salt diet using two-way ANOVA (SigmaSTAT ®). The statistical test was two sided to identify strain and dietary differences, the data were expressed as means and ranges, and differences of *P* values of less than 0.05 were considered to be significant.

Results

Presence of α ENaC-a, -b, -c and -d in Dahl rats kidney cortex on normal and high salt diet

Amplification of α ENaC-a, -b, -c and -d was done in Dahl S and R rats kidney cortex on normal and high salt diet (Figure 3.1A, 3.1B, 3.1C, 3.1D). The three alternatively spliced α ENaC-a, -b, -c forms share a common 5' donor splice site (GCTCCTGGG). PCR signals for α ENaC-a, -c and -d were lower than those for α ENaC-wt. In contrast, α ENaC-b signal was higher than α ENaC-wt.

Screening Dahl rat kidney cortex for the previously reported lung spliced forms and identification of α ENaC-c and -d spliced forms

Using PCR primers to amplify the 5 previously reported spliced forms; we did not find any in Dahl rat kidney cortex (Table 3.1). However, we obtained and characterized 2 novel C-terminus spliced forms from Dahl rat kidney cortex. The first novel spliced form, referred to as α ENaC-c is a 59 bp RNA that was confirmed by complete nucleotide sequencing, while the second spliced form, referred to as α ENaC-d is a 99 bp RNA that was confirmed by complete nucleotide sequencing (Figure 3.2).

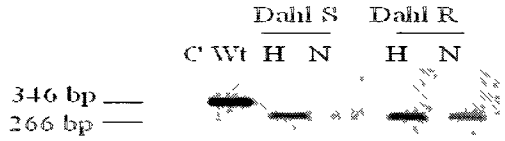
To confirm that α ENaC-c and -d are not cloning artifacts, we conducted PCR analysis using selective primers that would amplify the 59 bp and 99 bp variants, and we cloned and subsequently sequenced the resulting amplicons. Sequencing results showed that α ENaC-c is the shortest isoform, made up of 9 nt of exon 7 (GCTCCTGGG), 12 nt of exon 9 (TGTGATCAACT) and 20 nt of exon 12 (ACATCCCCACCACCTTCTTT). α ENaC-d is the second shortest variant and included 20 nt of exon 12.

Figure 3.1: PCR analysis of α ENaC-a, -b, -c and -d variants *versus* α ENaC-wt in kidney cortex of Dahl S *versus* R rats on normal and high salt diet. Figure 3.1A) Specific sense and antisense primers were used for amplifying α ENaC-wt. α ENaC-wt fragment of 325 bp was amplified from kidney cortex of Dahl S and R rats fed normal and high salt diet. Figure 3.1B) Specific sense primers (missing the 23 bp unique to α ENaC-a) were utilized to amplify α ENaC-a. α ENaC-a fragment of 266 bp was amplified from kidney cortex of Dahl S and R rats fed normal and high salt diets. For a negative control, water without a cDNA template was used and for a positive control α ENaC-wt primers were utilized to amplify a α ENaC-wt fragment of 346 bp. Figure 3.1C) Specific antisense primers (missing the 79 bp unique to α ENaC-b) were utilized. α ENaC-b fragment of 278 bp was amplified from kidney cortex of Dahl S and R rats fed normal and high salt diet. Figure 3.1D) Specific primers for α ENaC-c were used to amplify a fragment of 59 bp. α ENaC-c was amplified from kidney cortex of Dahl S and R rats fed normal and high salt diet. Figure 3.1E) Specific primers for α ENaC-d were used to amplify a fragment of 99 bp from kidney cortex of Dahl S and R rats. α ENaC-d was amplified from kidney cortex of Dahl S and R rats fed normal and high salt diet. *C (Control):* water without cDNA template; *wt:* wildtype α ENaC primers; *H:* High salt diet; *N:* Normal salt diet. The sizes of the expected PCR products are indicated.

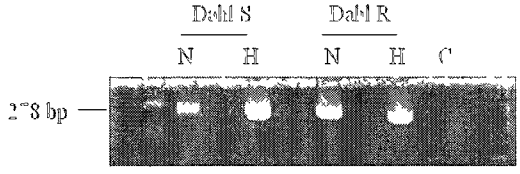
A.


Dahl S
Dahl R
H
N
H
N
325 bp

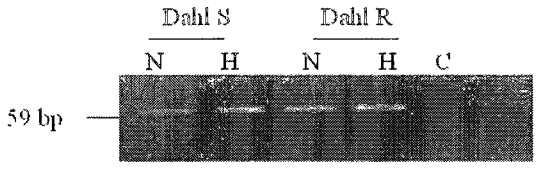
B.


Dahl S
Dahl R
C' Wt
H
N
H
N
346 bp
266 bp

C.


Dahl S
Dahl R
N
H
N
H
C
278 bp

D.


Dahl S
Dahl R
N
H
N
H
C
59 bp

E.

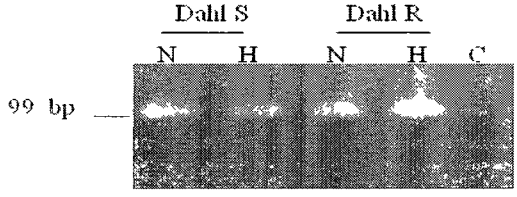

Dahl S
Dahl R
N
H
N
H
C
99 bp

Figure 3.2: Schematic representation of novel α ENaC-alternatively spliced forms (-c and -d). A) A schematic illustration of the mRNA sequences of α ENaC-c and -d forms. α α ENaC-c and -d are small non coding RNA. α ENaC-c is composed of portions of exon VII, IX and XII, while that of α ENaC-d is formed of a portion of exon XII and an intronic fragment. B) The genomic sequence of α ENaC-c and -d forms. α ENaC-c share the same splicing site (CCTGGG) with α ENaC-a and -b spliced forms, which is located within exon VII. α ENaC-c and -d are made up of 59 and 99 bases respectively (see appendices O and P).

A.

B. Nucleotide sequence of α ENaC-c

59 bp

gctcctgggtgtgatcaactaaggtgaaggtcggagaagacatccccaccaccttcttt

Exon VII IX XII

Nucleotide sequence of α ENaC-d

99 bp

Ccacgcgtccgaagaaactgaagatggtgatgggattaataacttcaaataaagtcataaaagttaatgcaatgaaag

ca*catccccaccaccttcttta*

ExonXII

Examining α ENaC-b, -c and -d translation in silico

α ENaC-b is translatable as predicted by Transeq ® web tool, and as previously reported [15]. Both newly identified spliced forms α ENaC-c and -d were non coding RNA. The peptide sequences as predicted by Transeq ® computer software were interrupted with multiple stop codons in each of the three forward and three reverse sense frames, and this in turn hinders the translation of α ENaC-c and -d into a polypeptide sequence.

Comparison of mRNA expression levels of α ENaC -a, -b, -c and -d to α ENaC-wt using quantitative RT-PCR strategy

Using PCR primers that were designed to amplify α ENaC-a and -b, we amplified and subsequently cloned 266 bp and 278 bp of alternatively spliced forms α ENaC-a and -b respectively. We confirmed the deletions in α ENaC-a and -b by nucleotide sequencing of the resulting amplicons.

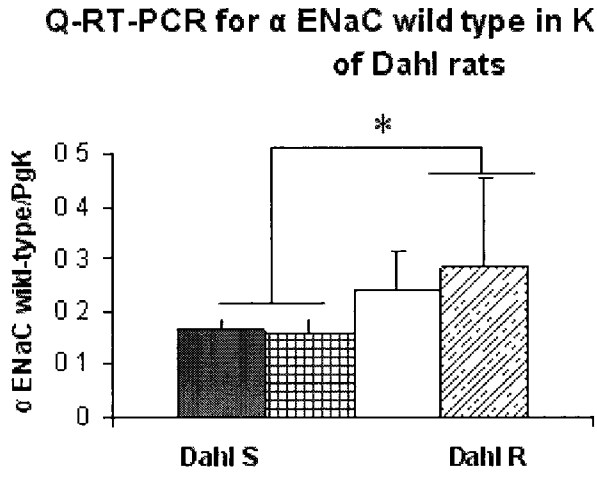
α ENaC spliced forms -a, and -b were significantly higher in Dahl R *versus* S rats ($P<0.05$), a pattern similar to α ENaC-wt mRNA expression (Figure 3.3). A statistically significant increase of $P<0.05$ was seen only in Dahl R rats for α ENaC-b spliced form on four-week high salt diet. α ENaC-c and -d expression levels were comparable in Dahl S and R rats, with no significant dietary impact on their corresponding mRNA expression levels.

α ENaC-a, -c and -d were respectively 4 +/-2 fold, 110+/-20 fold, and 10+/-2 fold lower in abundance than α ENaC-wt (Figure 3.3), while α ENaC-b transcript was 32+/- 3 fold higher than α ENaC-wt.

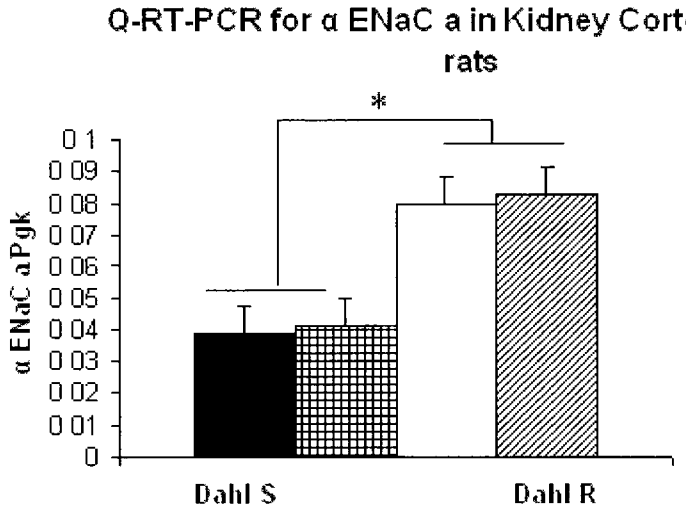
Figure 3.3: Quantitative RT-PCR analysis of RNA expression of α ENaC-wt and the alternatively spliced forms -a, -b, -c and –d in kidney cortex of Dahl S and R rats fed normal and high salt diet. Figure 3.3A) Specific primers for α ENaC-wt were used to amplify cDNAs from kidney cortex of Dahl S and R rats on normal and high salt diet. A significant increase ($P<0.05$) in α ENaC-wt expression was observed in Dahl R *versus* S rats. No significant dietary impact on the expression levels of α ENaC-wt was noticed in Dahl S or R rats. Figure 3.3B) Specific primers unique to α ENaC-a were used to amplify cDNAs from kidney cortex of Dahl S and R rats on normal and high salt diet. A significant increase ($P<0.05$) in α ENaC-a expression was observed in Dahl R *versus* S rats. No significant dietary impact on the expression levels of α ENaC-a was noticed in Dahl S or R rats. Figure 3.3C) Specific primers unique to α ENaC-b were used to amplify cDNAs from kidney cortex of Dahl S and R rats on normal and high salt diet. A significant increase ($P<0.05$) in α ENaC-b expression was observed in Dahl R *versus* S rats. Additionally, a significant increase in the expression levels of α ENaC-b was observed on high *versus* normal salt diet in Dahl R, but not S rats. Figure 3.3D) Specific primers unique to α ENaC-c were used to amplify cDNAs from kidney cortex of Dahl S and R rats on normal and high salt diet. Only a slight, insignificant increase in α ENaC-c expression was observed in Dahl R *versus* S rats. No significant dietary impact on the expression levels of α ENaC-c was noticed in Dahl S or R rats. Figure 3.3E) Specific primers unique to α ENaC-d were used to amplify cDNAs from kidney cortex of Dahl S and R rats on normal and high salt diet. Only a slight, insignificant increase in α ENaC-a expression was observed in Dahl R *versus* S rats. α ENaC-a, -c and -d are lower abundant transcripts. Only α ENaC-b exceeded α ENaC-wt expression by ~32 fold. Bars represent

mean +/- SEM, *: denotes $P<0.05$ between Dahl R and Dahl S, #: denotes $P<0.05$ in transcript abundance on normal and 4 week high salt diet in Dahl R, Dahl R: Dahl salt-resistant, Dahl S: Dahl salt-sensitive. Normal salt diet treatment: is represented by the black and white bars for Dahl S and R rats respectively. High salt diet treatment: is represented by the chequered and diagonally highlighted bars for Dahl S and R rats respectively. All α ENaC transcript levels were normalized against the house keeping gene PgK. N=6 rats/group and the results are the average of 3 independent experiments (see appendix S for numerical values).

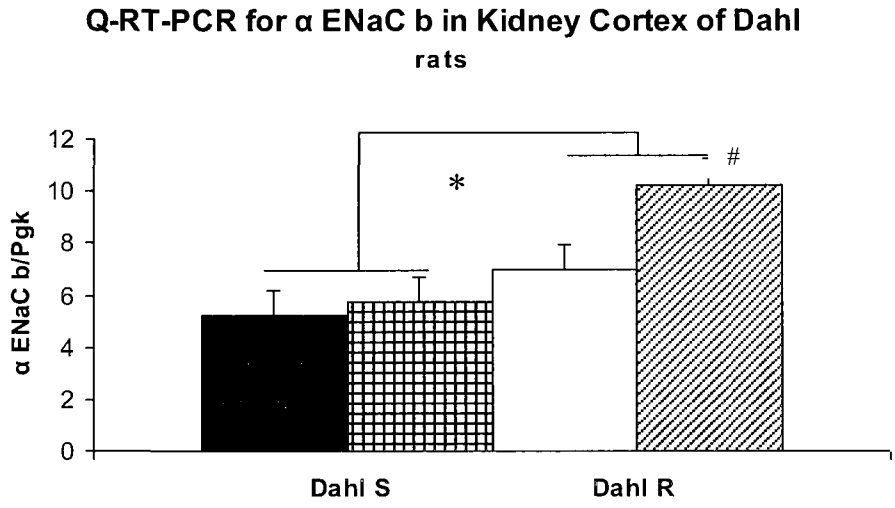
A.


Q-RT-PCR for α ENaC wild type in Kidney Cortex of Dahl rats
α ENaC wild-type/PgK
0 5
0 4
0 3
0 2
0 1
0
*
Dahl S
Dahl R

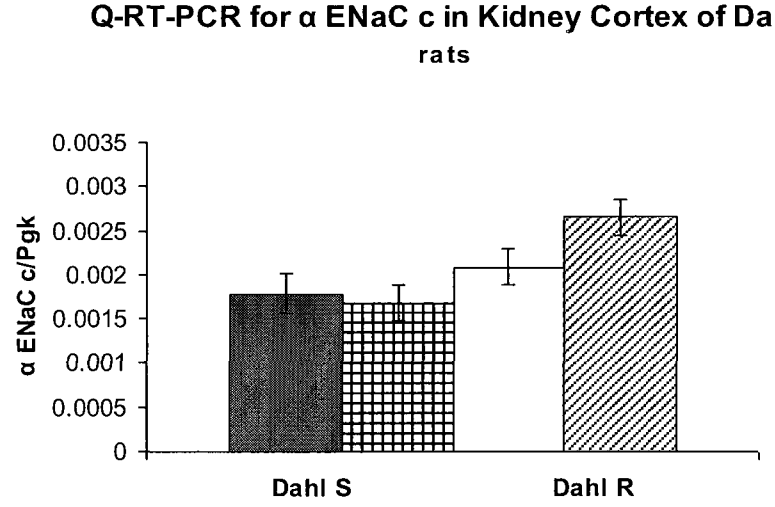
B.


Q-RT-PCR for α ENaC a in Kidney Cortex of Dahl rats
α ENaC aPgk
0 1
0 09
0 08
0 07
0 06
0 05
0 04
0 03
0 02
0 01
0
*
Dahl S
Dahl R

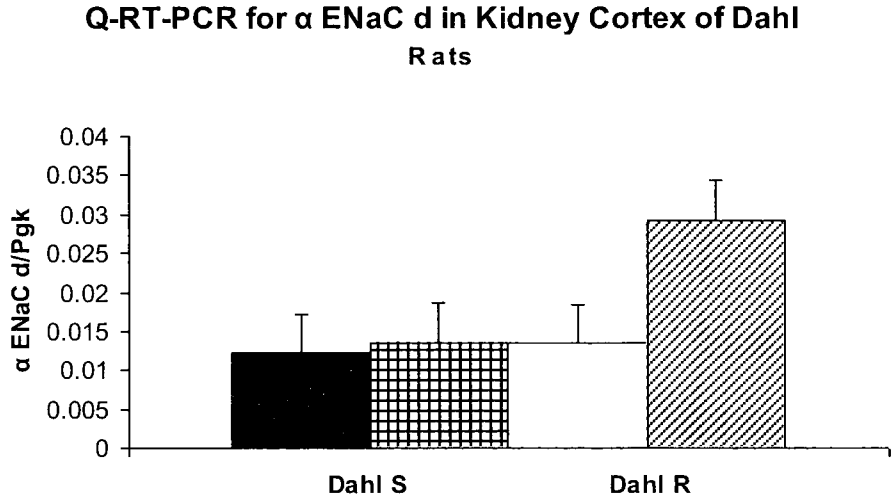
C.


Q-RT-PCR for α ENaC b in Kidney Cortex of Dahl rats
α ENaC b/Pgk
12
10
8
6
4
2
0
*
#
Dahl S
Dahl R

D.


Q-RT-PCR for α ENaC c in Kidney Cortex of Dahl rats
α ENaC c/Pgk
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
Dahl S
Dahl R

E.


Q-RT-PCR for α ENaC d in Kidney Cortex of Dahl Rats
α ENaC d/Pgk
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
Dahl S
Dahl R

Discussion

The present study demonstrates the following: α ENaC-a, -b, -c and -d are expressed in the kidney cortex of Dahl S and R rats on high and normal salt diet, and the mRNA expression levels of α ENaC-alternatively spliced forms α ENaC-a and -b are significantly higher ($P<0.05$) in Dahl R *versus* S rats. Compared to normal salt diet, four-week high salt diet caused only a slight, insignificant elevation in α ENaC-a, whereas a moderate increase in the abundance of α ENaC-b ($P<0.05$) in Dahl R rats was determined. Two novel non coding spliced forms referred to as α ENaC-c and -d were characterized, cloned and sequenced from Dahl rat kidney cortex. α ENaC-c and -d mRNA abundance were insignificantly higher (up to 1.6 fold) in Dahl R *versus* S rats. None of the previously reported lung spliced forms (CK479583, CK475461, CK364785, CK475819, and CB690980) were identified in kidney cortex of Dahl S, nor R rats on normal or four- week high salt diet. Comparing α ENaC-alternatively spliced forms to α ENaC-wt transcript abundance, only α ENaC-b was 32 +/- 3 folds higher than α ENaC-wt, while α ENaC-a, -c and -d were respectively 4 +/-2 fold, 110+/-20 fold, and 10+/-2 fold lower in abundance than α ENaC-wt.

We chose to study α ENaC-alternatively spliced forms' abundance four weeks post high salt diet in kidney cortex and not the medulla of Dahl S and R rats, because of the significant strain differences already reported in α ENaC-wt expression levels. Kidney medulla α ENaC-wt mRNA was not affected by either diet or strain at any time point (two or four-week high salt diet) [20], whereas kidney cortex α ENaC-wt mRNA was significantly higher in Dahl R *versus* S rats kidney after four, but not two-week of high salt diet [20].

Alternative splicing is a major contributor to the structural and functional diversity of ion channels such as the Na^+, K^+, and Ca^{++} channels [21-25]. Formation of alternatively spliced forms of α ENaC have been previously reported in four species: humans [14-17], rats [14-17], mice [14-17] and chicken [14-17]. In rats, two alternatively spliced forms (α ENaC-a and -b) of the α ENaC subunit are currently published [14-17]. α ENaC-a and -b are identified in the rat kidney and tongue taste tissues [14-17]. Both α ENaC-a and -b encode for shorter proteins that lack the second transmembrane domain M2. α ENaC-a has been expressed in HEK293 and CV1 cells [14-17], while α ENaC-b translation *in vitro* is yet to be determined. When α ENaC-a is expressed in *Xenopus* oocytes, loss of channel function was reported suggesting that α ENaC-a polypeptide might hinder α ENaC-wt expression and/or function by direct physical interaction, or by disrupting channel assembly in vivo because of the critical involvement of the missing transmembrane domain in creating functional channels [14-17].

Our screening study yielded preliminary evidence that suggested the generation of additional spliced forms in kidney cortex (α ENaC-c and -d) besides the previously reported spliced forms (α ENaC-a and -b). α ENaC-a, -b, and -c spliced forms share the same 5' donor splice site (GCTCCTGGG) with the human α ENaC +22 variant [14-17] and the chicken 3399 bp variant [14-17]; suggesting that this 5'donor splice site is preferably utilized by various species to generate spliced forms of the α ENaC subunit [14-17]. There was an insignificant elevation (up to 2 fold) in α ENaC-c and -d mRNA in Dahl R *versus* S rats which might have possibly reached significance by increasing the number of rats screened (n>6). When α ENaC-c and -d were examined for translation in silico using

Transeq®, they appeared to be non coding. It is not certain at this point whether α ENaC-c and -d, are the result of aberrant splicing or the result of regulated alternative splicing.

Until very recently there were no reports on the regulation of ENaC in Dahl S and R rat models. Then, Aoi et al. reported an abnormal increase in α ENaC-wt mRNA (2.5-fold) in the kidneys of Dahl S, but not R rats (α ENaC mRNA is suppressed in Dahl R rats) on high *versus* regular salt intake for 4 weeks [26, 27]. It is worth mentioning that the sequences of the forward and reverse primers used by Aoi and co-authors [26, 27] are common among α ENaC-wt, α ENaC-a and -b spliced forms, and the altered levels reported for α ENaC should be carefully interpreted (because the primers would amplify both the major transcript and the two alternatively spliced forms, and hence cause false elevations of full-length α ENaC mRNA). Our results, on the other hand, using specific α ENaC-wt primers showed no significant changes in α ENaC-wt mRNA concentration in response to salt in Dahl S and R rats, but a constitutive increase in α ENaC-wt mRNA concentration was witnessed in Dahl R *versus* S rats.

Indeed, the increased levels of expression of α ENaC-a and -b transcripts in Dahl R *versus* S rats might be indicative of a putative regulatory effect on α ENaC expression/function. In conclusion, α ENaC-alternative splicing might play a role in ENaC regulation by the resulting coding (α ENaC-a and -b) and non coding (α ENaC-c and -d) alternatively spliced forms. α ENaC-a and -b coding transcripts allow the expression of shortened, but partially functional proteins. The highly abundant α ENaC-b transcript might possibly play a role as a dominant negative element in Dahl R rats rescuing the α ENaC from overactivity in high salt environment, either by direct interaction with α ENaC or by affecting α ENaC proper assembly and the formation of

functional channels. The non coding newly identified α ENaC-c and -d alternatively spliced forms might act as potential regulators for the RNA machinery possibly by facilitating RNA processing, assisting alternative splicing, regulating α ENaC-as well as each other or finally they might be an indicative of the cells' potential to tolerate a splicing process which generates frequent splicing errors. It is worth mentioning that at the time of submission of the current manuscript, preliminary evidence of the translation of α ENaC-b *in vitro* and its putative dominant negative effect on α ENaC-wt expression have been demonstrated [28, 29].

Acknowledgments

The author acknowledges Roche Diagnostics® Canada for generously providing free samples of the Fast Start DNA Master SYBR Green I, as well as free samples of Taq polymerase enzyme. Additionally, the author is grateful to Qiagen® Canada for providing free samples of the DyeEx 2.0 spin kit columns used for sequencing. MF Shehata is the recipient of the Memorial University of Newfoundland Horizon Award for 2007. MF Shehata has received numerous awards including the Canadian Institutes of Health Research/Pfizer/Canadian Hypertension Society Doctoral Research Award, Ontario Graduate Scholarship and the Ontario Graduate Scholarship for Science and Technology.

Competing interests

The author declares no competing interests.

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Prelude to Manuscript IV

Statement of the rationale, hypothesis and objectives

Rationale

Following the initial study on α ENaC-alternatively spliced forms, their existence in Dahl rats' kidney cortex and the impact of dietary salt on their levels of expression; we extended our investigation to studying α ENaC-b further for the following reasons:

1) Alternatively spliced form α ENaC-b mRNA levels exceeded α ENaC-wt by ~32 +/- 3 times.

2) α ENaC-b mRNA is significantly higher in kidney cortex of Dahl R four-week post high salt diet *versus* normal salt diet ($P<0.05$).

3) α ENaC-b mRNA is significantly higher in Dahl R *versus* S rats ($P<0.05$).

The above findings suggest that alternative splicing might offer an additional mechanism for ENaC regulation in Dahl rats, and might explain the increased ENaC activity in Dahl S *versus* R rats.

Hypothesis

α ENaC-b is a dominant negative expression regulator for α ENaC-wt in Dahl R rats to possibly rescue the channel from overactivity in high salt environment, and potentially explain their salt-resistance.

Objectives

Objective 1. Is the alternatively spliced form α ENaC-b translated *in vitro*? Using COS-7 cells, we will examine the *in vitro* translation of α ENaC-b.

Objective 2. What would be the impact of α ENaC-b polypeptide on α ENaC-wt when co-expressed in COS-7 cells? Using COS-7 cells and western blot analysis, we will

examine the co-expression of α ENaC-b with α ENaC-wt.

Objective 3. Does α ENaC-b binds to α ENaC-wt in vitro? Using native western blot analysis, we will determine if a physical interaction of α ENaC-b with α ENaC-wt does occur *in vitro.*

Objective 4. Does α ENaC-b sequester α ENaC-wt to enhance its proteolytic degradation in a dose-dependent manner? Using COS-7 cells and denaturing western blot analysis, we will examine the impact of increasing doses of α ENaC-b on α ENaC-wt expression *in vitro.*

Corrigendum note: We have included a "study limitations" section following the conclusion of chapter 4. This study limitations section lists some of the important controls that were missing and might potentially impact the results presented in this chapter. Moreover, appendix Q and R (pages 191 and 192) included a more accurate representation of the results along with the appropriate loading controls, densitometric analysis, graphical and statistical analysis of the last set of experiments presented in this chapter.

4.0 Chapter 4: Manuscript IV

A Novel Mechanism in Regulating the alpha-subunit of the Epithelial Sodium Channel (α ENaC) by the Alternatively Spliced Form α ENaC-b

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Published Manuscript

Biochemistry Insights 2009:2 21-27

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Abstract

INTRODUCTION: In Dahl rats' kidney cortex, the alternatively spliced form of the epithelial sodium channel α subunit (α ENaC-b) is the most abundant mRNA transcript (32+/-3 fold > α ENaC-wt) as was investigated by quantitative RT-PCR analysis. α ENaC-b mRNA levels were significantly higher in Dahl R *versus* S rats, and were further augmented by high salt diet. **OBJECTIVES:** In the present study, we described the molecular cloning and searched for a possible role of α ENaC-b by testing its potential expression in COS7 cells as well as its impact on α ENaC-wt expression levels when co-expressed in COS7 cells in a dose-dependent manner. **METHODS:** Using RT-PCR strategy, the full-length wildtype α ENaC transcript and the alternatively spliced form α ENaC-b were amplified, sequenced, cloned, subcloned into PCMV-sport6 expression vector, expressed and co-expressed into COS7 cells in a dose-dependent manner. A combination of denaturing and native western blotting techniques was employed to examine the expression of α ENaC-b in vitro, and to determine if an interaction between α ENaC-b and α ENaC-wt occurs in vitro, and finally to demonstrate if degradation of α ENaC-wt protein does occur. **RESULTS:** α ENaC-b is translated in COS7 cells. Co-expression of α ENaC-b together with α ENaC-wt reduced α ENaC-wt levels in a dose-dependent manner. α ENaC-wt and α ENaC-b appear to form a complex that enhances the degradation of α ENaC-wt. **CONCLUSIONS:** Western blots suggest a novel mechanism in α ENaC regulation whereby α ENaC-b exerts a dominant negative effect on α ENaC-wt expression. This is potentially by sequestering α ENaC-wt, enhancing its proteolytic degradation, and possibly explaining the mechanism of salt-resistance in Dahl R rats.

Keywords: α ENaC-wt, α ENaC-b, COS7 cells, transfection, expression, binding, dose-dependent response, salt-sensitive hypertension, Dahl rats

Introduction

The amiloride-sensitive epithelial sodium channels (ENaC) consists of at least three subunits denoted by α, β, & γ, each of which possesses two transmembrane domains [2-4]. The α ENaC subunit is a key molecule for the formation of a functional Na^+ channel complex in vivo, whereas the β & γ subunits greatly potentiate the channel activity [4]. α ENaC gene knockout in mice was lethal within 40 h of birth because of fluid-filled lungs, confirming the critical role of α ENaC in forming functional Na^+ channels in vivo [5]. Naturally occurring α ENaC-alternatively spliced forms have been identified in mice, chicken, humans and rats [6-9]. To date, there are two alternatively spliced forms of the α subunit of ENaC (α ENaC-a & -b) that have been published in rats, in addition to the major α ENaC transcript [9]. α ENaC-a transcript is a low abundance transcript compared to the full-length α ENaC-and has been studied in terms of expression, functionality and binding to ENaC blocker [9]. On the other hand, α ENaC-b is yet to be characterized and appears to play a role in modulating ENaC for the following reasons: a) it shares the same splice site with human and chicken spliced forms; b) our preliminary results showed that unlike α ENaC-a, α ENaC-b mRNA levels are ~32±3-fold higher than full-length α ENaC in kidneys of Dahl rats indicating an increased stability/synthesis of the former [10]; c) α ENaC-b mRNA concentrations are significantly higher in Dahl R *versus* Dahl S rats [10], suggesting a putative dominant negative effect on α ENaC-activity in a model of suppressed ENaC activity (Dahl R rats) [11]; d) α ENaC-b is a salt-sensitive transcript, because high *versus* normal salt diet caused a remarkable increase in α ENaC-b mRNA levels ($P<0.05$) in Dahl R rats [10].

Owing to the fact that non functional α ENaC-alternatively spliced forms, such as α ENaC-b, have been proposed to serve as negative regulatory components for ENaC activity [8], we were prompted to invest in basic research with a view to identifying novel targets for diagnosis, prevention and treatment of disorders related to ENaC dysfunction. The overall objectives of these studies were to investigate whether α ENaC-b is translated in vitro, whether it is co-expressed with α ENaC-wt, and whether α ENaC-b expression suppresses α ENaC-wt expression when co-expressed in COS7 cells in a dose-dependent manner.

Materials and Methods

A. RNA extraction, RT-PCR and PCR

Kidney cortex of Dahl S rats was cut out microscopically and homogenized with polytron. Poly $(A)^+$ mRNA from kidney cortex of Dahl S rats was isolated using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Isolated RNA was subjected to DNAse treatment for removing potential genomic DNA contamination using DNase treatment kit (Ambion, ON, Canada). First strand cDNA was synthesized from 2 μg of mRNA with superscript II RNase H- Reverse transcriptase (Invitrogen, CA, USA).

For α ENaC-wt and α ENaC-b, specific primers (Table 4.1) were designed to flank the open reading frames of α ENaC-wt and -b form. High fidelity Expand Long Range, dNTPack (kind gift from Roche Applied Science®, QC, Canada) is employed to amplify the full length α ENaC-and α ENaC-b. Touchdown PCR method was employed to improve the efficiency of gene amplification as follows (start temperature (Tm) at 68°C and the annealing Tm reduced 2°C per cycle for the next cycles up to Tm of 58°C, then the remaining cycles were performed at a 58°C annealing temperature). After amplification, the specific fragments of 2267 bp (α ENaC-wt) and 1586 bp (α ENaC-b) were visualized in 1% agarose gels (figure 4.1).

Table 4.1: Primers used for reverse transcription polymerase chain reaction and expected sizes of cDNA fragments

	Primers used	**Amplicon size**
α ENaC-wt	**Sense:** 5'-AGCTCAATACTGCTTGGTTGG-3' **Antisense:**5'-AGGGCTGGGTGAGAGGAT-3'	2.2 kb
α ENaC-b	**Sense:** 5'- AGCTCAATACTGCTTGGTTGG-3' **Antisense:**5'- AAAGCAGAGCTCCTGGGTGT-3'	1.6 kb

Sense and antisense primers were selected to amplify full length α ENaC-wt and -b transcripts. The expected amplicon size was 2.2 kb for α ENaC-wt and 1.6 kb for α ENaC-b.

B. Plasmids and Generation of Constructs

PCR products for α ENaC-wt and α ENaC-b were ligated with pCR®2.1-TOPO® vector using the TOPO TA® cloning kit (Invitrogen, CA, USA), with blue/white screening of DH5α competent cells on ampicillin selective plates. A total of 14 individual colonies for each species were isolated. Positive clones were picked and cultured in LB broth. The DNA extracted from these cultures using Qiagen DNA miniprep kit, was sequenced using the M13 universal forward and reverse primers, in addition to embedded primers within α ENaC-wt and-b forms to verify their full lengths sequences. Sequencing was performed using the DYEnamic ET Terminator kit according to the instructions provided by the manufacturer (PE Applied Biosystems, Foster City, CA). Sequencing products were purified (DyeEx 2.0 spin kit columns; Qiagen Canada, Mississauga, ON, Canada) and analyzed on 3100 DNA analyser (Applied Biosystems, Foster City, CA). The sequence of the cloned α ENaC-b is identical to the one previously reported [9], and the sequence of the α ENaC-wt is identical to the one previously reported by our group for Dahl rats [12].

Ligated pCR®2.1-TOPO®-α ENaC-wt clone was digested using EcoRI, while that of α ENaC-b was digested using EcoRV and HindIII. Digested α ENaC-wt and -b fragments were purified from 1% agarose gel (QIAquick Gel Extraction Kit, Qiagen ™, ON, Canada) and then subcloned overnight at 16 °C into PCMV-sport6 vector (Invitrogen, CA, USA) downstream the T7 promotor using the EcoRI site for α ENaC-wt and the EcoRV and HindIII sites for α ENaC-b. The positive clones were verified by restriction digestion, then PCR of the inserted construct and later by direct nucleotide sequencing as described above. DNA yield from positive clones was later increased for transfection

studies using Qiagen midi-prep kit (Qiagen ™, ON, Canada) for amplification of the yield.

C. Cell culture and transfection

COS-African green monkey kidney cells were maintained in culture at 37 °C/5% CO2 using Dulbecco's Modified Eagle's Medium (DMEM, Hyclone ® Thermo Fisher Scientific Laboratories, Logan, UT) supplemented with glutamine and 10% heat-inactivated fetal bovine serum (FBS, PAA Laboratories, Pasching, Austria) -See appendix L. Transfections were performed using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and up to 35 μg DNA per 10 cm plate in co-transfection experiments. COS7 cells were seeded at approximately one third confluency on 10 cm diameter plates 18 h before the transfection. Immediately before transfection, the culture medium was replaced with 5 ml of DMEM supplemented with 10% (v/v) fetal bovine serum. PCMV-sport6 /αENaC-wt and -b were transfected into COS7 cells separately at a concentration of 25 μg DNA /plate (figure 4.3), as well as co-transfected together in a dose-dependent manner as demonstrated in figures 4.4 & 4.5. The empty PCMV-sport6 vector at a concentration of 25 μg DNA /plate was transfected as a control. DNA was added to 250 μl TE buffer and lipofectamine was then added (Invitrogen®, ON, Canada). The mixture was incubated at room temperature for 20 min before addition to the COS7 cell culture. The cells were incubated overnight at 37 °C in an air/CO2 (19:1) atmosphere before the medium was aspirated and the COS7 cell culture osmotically shocked for 2 min with 10% (v/v) PBS.

D. Electrophoresis Analysis

We used two different Western blot analyses namely SDS-PAGE or "denaturing gels" and native PAGE or "non denaturing gels". While SDS-PAGE denatured α ENaC-wt and -b proteins, and allowed an easy access of the α ENaC-antibody to the reactive epitope and subsequent separation of the α ENaC proteins based on molecular mass, native gel electrophoresis, on the other hand, allowed us to study the α ENaC-wt and -b proteins oligomerization potential. Migration for both SDS-PAGE and native gels was performed under 150 V and for 120 min (appendix N).

D.1. SDS-PAGE and Western Blot Analyses

Cells from transiently transfected COS7 cells expressing α ENaC-wt, or α ENaC-b, or the empty vector along with COS7 cells equally co-transfected with α ENaC-wt and -b form were washed twice with ice-cold PBS, scraped and then maintained for >2 h at 4°C in RIPA lysis buffer (10 mM $NaPO_4$, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 7.2) supplemented with the protease inhibitor cocktail (Sigma, St Louis, MO). Protein concentrations were measured by Bradford. Proteins were then separated by SDS-PAGE resolving gels containing 10 % acrylamide, and 4 % of the same solution for stacking gels. Samples were mixed with Laemmli buffer (Bio-Rad, 0.5 M Tris-Hcl, pH 6.8, 10% glycerol, 0.02% bromophenol blue) containing 0.1% SDS [sodium dodecyl-sulphate (w:v)] and supplemented with β- mercaptoethanol (v:v). Before loading, SDS samples were heated at 100 °C for 5 minutes. Proteins were separated by SDS-PAGE and western blots were performed according to procedures recommended by the Bio-Rad Protein III system (Bio-Rad Inc.).

D.2. Native Gel Electrophoresis and Western Blot Analyses

Separation of proteins by PAGE under non denaturing conditions was performed similar to SDS-PAGE, except that SDS and β-mercaptoethanol were totally excluded and the samples were not boiled. This will preserve any protein interactions. Samples and protein markers were diluted in 0.5 M Tris-HCl, pH 6.8, 40% glycerol (v:v). and 0.02% bromophenol blue (w:v) (without SDS and β-mercaptoethanol), and without heating. In Native PAGE, 20 μg of proteins were separated on precast Novex ® 8% Tris-Glycine Midi Gel (Invitrogen, CA, USA). Before loading the samples, the gel was cooled to 4 °C. After addition of native sample buffer (5X), samples were directly applied to the gel without any preconditioning. Native gel electrophoresis (18 cm × 16 cm) was conducted in the same apparatus as above. The running buffer (pH 8.3) contained Tris base (25 mM) and glycine (192 mM).

Polyclonal antibodies raised against the N terminal aa 46-68 [NH2-LGKGDKREEQGLGPEPSAPRQPT-COOH] of the rat α ENaC were used to recognize the α ENaC-wt and -b proteins. Antibodies were purified from rabbit sera, concentrated to 1.3 ug/ul and diluted to 1:1000. Primary α ENaC-antibodies were a kind gift from Dr. M. Amin. In Western blot, protein samples were transferred to nitrocellulose membranes (Bio-Rad Inc.) using Trans-Blot Cell system (Bio-Rad Inc., CA, USA). After transfer, nitrocellulose membranes were blocked for 1 h with 5% skimmed milk in 0.1% Tris-buffered saline containing 0.1% Triton X-100 (TBST) and incubated with primary antibody overnight. Blots were probed with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Promega Inc., ON, Canada) for 2 h at room temperature.

Multiple washes for the membranes were performed using TBST after the primary and secondary incubations. Protein signals were detected using ECL substrate (Amersham Biosciences Inc. Chicago, IL) and quantified using Alpha Ease FC (FluorChem 9900) software. All Western blots were stripped in 100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl (pH 6.7), and 2% SDS for 30 min at 55°C with constant agitation. After removal of antibodies, nonspecific interactions were reblocked by incubation in Tris-buffered saline-Tween 20 and 5% milk for 2 h before the blots were reprobed with the house keeping gene (β-actin).

Results

Molecular cloning and Sequencing of α ENaC-wt and alternatively spliced form α ENaC-b

To determine whether α ENaC-b is translated in vitro, and if so, whether α ENaC-b binds to α ENaC-wt, we cloned α ENaC-wt and -b transcripts from Dahl S rats' kidney cortex, using RT-PCR. Using PCR primers that were designed to amplify full-length coding sequences of α ENaC-wt and α ENaC-b transcripts, α ENaC-wt and α ENaC-b were obtained and cloned (figure 4.1). Sequences for both α ENaC-wt and -b form were confirmed by complete nucleotide sequencing analyses. α ENaC-wt sequence was consistent with our previously reported Dahl rat coding sequence [12]. The present α ENaC-b sequence was consistent with the previously reported α ENaC-b sequence [9].

Expression of full length α ENaC-and truncated α ENaC-b alternatively spliced form in transiently transfected COS7 cells.

Full length α ENaC-and truncated α ENaC-b transcripts were studied in COS7 cells to confirm their ability to make a protein. In vitro translation of α ENaC-wt and α ENaC-b produced proteins of 85 and 53 kDa, respectively (figure 4.2).

Dimerization of α ENaC-wt and α ENaC-b

Using native Western analysis for lysates obtained from COS7 cells co-transfected with α ENaC-wt and -b forms, a dimer was formed at 140 kDa, equivalent to the summation of the molecular weights of α ENaC-wt and α ENaC-b. α ENaC-wt protein has a molecular mass of 85 kDa, while α ENaC-b protein has a molecular mass of 53 kDa (figure 4.3).

Dose-dependent suppression of full-length α ENaC expression by increasing doses of α ENaC-b

Western blot analysis using an α ENaC-antiserum revealed that co-expression of α ENaC-wt with spliced form α ENaC-b decreased α ENaC-wt protein levels by increasing doses of α ENaC-b protein (figure 4.4).

Figure 4.1: Amplification of ENaC-wt and alternatively spliced form from total RNA. Total RNA extracted from kidney cortex of Dahl S rats was utilized as a template to generate full lengths cDNAs of α ENaC-wt and alternatively spliced form α ENaC-b. High fidelity Expand Long Range, dNTPack was used to amplify α ENaC-wt and -b form. Touchdown PCR method was employed to improve the efficiency of gene amplification. After amplification, the specific fragments of 2267 bp (α ENaC-wt) and 1586 bp (α ENaC-b) were visualized in 1% agarose gels.

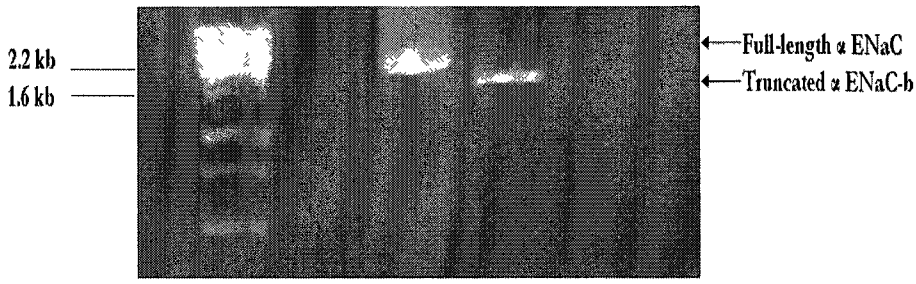
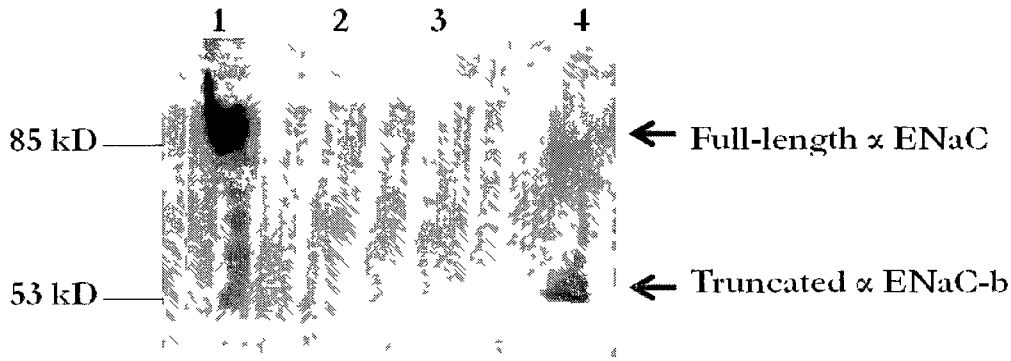
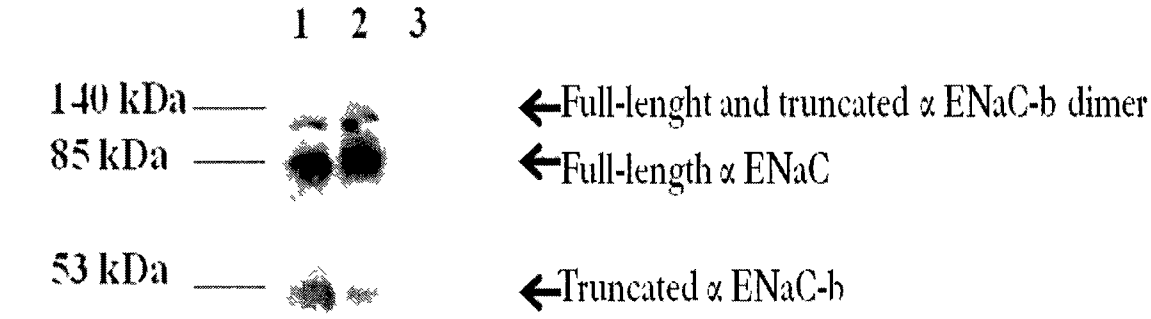

2.2 kb
1.6 kb
Full-length α ENaC
Truncated α ENaC-b

Figure 4.2: Expression of α ENaC-wt and α ENaC-b in COS7 cells. SDS/PAGE and Western blot analysis were performed using extracts from mammalian cells expressing either the alternatively spliced form α ENaC-wt or -b form or the empty vector in COS7 cells. Lane 1 represents proteins extracted from COS7 cells transfected with α ENaC-wt, lanes 2 and 3 represent COS7 cells transfected with the empty PCMV-sport6 vector, and lane 4 represents COS7cells transfected with α ENaC-b.



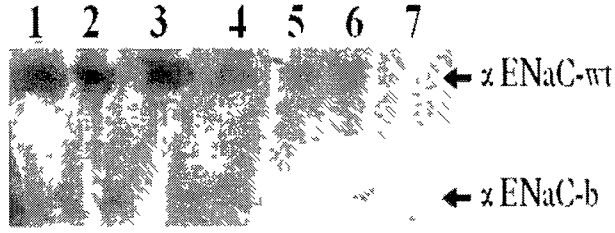
Full-length αENaC	+			
Truncated α ENaC				+
PCMV-Sport6 plasmid		+	+	

Figure 4.3: Analysis of α ENaC-wt and α ENaC-b expression by native Western analysis. α ENaC-wt and α ENaC-b interacts in vivo by the formation of a dimer of a molecular mass of 140 kDa. Lanes 1 and 2 represent proteins from COS7 cells co-expressing α ENaC-wt and α ENaC-b in equal proportions (1:1), lane 3 represents proteins from COS7 cells transfected with the empty PCMV-sport 6 vector. Bands are normalized against the house keeping gene β-actin and the experiment was done in triplicate.



Full-length αENaC	+	+	
Truncated α ENaC-b	+	+	
Sport-6 plasmid			+

Figure 4.4: Dose-dependent suppression of α ENaC-wt by increasing doses of α ENaC-b. Lanes 1-6 represent proteins of COS7 cells co-transfected by α ENaC-wt and α ENaC-b in the ratios provided. Denaturing Western analysis demonstrate a dose-dependent reduction in full-length α ENaC expression. Lane 7 represents proteins from cells transfected with the empty PCMV-sport 6 vector. A weak signal of α ENaC-b can be detected at 53 kDa, possibly indicating that dimerization of α ENaC-wt with α ENaC-b proteins degrades α ENaC-wt primarily and eventually degrades α ENaC-b. Bands are normalized against the house keeping gene β-actin and the experiment was done in triplicate.



αENaC-wt (μg/10-cm plate)	5	5	5	5	5	5	
α ENaC-b (μg/10-cm plate)	0	5	10	20	25	30	
Sport-6 plasmid (μg/10-cm plate)							25

Discussion

The present study reports the following a) α ENaC-b transcript is translated in COS7 cells to a peptide of apparent mass of 53 kDa, b) α ENaC-b transcript is co-expressed with α ENaC-wt transcript, c) α ENaC-b forms a complex with α ENaC-wt resulting in dimer formation of a molecular mass of approximately 140 kDa, d) Increasing doses of α ENaC-b suppressed α ENaC-wt expression in a dose-dependent manner when co-expressed in COS7 cells

Formation of alternatively spliced forms of α ENaC have been previously reported in four species rats, humans, mice and chicken [6 8 10 13] suggesting that alternative RNA splicing is possibly a mechanism for regulating α ENaC-activity, besides transcriptional regulation The biological impact of alternatively spliced forms, particularly those lacking functional domains such as α ENaC-b lacking the second transmembrane domain, may go as far as a drastic switch-off effect [14 16]

Our previous findings showed that α ENaC-b mRNA levels were significantly higher in Dahl salt-resistant (R) *versus* salt-sensitive (S) rats [10] Overrepresentation of α ENaC-b in Dahl R rats could be indicative of a dominant negative effect on α ENaC expression/function in this model Additionally, owing to the fact that α ENaC-b is a salt-sensitive transcript in Dahl R rats [10], we were motivated to search for a biological role of α ENaC-b in vitro that might be in progress to protect the channel from over-activity in high salt environment in Dahl R rats, possibly by a dominant negative effect on α ENaC-wt Therefore, α ENaC-b was amplified, sequenced and cloned Expression of α ENaC-b

alone and in the presence and absence of α ENaC-wt was examined.

Results show that the truncated α ENaC-b protein is translated in vitro and its co-expression with α ENaC-wt appeared to substantially reduce α ENaC-wt expression in a dose-dependent manner. α ENaC-b acts as a dominant inhibitor of α ENaC-wt by forming a complex with α ENaC-wt. These results support an important role for alternative splicing in α ENaC regulation, and suggest that α ENaC-can form heteromers which may be important in regulating α ENaC expression and/or activity.

The present study is the first to demonstrate the expression of alternatively spliced α ENaC-b form in mammalian COS7 cells and expands our understanding of the putative mechanism of action of α ENaC-b as a negative expression regulator of α ENaC-wt; by directly interacting with α ENaC-wt as a dominant negative protein that ultimately hinder α ENaC-wt expression and potentially its activity. In co-expression studies (figure 4.4), a weak signal of α ENaC-b can be detected at 53 kDa, possibly indicating that dimerization of α ENaC-wt with -b protein degrades α ENaC-wt primarily and eventually degrades α ENaC-b. Our findings therefore uncover a new mechanism that may control the regulation of α ENaC expression in vivo by a potential spliced form α ENaC-b. A detailed understanding of the spectrum of alternatively spliced α ENaC-b is of importance not only for the understanding of ENaC regulation in Dahl rats, but also for the future drug development efforts. It will also provide insight into the beneficial impact of increasing α ENaC-b to possibly prevent salt sensitivity.

To this end, the present study suggests a dominant negative effect imposed by α ENaC-b on full length α ENaC protein expression in Dahl R rat kidneys. Our preliminary results suggest the intriguing possibility of a "genotoxic" effect of α ENaC-b on full-length α ENaC, potentially by accelerating proteolytic degradation of the latter in a dose-dependent fashion. There are at least two areas of potential clinical importance for these studies. First, identification of spliced forms that impair the hyperactivity of ENaC to sodium in the kidney of Dahl S rats would identify specific targets for the development of novel antihypertensive drug therapy for salt-dependent hypertension. Second, as gene therapy delivery systems continue to evolve, we will be examining in vivo delivery of adenoviruses carrying α ENaC-b and then assessing the resultant blood pressure effects. This may eventually gain relevance as a possible "proof-of-concept" gene therapy.

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Addendum to Chapter 4

Study Limitations

It should be noted that despite the demonstration of a dose-dependent reduction in α ENaC-wt protein expression by increasing doses of α ENaC-b, the following two major study limitations warrant a careful interpretation of the results.

First, the native gel presented in figure 4.3 lacks two important controls (α ENaC-wt and α ENaC-b). The lack of these controls on the native western gel brings into question whether or not the high molecular weight complex is a complex of α ENaC-wt and α ENaC-b, or rather a complex of α ENaC-wt and other α ENaC regulators. Although we have shown the independent protein expression of α ENaC-wt and α ENaC-b on the denaturing gel at the expected sizes (figure 4.2), it is equally important to have been able to show the same controls on the native gel, because of potentially different running patterns for the two gels.

Secondly, the western blot results presented in figure 4.4 in light of the missing transfection efficiency control should be carefully interpreted. Lack of a transfection efficiency control questions if the dose-dependent reduction in α ENaC-wt expression is induced by increasing doses of α ENaC-b or rather variability in the efficiency of transient transfection experiments. It is worth mentioning at this point that we were able to detect an increased proteolytic product formation (~15 kDa) concomitant with increasing doses of α ENaC-b and disappearance of α ENaC-wt (data not currently presented in the thesis). This proteolytic product formation underscores the putative role of α ENaC-b as a dominant negative expression regulator for α ENaC-wt.

5.0 Final Discussion

The importance of preventing and controlling salt-sensitive hypertension via a restriction in dietary sodium intake and/or regulating the amount of Na^+ entering the cell is heightened by the existence of clinical studies demonstrating that even the presence of modest blood pressure reductions (6.3 mm Hg in systolic blood pressure) can be achieved by decreasing daily dietary salt intake by 100 mmol (Fodor et al 1999). Modest blood pressure reductions result in a significant improvement of co-morbidity factors (*i.e.,* diseases associated with high blood pressure such as stroke, congestive heart failure, and kidney disease).

As it is, the direct cost of high blood pressure is estimated to be over 7.4 billion per year manifested in health care costs and money spent on blood pressure lowering medications (center for chronic diseases control at http://www.phac-aspc.gc.ca/ccdpc-cpcmc/cvd-mcv/publications/pdf/strate.pdf). With new products and potential lifestyle solutions being reported everyday, it is apparent that the scientific community has not yet solved the blood pressure dilemma. There is no doubt that both genetic and environment factors are jointly responsible for the hypertension statistics that have generated a North American health care crises. What is often lost in these discussions is that there is no single solution that can be mass-produced for everybody as we are each genetically unique and hence react differently to environmental challenges.

Several variations in genes that control blood pressure can predict the risk for salt-induced high blood pressure. Variations might be at the level of sequence variability, or excessive formation of alternatively spliced forms in one population and not the other, or at the level of gene regulators. Identifying these genetic variations contributing to salt-

induced high blood pressure together with the putative underlying mechanisms associated with sodium retention will provide new insights into how salt-sensitive hypertension develops, how it can be predicted or diagnosed and how it can be treated. Dahl rats provide the opportunity to test the effect of genetics and environmental factors, like salt intake, on the development of salt-sensitive hypertension. Although Dahl S and R rats share 98% of their genetic background (St Lezin et al 1992), high salt intake increases blood pressure in Dahl S, but not R rats.

The essence of the questions addressed in this thesis through three published manuscripts can be summarized as follows: Do sequence variation in the epithelial sodium channel α, β and γ genes explain salt-sensitivity in Dahl S *versus* R rats? Are there alternatively spliced forms associated with α ENaC? If yes, are their levels different in Dahl S *versus* R rats? Are their levels altered by dietary salt intake? What might be the potential mechanism by which α ENaC-alternatively spliced forms regulate α ENaC? The contributions to the literature of the first piece of work include:

i) Comprehensive sequence analysis of ENaC genes 5' and 3' flanking regions, intron-exon junctions and complete coding regions did not reveal any differences between Dahl S and R rats that were isogenic in the regions screened.

ii) Differences between strains were identified. Dahl and Wistar rats exhibited differences in the coding regions and 5' flanking regions screened.

The conclusion withdrawn from the first piece of work is that the enhanced ENaC activity in Dahl S rats can not be attributed to sequence variability in the 5' and 3'flanking regions, exon-intron junctions and complete coding regions in Dahl S *versus*

Dahl R rats. Sequence variations can still exist in the intronic regions that were not screened in the above study. On the other hand, enhanced ENaC activity in Dahl S rats can be attributed to differential ENaC regulation via RNA alternative splicing in Dahl S *versus* their sister salt-resistant strain (Dahl R rats).

Knowing the importance of α ENaC in the functionality of the channel, the purpose of the second research study was to determine if α ENaC is associated with alternatively spliced forms in Dahl rats. Because alternative splicing is a major contributor to the structural and functional diversity of ion channels such as the K^+, and Ca^{++} channels, we chose to determine the existence and subsequently measure the levels of alternatively spliced forms (should they exist) in Dahl S *versus* R rats on normal and high salt diet.

Through the second piece of work, we established that:

i) Previously reported ENaC-a and -b alternatively spliced forms are present in Dahl rats' kidney cortex and are significantly higher in Dahl R *versus* S rats ($P<0.05$).

ii) Four-week of high salt dietary treatment significantly increased α ENaC-b ($P<0.05$), but not α ENaC-a transcript abundance in Dahl R, but not S rats.

iii) Two non coding α ENaC spliced forms -c and -d are newly identified in the present study, whose levels were comparable in Dahl S *versus* R rats.

iv) Compared to α ENaC wt, α ENaC-a, -c and -d were low abundance transcripts (4 +/-2, 110+/-20, and 10+/-2 fold less respectively), in contrast to α ENaC-b transcript abundance that was 32 +/- 3 folds higher than α ENaC wt.

v) We could not identify any of the five previously reported lung-specific α ENaC spliced forms (CK479583, CK475461, CK364785, CK475819, and CB690980) in Dahl rats' kidney cortex.

The conclusion for the second piece of work is that α ENaC-alternative splicing might regulate ENaC by the formation of coding RNA species (α ENaC-a and -b) and non coding RNA species (α ENaC-c and -d). Results have shown α ENaC-a and -b mRNA levels to be higher in Dahl R *versus* S rats and in particular the salt-sensitive α ENaC-b transcript that was highly abundant 4-weeks post high salt diet compared to normal salt diet in Dahl R rats. Of the four α ENaC transcripts, only the salt-sensitive α ENaC-b was the most abundant species that exceeded α ENaC-wt abundance by 32 fold. α ENaC-b might act as a dominant negative protein for ENaC activity, to rescue Dahl R rats from developing salt-sensitive hypertension on high salt diet.

The above conclusions prompted us to examine the physiological role of α ENaC-b. Knowledge of the mechanism by which α ENaC-b regulates full-length α ENaC-and possibly reduces expression/activity of α ENaC in Dahl R rats and the subsequent genesis of salt-dependent hypertension [a disease that comprises a large subgroup (over 50%) of Canadian adults] would enhance the understanding of the basic regulation of ENaC and the pathophysiology of ENaC-associated disorders such as salt-sensitive hypertension. It may also create one or more specific targets for the development of novel anti-hypertensive drug or gene therapy.

Our final piece of work showed the following:

i) α ENaC-b is translated in COS7 cells.

ii) Co-expression of alternatively spliced α ENaC-b form together with α ENaC-wt reduced α ENaC-wt levels in a dose dependant manner.

iii) α ENaC-b binds to α ENaC-wt when co-expressed in COS7 cells.

iv) Western blots suggest that α ENaC-b might possibly exert a dominant negative effect on α ENaC-wt expression potentially by sequestering α ENaC-wt to enhance its proteolytic degradation. This provides an insight into the putative mechanism of salt-resistance in Dahl R rats

Our final piece of work has two major limitations that can be summarized as follows:

First of all, two important controls (α ENaC-wt and α ENaC-b) are missing on the native western gel (figure 4.3). This in turn questions the nature of the high molecular weight complex (if it is a complex of α ENaC-wt and α ENaC-b or rather a complex of α ENaC-wt and α ENaC regulators). We have shown the independent protein expression of α ENaC-wt and α ENaC-b forms on the denaturing gel at the expected sizes (figure 4.2). However, it is equally important to demonstrate the same controls on the native gel, because of potentially different running patterns for the two gels.

Secondly, the western blot result presented in figure 4.4 lacks a transfection efficiency control which warrants a careful interpretation of the results. Lack of a transfection efficiency control questions if the dose-dependent reduction in α ENaC-wt with increasing doses of α ENaC-b is actually specific to the α ENaC-b or rather a variability in efficiency of transient transfections. It is worth mentioning that when our western gels were run for shorter durations, we detected an increased proteolytic product formation (~15 kDa) simultaneously with increasing α ENaC-b doses and disappearance of α ENaC-wt (data not currently presented in the thesis). The enhanced formation of the

above proteolytic fragment of ~15kDa again highlights the putative role of α ENaC-b as a dominant negative expression regulator for α ENaC-wt. Similarly, a transfection efficiency control in figure 4.4 would have been crucial for confirming the above findings.

What we can conclude from these studies is that RNA alternative splicing constitutes an additional mechanism for ENaC regulation by the formation of coding and non coding transcripts. Coding transcripts, particularly, α ENaC-b is a putative dominant negative expression regulator for α ENaC. Increased α ENaC-b levels in Dahl R rats might explain their suppressed ENaC activity compared to Dahl S rats. α ENaC-b might ultimately be used as a diagnostic tool for defining rats that are salt-resistant and might eventually be used as a therapeutic target for achieving lower blood pressure in salt-sensitive subjects.

It would be interesting to continue the above studies by examining the *in vivo* levels of protein expression of α ENaC-b in kidney tissues of Dahl S *versus* R rats pre- and post-high salt diet. Should the *in vivo* protein expression levels of α ENaC-b (*versus* α ENaC-wt) be elevated in Dahl R *versus* S rats post-high salt diet, a conclusion can be drawn on the putative dominant negative effect of increased levels of α ENaC-b on α ENaC-wt post-high salt diet. Hence, the above proposal would be confirmatory to the *in vitro* findings obtained from the present studies. A detailed outline of future experiments further examining α ENaC-b is appended in the form of a published manuscript (appendix T).

Within the next decade, researchers will begin to correlate RNA levels with individual responses to medical treatments, identify particular subgroups of patients, and

develop drugs customized for those populations. Consequently, the study of RNA splicing is the focus of a great deal of pharmacogenetic research aimed at the optimization of drug therapy based on an individual's genetic profile. Genetic tailored treatment recommendations are undoubtedly on the horizon as we discover that some individuals require more stringent and stronger treatment intervention than others based on their genetic predisposition.

Proposed mechanism of salt-resistance in Dahl R rats and the proposed mechanism of salt-sensitive hypertension in Dahl S rats.

Our proposed model of salt-resistance in Dahl R rats and salt-sensitivity in Dahl S rats can be summarized in the following: α ENaC-b suppresses α ENaC-wt expression in a dose-dependent manner. A dominant negative effect is caused by the highly abundant α ENaC-b in high salt-fed Dahl R *versus* S rats (figure 5.1). By acting as a negative expression regulator of α ENaC-wt, α ENaC-b allows less Na^+ to be absorbed into the blood stream. Less Na^+ means less water to follow by osmosis, which in turn means a lower blood volume and ultimately a lower blood pressure. On the other hand, enhanced ENaC activity in high salt-fed Dahl S rats might be owing to a putative lower α ENaC-b abundance below the threshold required for exerting a dominant negative effect on α ENaC-wt (figure 5.2). Lower α ENaC-b will not affect α ENaC-wt expression on high salt diet and therefore will allow a higher Na^+ concentration to be absorbed into the blood stream followed by an increase in the water absorbed by osmosis. Higher blood volume and higher blood pressure will ultimately result.

Figure 5.1: Proposed mechanism of salt-resistance in Dahl R rats. An illustration of the proposed role of α ENaC-b as a negative expression regulator of α ENaC-wt. α ENaC-b is the predominant species in Dahl R, and its abundance might be a contributor to the decreseaed activity of ENaC in high salt-fed Dahl R rats. Decreased ENaC activity in Dahl R rats will result in a lower amount of Na^+ that will be absorbed, a smaller volume of water to be absorbed accordingly by osmosis, and a diminished blood volume will follow and ultimately a lower blood pressure will be the net result.

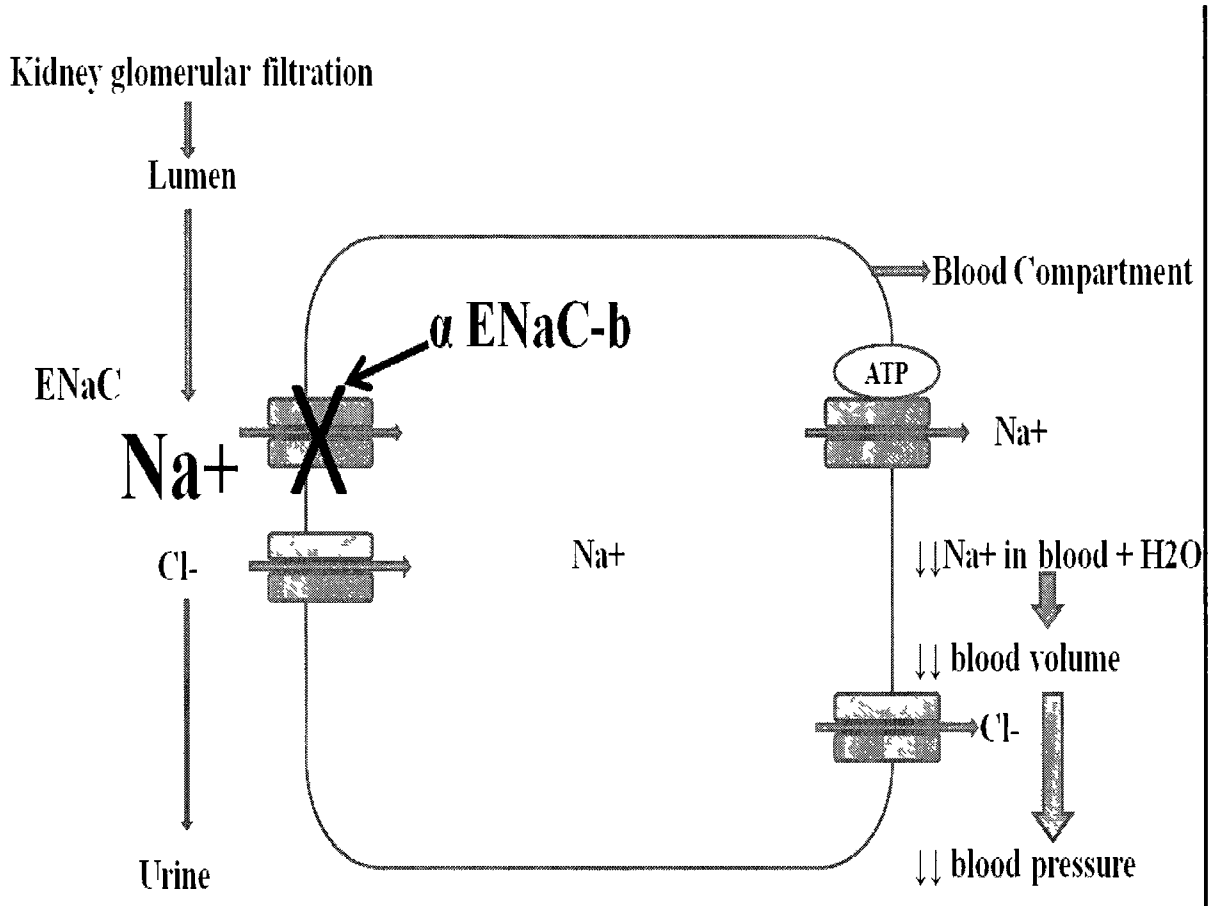
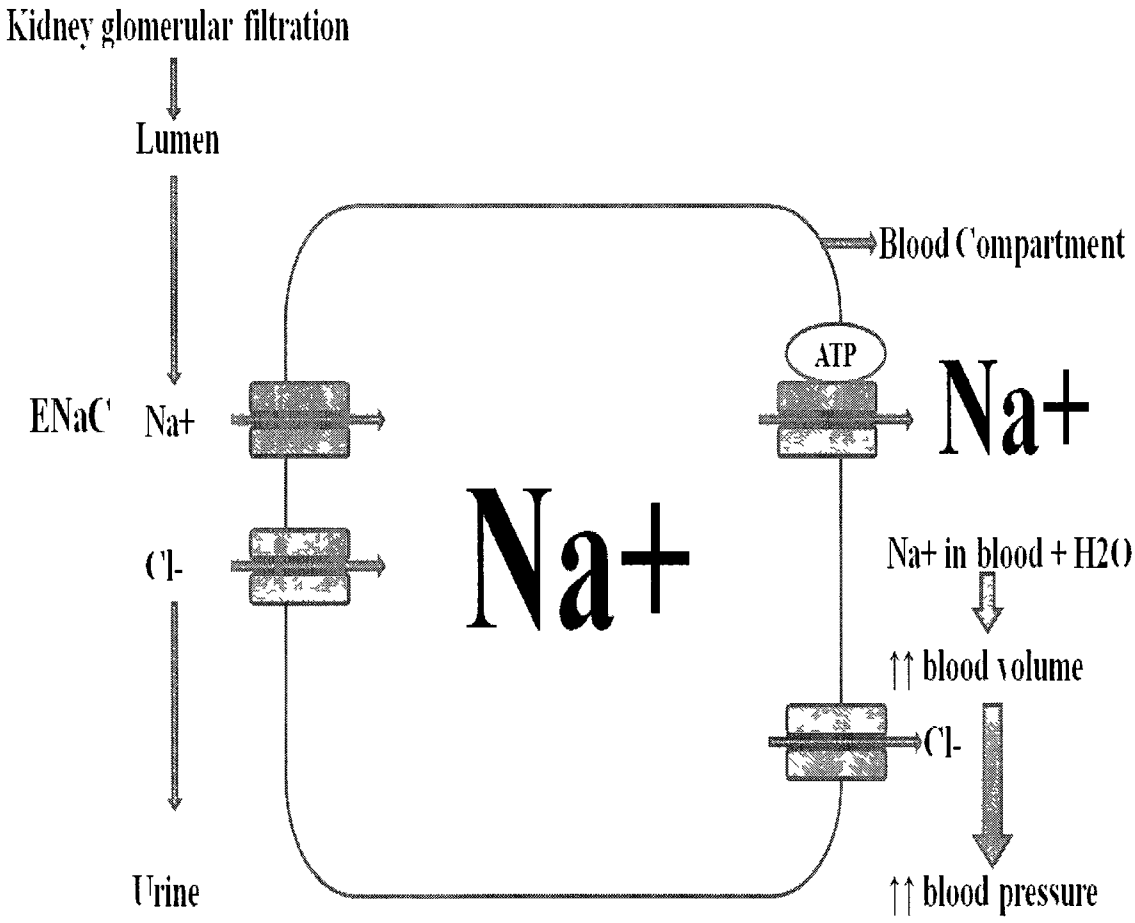

Kidney glomerular filtration
Lumen
ENaC
Na+
Cl-
Urine
α ENaC-b
Blood Compartment
ATP
Na+
Na+
↓↓Na+ in blood + H2O
↓↓ blood volume
Cl-
↓↓ blood pressure

Figure 5.2: Proposed mechanism of salt-sensitivity in Dahl S rats. α ENaC-b abundance is lower in Dahl S *versus* R rats, and its lower abundance might be a contributor to the enhanced activity of ENaC in Dahl S rats. Enhanced ENaC activity in Dahl R rats will result in a higher amount of Na^+ to be absorbed, larger volumes of water to be absorbed accordingly by osmosis, a larger blood volume will follow and ultimately a higher blood pressure will be the net result.


Kidney glomerular filtration
Lumen
ENaC
Na+
Cl-
Urine
Blood Compartment
ATP
Na+
Na+
Na+ in blood + H2O
↑↑ blood volume
Cl-
↑↑ blood pressure

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7.0 Appendices

Appendix A: Permission

00071112: Request a Journal Article to be Included in my Doctoral Dissertation [ref:00D28Yu.50023nUao:ref]

Wednesday, November 12, 2008 8:29 AM
From:
"Ben Weston" <ben.weston@biomedcentral.com>
To:
"marlenefouad@yahoo.com" marlenefouad@yahoo.com

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----------Your Question/Comment ----------

Dear the Editor in Chief

I am a Doctoral Student at the University of Ottawa, Faculty of Medicine. I am in the process of writing up my doctoral thesis. I am requesting your permission to include the full-version of the article entitled "Sequence analysis of coding and 3' and 5' flanking regions of the epithelial sodium channel alpha, beta, and gamma genes in Dahl S versus R rats" in my doctoral thesis. The article was published in BMC Genetics in 2007 Jun 25;8:35. I served as the primary author for the above original investigation.

Thank you for considering my request.

Marlene Shehata
PhD candidate
University of Ottawa

"Be strong and courageous. Do not be afraid or terrified because of them, for the LORD your God goes with you; he will never leave you nor forsake you." Deuteronomy 31:6

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Appendix B: Permission

Re: Request a Journal Article to be Included in my Doctoral Dissertation

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From:
This sender is DomainKeys verified
"Manuel Menéndez" <editorial.archmed@gmail.com>
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To:
"Marlene Fouad" <marlenefouad@yahoo.com>

Dear Marlene Fouad,

Yes, of course, you have permission to include the full-version of ther article in your doctoral thesis. What is more, you as author are the owner of the rights since the article was published under the open access policy.

Best wishes,
Manuel Menéndez
Editor in Chief

On Wed, Apr 29, 2009 at 5:55 AM, Marlene Fouad <marlenefouad@yahoo.com> wrote:

Dear the Editor in Chief

I am a Doctoral Student at the University of Ottawa, Faculty of Medicine. I am in the process of writing up my doctoral thesis. I am requesting your permission to include the full-version of the article entitled "Characterization of Epithelial Sodium Channel Alpha Subunit Transcripts and Their Corresponding mRNA Expression Levels in Dahl S *versus* R rats' Kidney Cortex On Normal and High Salt Diet" in my doctoral thesis. The article was published in International Archives of Medicine 2009 Mar 13; 2(1):5. I served as the sole author for the above original investigation.

Thank you for considering my request.

Marlene Shehata
PhD candidate
University of Ottawa

Appendix C: Permission

RE: Request a Journal Article to be Included in my Doctoral Dissertation
Wednesday, April 29, 2009 7:01 PM
From:
"Sandi McIver" <sandi.mciver@libertasacademica.com>
Add sender to Contacts
To:
"Marlene Fouad" <marlenefouad@yahoo.com>

Dear Dr Shehata,

You are welcome to include the article "A Novel Mechanism in Regulating the alpha-subunit of the Epithelial Sodium Channel (α ENaC) by the Alternatively Spliced Form α ENaC-b" as authors retain copyright of their work.

Kind regards,

Sandi

Sandi McIver
Managing Editor

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From: Marlene Fouad [mailto:marlenefouad@yahoo.com]
Sent: Wednesday, 29 April 2009 3:49 p.m.
To: Sandi McIver
Subject: Request a Journal Article to be Included in my Doctoral Dissertation

Dear the Editor in Chief

I am a Doctoral Student at the University of Ottawa, Faculty of Medicine. I am in the process of writing up my doctoral thesis. I am requesting your permission to include the full-version of the article entitled "A Novel Mechanism in Regulating the alpha-subunit of the Epithelial Sodium Channel (α ENaC) by the Alternatively Spliced Form α ENaC-b" in my doctoral thesis. The article was published in Biochemistry Insights 2009:2 21-27. I served as the sole author for the above original investigation.

Thank you for considering my request.

Marlene Shehata
PhD candidate
University of Ottawa

Appendix D: Primers used for ENaC genotyping

Primers designed to screen the coding sequence of ENaC α, β, γ genes. Optimum temperatures for both PCR and DHPLC are included in the table along with the optimum theoretical melting temperatures (Tm) provided by Qiagen.

A. Primers designed to screen the coding sequence of ENaC α, β, and γ genes.

Optimum temperatures (°C) for PCR and DHPLC are included in the table.

		Sequence	PCR	DHPLC
ENaC α				
Exon 1	Forward	GGTAGCACGGAGCTGGAG	64	63
	Reverse	CCCAGGCTGAGTTCACTCTC		
	Forward	AGCTCAATACTGCTTGGTTGG	61	63
	Reverse	GAAGAGCTCCCGGTAGGAG		
	Forward	GGGACAAACGTGAAGAGCAG	61	63
	Reverse	CTTGCTTTTGTGCTGCTGAG		
Exon 2	Forward	CACTGATCCCCTCCGTGTTA	60	64
	Reverse	TCCATCAGGCCTCTATCTGAA		
Exon 3	Forward	GCTCTCTGTCCCTCACCTTG	63	62
	Reverse	ACCACCAAGCATTTCCTGAG		
Exon 4	Forward	CACATTGGAGGTGACAGGAA	63	61
	Reverse	CCAAGGTGAGACCCAGAAGA		
Exon 5	Forward	GCTGGCCTTGTTCTATCAGG	62	62
	Reverse	CTCCCTTCAGTCTCCTGCTG		
Exon 6	Forward	GGTGAAGCCTGAGTCATTCC	63	62

	Reverse	CCAACCTACTTCCCCTCCAT		
Exon 7	Forward	GAGGGATGGAGGGGAAGTAG	61	61
	Reverse	AGCCAGCACCTAGGGAAAA		
Exon 8	Forward	GGCACCATTGAAATGCTCTT	59	61
	Reverse	ATCAAAGTGCCCAGTTACGG		
Exon 9	Forward	GCAGCTGCTTAACCTGGTAGA	61	61
	Reverse	ATGTCCACTTGTGCGTGTGT		
Exon 10	Forward	CCATCCCTGTAAACATGAGG	61	61
	Reverse	CCCCAATATCTCCACCAGAA		
Exon 11	Forward	TGGTGGAGATATTGGGGAGA	61	61
	Reverse	CAAACCCTTCTGACCCTTCA		
Exon 12	Forward	TGACAGGAGGCGCTAGAGT	62	63
	Reverse	AGTAGCATAGGCAGGTGGAG		
	Forward	CTCCACTCCAGCTTCCTCCT	62	63
	Reverse	ATCGTTAGCCCCTGTCCTCT		
	Forward	TCTCACTTCAGCACATCTTCC	61	63
	Reverse	GGCCTACCCTGGTCTGTCTT		
	Forward	ACCCAAAAGCCCCCTTGT	61	61
	Reverse	AGTACACTGTGGGGGTGAGG		
	Forward	CCAAAGGCACCATTTCTTTT	59	61
	Reverse	ATGTAGGCGGTGCCTCAG		

ENaC β

Exon 2	Forward	CTAGTCTCCAGGCCCATGAC	62	64
	Reverse	CTACTGGAAGGGGCTGGAAT		
Exon 3	Forward	CCCCATGTTCCACACTCTTT	60	62
	Reverse	AACAAAAATCGATTGCTACCAG		
Exon 4	Forward	TAATACGGTGCTGCCATTCC	61	63
	Reverse	GCATAGATCAGCCTGTGTGC		
Exon 5	Forward	CTCCAGCAGAGCAGGACAAT	62	61
	Reverse	GGTCTTTCCGCCCTGTGT		
Exon 6	Forward	GGTGATGGCCTCCCTTCTAT	61	61.5
	Reverse	AGGCAGCCTGAACACAAGAG		
Exon 7	Forward	GCTCCATGGGGAGGTACATA	63	63
	Reverse	GTCCGGCCCTCATAGGTAAG		
Exon 8	Forward	AAAGTCTCTGGGCTCCAAGG	63	61.5
	Reverse	AGAGGCCCCCTTGCCTAAC		
Exon 9	Forward	GAGGGAAGACCCCTGGAAG	62	63.5
	Reverse	ATACTGGGTGTCGCTGGAAG		
Exon 10	Forward	TCCTGCAAGTGAGTGTGTCC	62	63
	Reverse	AGGGGGAGAAGACCCTCTTT		
Exon 11	Forward	AGCATGTGTGTGCGTGTGTA	60	63
	Reverse	CAGCAAGAGCAGTTTGGACA		
Exon 12	Forward	CAACTCAGACTGGAGATGAGCA	62	62.5
	Reverse	GGAACCATAACCCCCACCTA		
Exon 13	Forward	CCTAGGTGGGGGTTATGGTT	62	64

	Reverse	GCAGCCTCAGGGAGTCATAG		
	Forward	CTTCCAGCCTGACACAACC	62	63.5
	Reverse	GCCTGTCTGCTAGGTCAACA		
	Forward	CTGAGGGGTTCATAGGGTCA	62	60.5
	Reverse	ATCCTACACCCCAGACATGC		
ENaC γ				
Exon 2	Forward	AGTCGCAGGCTCCAGAGAT	62	64
	Reverse	GAGGGGCTTTGACATCCAT		
Exon 3	Forward	GGAAGGCAACATGAAGAAGC	61	60
	Reverse	ACTGTGAGCCCACCAGCTC		
Exon 4	Forward	AAGGACCTACCCTGGCATCT	63	60
	Reverse	CTCAAGGCCTACAGGTGAGC		
Exon 5	Forward	ACGCAATGCTTCCTGACTTC	60	60
	Reverse	TGCACTGCTGCTGTCAAGAT		
Exon 6	Forward	CCCTGGGGACTGCTTTTT	60	60
	Reverse	TCCATTAGCAGCACCTCCTT		
Exon 7	Forward	AGGGGAATCCTCCTATCTGG	61	62
	Reverse	CCTTGGCCTAGATATAGCTTCA		
Exon 8	Forward	AGGGAGTTCCCGGTCTCTAC	63	61
	Reverse	GCGTGGCCAAGCTGATTC		
Exon 9	Forward	AGATGGTGGAGGTTCCACAG	63	60
	Reverse	GGGAGAAAGGCACAGAGTGA		

Exon 10	Forward	ACTGGGGCAGGTAGGACTTT	62	60
	Reverse	GCTTTGGCTGTGTTGCTGTA		
Exon 11	Forward	CCAAAGCCAGAGACAGGTTG	59	61
	Reverse	TCGAATGAACGAAAAGGTGA		
Exon 12	Forward	ACCTGGCAGGAAGCCAAG	62	61
	Reverse	CCCTCTGGCAGCAAAACTAC		
Exon 13	Forward	CCCTGAGTGCAGGATTTATCA	61	64
	Reverse	GTATCTGGGAGGTGGTGTGC		
	Forward	GTCAGTGGCACAAAGCCAAG	62	64
	Reverse	AGCTCATAAGTGCCAAGTCCA		
	Forward	CCTGCTGTGAACCGGATA	60	60
	Reverse	GGCCAACTGTCTGTCTGAGG		
	Forward	GCCAGCTATTGCCTGACAT	60	60
	Reverse	CGCATACTCTCAGTTCAAAGACA		
	Forward	GCCAAATGGTATTCCCACAA	59	57
	Reverse	ATTGGACTAGCCTGGGTGCT		

Primers designed to screen the 5'flanking sequence of ENaC α, β, and γ genes. Optimum temperatures (°C) for PCR and DHPLC are included in the table.

	Sequence	PCR	DHPLC
ENaC α			
Forward	CAGATTCAGCTGCCATGC	60	62
Reverse	AGGCCCTGCAGAACTTGCT		
Forward	GGTAGGAAGAGGCAGTGTGC	61	62
Reverse	ACAGGGTTGCAGGAATGTG		
Forward	GCTGGTGGTCTCTCCCAGTA	64	62
Reverse	GGTCCCTTCTGATGGAGGTC		
Forward	GGGGGACCCATTCTCCTT	62	59
Reverse	GGACTGATGGCGAACTGACT		
Forward	CCTTCCTGGCTTTTGTGTGT	61	62
Reverse	GTGTGGCTGGGCTGTGAC		
Forward	CAGGCACTGCACCTGTCA	62	62
Reverse	GGCTTAGCGTCTCTCCCTCT		
Forward	CACAGAAAGGGAGACCCAAA	60	63
Reverse	GGTCTTGCTCCTTGAATTGG		
Forward	GAGGGCAGCCTGGGATGCGG	61	62
Reverse	TTATAATAGCAATAGCCCCA		
Forward	TCCAAGGAGAAGGCGCCCCCA	61	62
Reverse	AGGGCTGGGTGAGAGGAT		

	Forward	AGCTCCTGGGGCTATTGCTA	61	61
	Reverse	TGTTCTGCAAGGACAGCATC		
	Forward	CAGAGCTCCTGGGGGCGCCTT	61	61
	Reverse	AGGGCTGGGTGAGAGGAT		
	Forward	TGTGCATTCACTCCTGCTTC	61	61
	Reverse	TGTTCTGCAAGGACAGCATC		
	Forward	CATCTTCACCTGTCGCTTCA	61	61
	Reverse	TGTTCTGCAAGGACAGCATC		
ENaC β				
	Forward	GGTCTTCTGGGAAGAGCAAA	61	62
	Reverse	CACGGTTCCATTTCCTGTGT		
	Forward	CAGCCATGCACATGAAGACT	61	57
	Reverse	TTGCAGCCAACTTGACTAGAT		
	Forward	TGGTGATACCCTTCTTGTTGG	61	60
	Reverse	CCTCCGTGTATCCTCACTCC		
	Forward	AAAATAGAACGGGGCTGTGA	61	62
	Reverse	GGAGCTGGGTCACCTGTAGA		
	Forward	TGACCTGGAGCCTGTCAACT	62	64
	Reverse	GACGGAACTGCGGTCATT		
ENaC γ				
	Forward	CTTGACATGTTTCTACCCACCA	61	58
	Reverse	TTAGAACGCTGAAACCGTGA		
	Forward	CATCCTCAACTCAAGAATCTGC	60	60

Reverse	CCACTCTGCAAGCTGCATTA		
Forward	TGCAGAAGCAGCAGTAAGAGA	61	62
Reverse	CTGGCGTGTGTACAGTCTGG		
Forward	CTTCTGGCCGTGCCACTT	61	62
Reverse	TCGGGCTCCTCTTTCAACTA		
Forward	CGCGGGAAAGCTTTTCTTTA	61	63
Reverse	CTTGCTCCCTTCCCTCTCAC		
Forward	ATGAACAGAGCCTCGCAAAC	61	62
Reverse	GATAGTGGTCGCAGGTGACA		
Forward	GGTGGAAACGTTGGAATAGG	62	58
Reverse	AAAGCAAGGCTGTCGCTCTA		

Appendix E: Protocol for DNA Extraction using FlexiGene DNA Kit

For buffy coat

1. Mix 500 µl of Buffer FG2 with 5 µl of protease.
2. Add 500 µl Buffer FG1 to 200 µl of buffy coat and invert 5 times.
3. Centrifuge for 40 sec 14 000 rpm.
4. Discard supernatant and leave the tube inverted on a kimwipe for 2 min. (pellet may be lose…pour slow)
5. Add 500 µl Buffer FG2/protease to tube and vortex IMMEDIATELY until pellet is completely homogenized. (if traces still remain add 30 µl FG2 and vortex)
6. Invert the tube 3 times and place in a heating block at 65°C for 5 min.
7. Add 500 µl isopropanol and mix thoroughly by inversion until precipitate becomes visible.
8. Centrifuge for 4 min at 14 000 rpm.
9. Discard the supernatant and invert the tube on a kimwipe.
10. Add 500 µl 70% ethanol and vortex for 5s.
11. Centrifuge for 4 min at 14 000 rpm.
12. Discard the supernatant and leave the tube inverted on a kimwipe for at least 5 min.
13. Air-dry the DNA pellet for 5 min until all the liquid has evaporated.
14. Add 200 µl (or less for higher DNA concentration) buffer FG3 and vortex for 5 sec on low and incubate at 65°C for 5 min or until dissolved.

Appendix F: PCR Protocol

Reagents

Prepare the following reagents before beginning PCR:

• 1 mM dNTPs:	dATP 100 mM	10 μL
	dTTP 100 mM	10 μL
	dCTP 100 mM	10 μL
	dGTP 100 mM	10 μL
	sterile H_2O	1000 μL

• 20 μM primers (forward and reverse):	forward primers 200 μM	20 μL
	reverse primers 200 μM	20 μL
	sterile H_2O	200 μL

Procedure

1. Turn on PCR machine and set up PCR program. The standard PCR program is as follows:
   1) 1 cycle: 94°C for 4 min.
   2) 30 cycles: 94°C for 1 min.
   (optimal annealing temperature)°C[1] for 1 min.
   72°C for 1 min.
   3) 1 cycle: 72°C for 10 min.
   4) hold at 6°C

   The robocyclers will take approximately 30 min. to warm up to the correct temperature.
2. Pipette 6 μL of DNA from dilution plate into each well of a 96-well plate or into PCR tubes.
3. Prepare PCR mix by pipetting the following reagents into an Eppendorf tube. The standard PCR mix (Gibco Taq) for **1 DNA sample** is as follows:

   1 mM DNTPs - 5 μL
   20 μM primers (forward and reverse) – 1 μL
   buffer – 2.5 μL
   $MgCl_2$ – 0.75 μL
   sterile H_2O – 9.5 μL
   Gibco Taq – 0.25 μL

   For sequences that are difficult to amplify, a PCR mix using Qiagen Taq may be substituted for the standard mix:

   1 mM DNTPs - 5 μL
   20 μM primers (forward and reverse) – 1.4 μL

[1] Optimal annealing temperature can be determined by performing PCR at a range of temperatures surrounding the suggested primer annealing temperature on a gradient robocycler.

Q buffer[2] – 2.5 μL

Q solution – 5 μL

sterile H_2O – 4.8 μL

Qiagen Taq – 0.25 μL

Multiply these concentrations by the number of DNA samples to obtain the final values needed for each reagent in the PCR mix. Vortex.

4. Pipette 19 μL of PCR mix into each well or PCR tube containing DNA. Seal plates with foil (either using the heat sealer or the adhesive foil) or cap the tubes. Place samples in PCR machine and press "Enter". PCR will take approximately 2 hours to complete.

[2] Contains 15 mM of $MgCl_2$

Appendix G: Verification of PCR Products on Agarose Gel

Preparation of Agarose Gel (for mini-gel system)

1. Pour 100 mL 1X TBE into small glass beaker and cover with tinfoil. Bring agarose powder, metal spatula, red rubber holder, and beaker full of TBE upstairs (4^{th} floor).
2. Weigh out 1 g of agarose and put into beaker of 1X TBE.
3. Take off tinfoil and microwave beaker for 1 min. and 30 s. Take beaker out when 10 s are remaining and swish contents around for a few seconds. Put beaker back in microwave and heat for another 10 s until boiling. Take beaker out of microwave with red rubber holder and cover with tinfoil. Go back downstairs.
4. Stir contents of beaker with magnetic stir bar for about 10 min.
5. Pipette 2 μL of ethidium bromide into agarose mixture and stir for another minute.
6. Pour hot agarose into mini-gel apparatus. The two "big" gels take about 50 mL each while the "small" gels require about 25 mL of agarose each. Let stand for about 1 hour in order for the agarose to set. Make sure there are no bubbles in the gels.

Loading of the Gels

1. Prepare DNA markers using reagents in little blue MBI Fermentas box.
2. In one PCR tube, prepare "Mix A" by pipetting 1 μL loading dye into 5 μL of 30% glycerol. Mix by pipetting up and down.
3. Transfer 5 μL of "Mix A" into a second PCR tube and add 1 μL of 50 bp ladder. Mix by pipetting up and down. This is the DNA ladder that will be used as a marker.
4. Mix 5 μL of your PCR products with 5 μL of 1X agarose blue dye.
5. Place mini-gel into electrophoresis apparatus and fill reservoir up to the line with TBE 1X.
6. Load 10 μL of each sample onto gel and 2 μL of the DNA ladder mix into separate lanes.
7. Run the gel at 100 V for about 20-30 min.
8. Check gel upstairs under UV light. If the PCR was successful, you should see only one band in each lane (except for the water control, which should be blank!).
9. Use GelDoc (computer software) to take a picture of the gel, save in TIF format under the I: drive so it can be exported into PowerPoint for future reference.

Appendix H: Determination of DNA Concentration by Spectrophotometry (U.V)

Use 0.5 ml quartz cuvettes
Blank should be made on ddH_2O
Following extraction from ± 10 ml of whole blood, DNA samples may be diluted 1/40 i.e.

10 µl in 390 µl

Mix by turning inside out after covering the cuvette with a piece of parafilm
Don't touch the clear face of the cuvette
Read the OD at **260 nm**. It allows calculation of the concentration of nucleic acid (DNA & RNA) in the sample. An OD of 1 corresponds to approximately:
50 ng/µl for double-stranded DNA,
37-40 ng/µl for single-stranded DNA and RNA
~ 20 ng/µl for single-stranded oligonucleotides.

For a 1/40 dilution, genomic DNA concentration is:

$$[DNA](ng/\mu l) = DO_{260} \times 50 \times 40$$

The normal yield is 150 µg/7.5 ml whole blood.

Read the OD at **280 nm**. Phenol exhibits a large peak at 270 nm and a smaller peak at approximately 272 nm. Proteins, which possess tyrosine residues (a common occurrence) absorb light at 280 nm.
The ratio OD_{260}/OD_{280} provides an estimate of the purity of the nucleic acid.

$$OD_{260}/OD_{280} \text{ for pure DNA} = 1.7\text{-}2.0$$

OD_{260}/OD_{280} for pure RNA = 2.0-2.1
If $OD_{260}/OD_{280} < 1.75$, there is a contamination by protein and/or phenol, an accurate determination of the amount of nucleic acid will not be possible. However, DNA sample ratios lower than 1.4 can be amplified in most cases without problems.

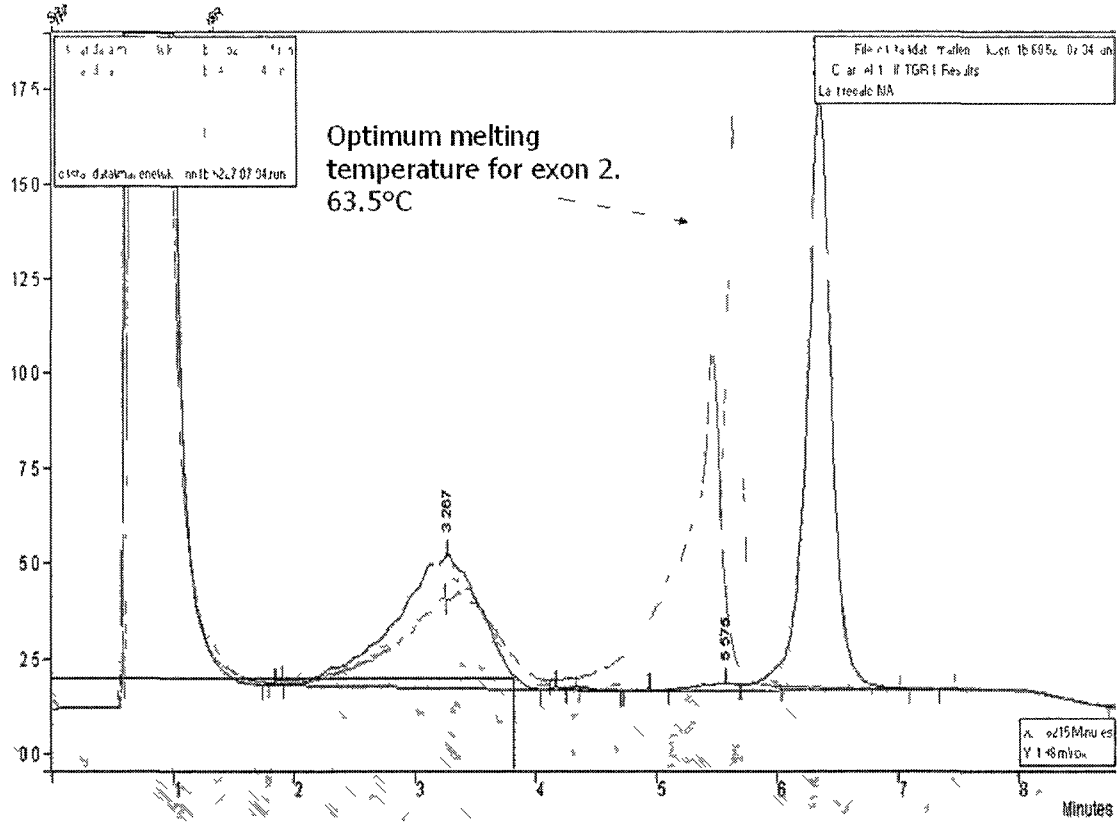
Read the OD at **230 nm**. Peptides, amino acids and salts exhibit a large peak in the spectrogram at a wavelength of 210 nm. If a sample is contaminated by any or all of these molecules, the abnormally high peak could distort the A_{230} reading, resulting in an abnormal higher absorbency value.
The ratio OD_{230}/OD_{260} provides an estimate of the purity of the nucleic acid.

$$OD_{230}/OD_{260} \text{ for pure DNA} = 0.3\text{-}0.5$$

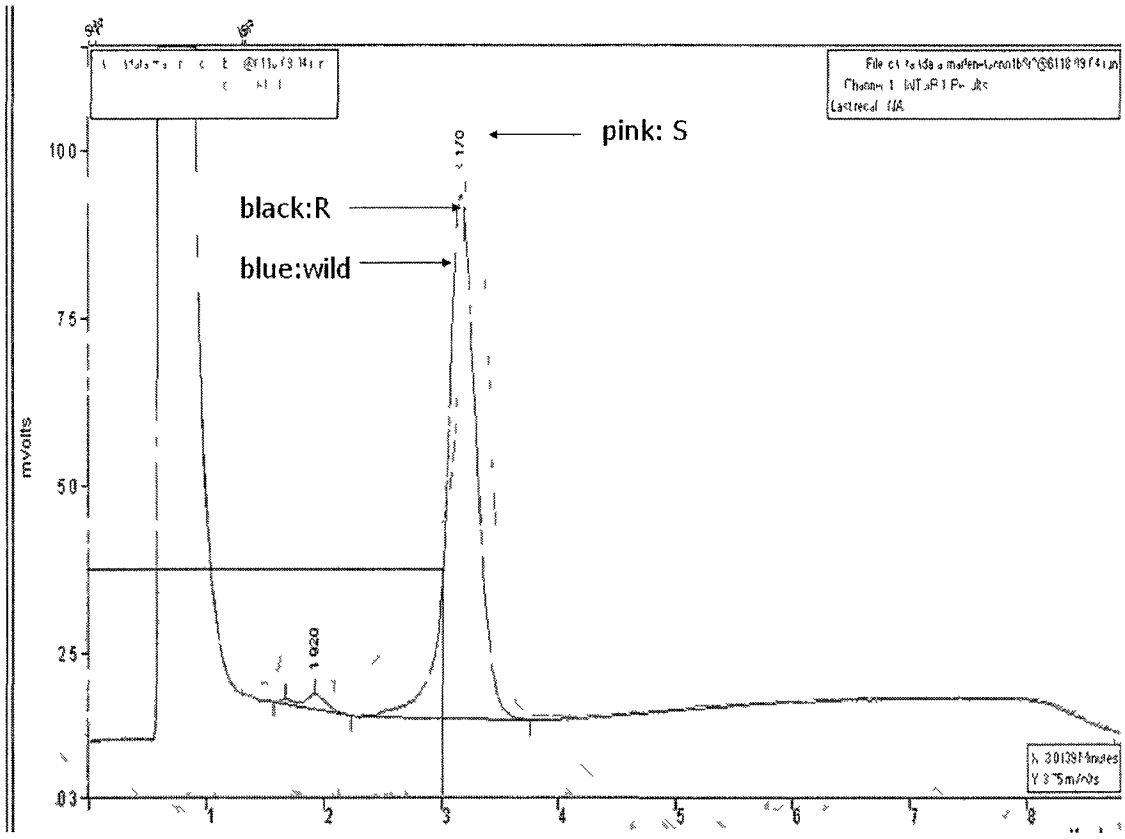
If $OD_{260}/OD_{230} > 0.5$, there is a contamination by peptides, amino acids and/or salts.

Appendix I: Denaturing High Performance Liquid Chromatography (DHPLC)

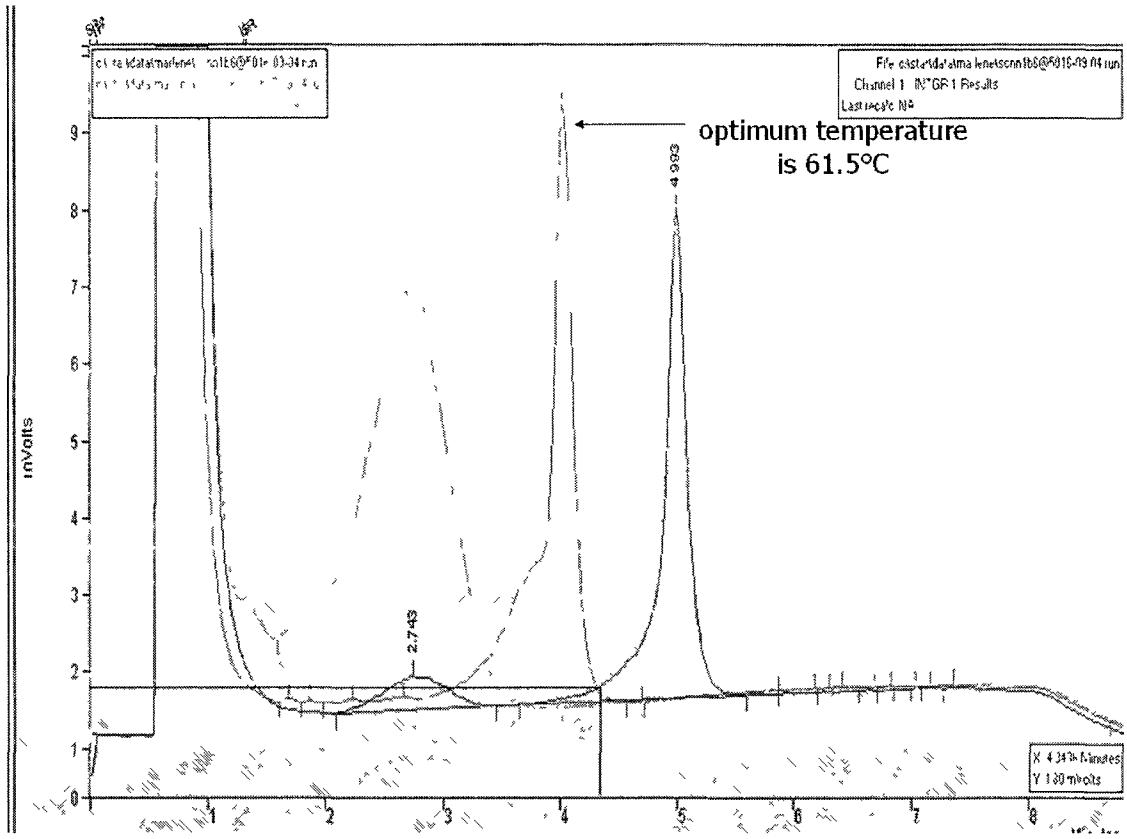
1) DNA fragment is amplified by PCR
2) Amplicon undergoes a post-PCR denaturing/reannealing step in which the sample is gradually cooled from 95 ° to 65 ° C
3) If sample contains a SNP – 4 species are formed 2 contain a sequence mismatch and 2 matched
4) To determine the optimum temperature for DHPLC insert given sequence into the Stanford Melt program software http //insertion stanford edu/melt html
5) Optimise annealing temperature
6) At partial denaturing temperatures, less stable amplicons form a bubble in the double stranded structure The more unstable the species the larger the bubble
7) The least stable elute first and most stable elute last The chromatogram will show up to 4 peaks (2 heteroduplex peaks and 2 homoduplex peaks) Depending on the amplicon, species can coelute forming 2 or 3 peaks rather than 4
8) A single peak indicates no sequence mismatch
9) To test for homozygote mutants-must re-run samples with control DNA (wildtype) added



DHPLC profile showing the optimum temperature for exon 2 in Scnn1A



DHPLC profiles of exon 2 in Scnn1A in Dahl S (black), R (blue) and wistar (pink) rats


optimum temperature
is 61.5°C
4.993
2.743
nVolts

Appendix J: Sequencing Reaction

Preparing DNA

Ready Reaction Mix

For PCR products:

If your fragment is:	Use:
100-200 bp	1-3 ng
200-500 bp	3-10 ng
500-1000 bp	5-20 ng

- Prepare Reaction Mix

PCR Product (diluted 1/20)		8 µl
Ready Reaction Mix		2 µl
5X Sequencing Buffer	2 µl	
10µM primer		3.2 µl
ddH_2O		4.8 µl
Final Volume		20 µl

- Mix and spin briefly

- Place PCR samples in machine and use the following cycle protocol:

96° C	10 sec	25 X
50° C	15 sec	
60° C	4 min	
4° C	HOLD	

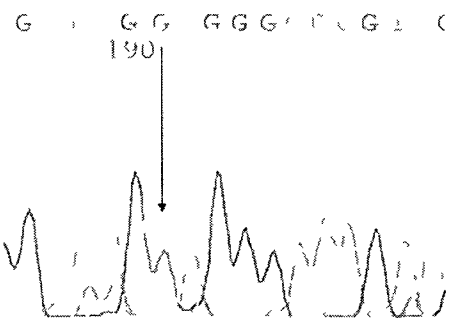
Purifying DNA (Dye-Ex spin columns)

1. Gently vortex the spin column to resuspend the resin.
2. Loosen the cap of the column a quarter turn.
3. Snap off the bottom closure of the spin column and place the spin column in a 2-ml collection tube.
4. Centrifuge for 3 minutes @ 750g.
5. Carefully transfer the spin column to a clean centrifuge tube. Apply the sequencing reaction (10-20 µl) directly onto the center of the slanted gel bed.
6. Centrifuge for 3 minutes @ 750g.
7. Remove the spin column from the microcentrifuge tube.
8. Dry the sample by placing on a heating block @ 95°C for 10 minutes.
9. Store @ -20° C or continue

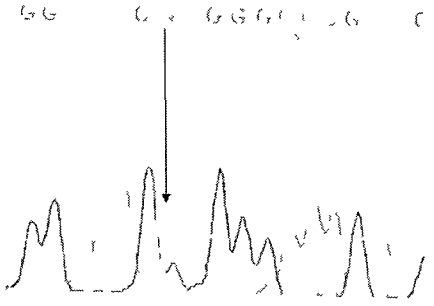
When you are ready to begin sequencing:

- Resuspend the sample in 25 µl formamide
- Vortex thoroughly
- Transfer to an ABI plate
- Denature for 5 min @ 95° C
- Place on ice until ready to run sequencer

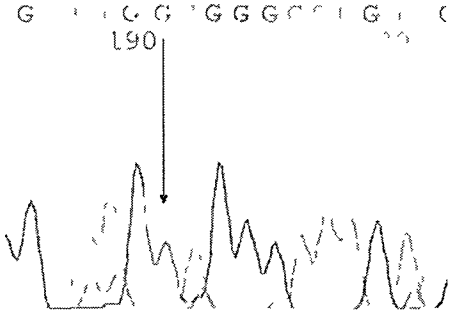
Appendix K: Sequencing Profiles



Genotype: Homozygous TT



Genotype: Heterozygous TG



Genotype: Homozygous GG

Appendix L: Cell Culture and Transfection Protocol

Media preparation

Complete growth medium:

D-MEM
10 % fetal bovine serum (FBS)
1:100 dilution of Penicillin/Streptomycin
1:100 dilution of glutamine (Addition of more glutamine is recommended because it degrades quickly)

Complete growth medium without antibiotic

D-MEM
10 % fetal bovine serum (FBS)
1:100 dilution of glutamine

Cell lines are stored at -140°C, in liquid nitrogen or at -80

- Cells are in 1.5ml tubes.
- Thaw the cells @ 37°C in a water bath.
- Add 10ml of medium to two 10cm plates.
- Divide the cells from the tubes equally into both these plates
- Check cells under microscope, cells should be floating.
- Incubate overnight at 37°C.

Check under the miscroscope for any contamination.

- Change the medium in the cells. This helps to remove DMSO. If the cells are kept too long in the original medium, they will die.

Check the cells again. If they are 80% confluent split them, otherwise leave them to grow.
Once cells are 80% confluent, add 2ml of trypsin, take it out. Trypsin neutralizes anti-trypsin activity of the serum.
Add 7 ml of complete medium.
Transfer solution to a tube, centrifuge at 1200 rpm for 5minutes.
Discard supernatant
Resuspend pellet in 12 ml of complete medium. Divide each of the plates into 5 plates.

For COS7 Cells, transfection was done at a cell density of $3*10^{\wedge 4\ cells}$/ml, in 60mm plates.

-Change medium in the plates. Do it in two motions otherwise cells will dry.

- Using serum free medium, add 5ml of the incomplete medium(without antibiotics and serum) to rinse the cells.
- Then add 4ml of the normal growth without antibiotics.
- Put the plate back in the incubator at 37°C.

- Dilute between 16ug of total DNA into 500ul DMEM without serum and antibiotic.
- Dilute 20ul of lipofectamine™ 2000/plate to 500ul of DMEM without serum and antibiotic and incubate for 5min at room temperature.

Note: Longer incubation times may decrease activity.

- Combine the diluted DNA with the diluted lipofectamine™ 2000. Total volume is 1ml. Incubate at room temperature for 20 min to allow the DNA-Lipofectamine™ 2000 complexes to form. The solution may appear cloudy, but this will not inhibit the transfection. The DNA-Lipofectamine™ 2000 complexes are stable for 6h.
- Add the 1ml of the DNA-Lipofectamine™ 2000 complexes to each of the plates.
- Mix gently by rocking the plate back and forth.
- Incubate the cells at 37°C in a CO_2 incubator for 24-48 hours until they are ready to assay for gene expression. It is not necessary to remove the complexes or change the medium.

Protein Extraction

- Take out the transfected plates from he incubator
- Wash the plates with PBS 1X
- Add 100ul of Ripa Buffer(mixed with 1:100 dilution of protease inhibitor cocktail)
- Scrape the plates, and collect the solution in 1.5ml eppendorrf's
- Centrifuge at 10000 RPM for 10 min
- Collect the supernatant

NOTES

You need 1.5 µl of lipofectamine for 1µg of DNA
Example:

[DNA] =765.5ng/ul
You need 0.5 µg:
765.5 ng ----- 1ul
500 ng -------500 x 1/765.5= 0.65 µl

To freeze the cells

- Tryspine the plates of cells (1 plate per vials)
- Centrifuge and take out supernatant
- Resuspend in 1 ml of DMEM(50%)+FBS(40%)+DMSO(10%). (1ml/plate)
- Distribute in cryo-vials (1ml/vial)
- Freeze at -80°C in isopropanol container

Lipofectamine 2000

· Do not add antibiotics to media during transfection as this causes cell death.
· Maintain the same seeding conditions between experiments

Plasmid DNA Transfection

Use the following procedure to transfect DNA into mammalian cells in a **24-well format.** For other formats, see Scaling Up or Down Transfections. All amounts and volumes are given on a per well basis. Prepare complexes using a DNA (ug) to Lipofectamine 2000 (ul) ratio of 1:2 to 1:3 for most cell lines. Transfect cells at high density for high efficiency, high expression levels, and to minimize cytotoxicity. Optimization may be necessary.

1. **Adherent cells:** One day before transfection, plate 0.5-2.0x10^5 cells in 500ul of growth medium without antibiotics so that cells will be 90-95% confluent at the time of transfection.

   Suspension cells: Just prior to preparing complexes, plate 4-8x10^5 cells in 500ul of growth medium without antibiotics.

2. **For each transfection sample**, prepare complexes as follows:
   a. Dilute DNA in 50ul 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 the appropriate amount in 50ul of Opti-MEM I Medium. Incubate for 5 minutes at room temperature. NOTE: Proceed to Step c within 25 minutes.
   c. After the 5 minute incubation, combine the diluted DNA with diluted Lipofectamine 2000 (total volume=100ul). Mix gently and incubate for 20 minutes at room temperature (solution may appear cloudy). NOTE: Complexes are stable for 6 hours at room temperature.

3. Add the 100ul 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 18-48 hours prior to testing for transgenic expression. Medium may be changed after 4-6 hours.

5. **For stable cell lines:** Passage cells at a 1:10 (or higher dilution) into fresh growth medium 24 hours after transfection. Add selective medium (if desired) the following day.

Optimizing Plasmid DNA Transfection

To obtain the highest transfection efficiency and low cytotoxicity, optimize transfection conditions by varying cell density as well as DNA and Lipofectamine 2000 concentrations. Make sure that cells are greater than 90% confluent and vary DNA (ug) : Lipofectamine 2000 (ul) ratios from 1:0.5 to 1:5.

Scaling Up or Down Transfections

To transfect cells in different tissue culture formats, vary the amounts of Lipofectamine 2000, nucleic acid, cells, and medium used in proportion to the relative surface area, as shown in the table. With automated, high-throughput systems, a complexing volume of 50ul is recommended for transfections in 96-well plates. NOTE: You may perform rapid 96-well plate transfections by plating cells directly into the transfection mix. Prepare complexes in the plate and directly add cells at twice the cell density as in the basic protocol in a 100ul volume. Cells will adhere as usual in the presence of complexes.

Culture vessel	**Surf. Area per well[1]**	**Shared reagents**		**DNA transfection**	
		Vol. of plating medium	**Vol. of dilution medium[2]**	**DNA**	**Lipofectamine 2000**
96-well	$0.3cm^2$	100ul	2x25ul	0.2ug	0.5ul
24-well	$2cm^2$	500ul	2x50ul	0.8ug	2.0ul
12-well	$4cm^2$	1ml	2x100ul	1.6ug	4.0ul
6-well	$10cm^2$	2ml	2x250ul	4.0ug	10ul
60-mm	$20cm^2$	5ml	2x0.5ml	8.0ug	20ul
10-cm	$60cm^2$	15ml	2x1.5ml	24ug	60ul

[1] Surface areas may vary depending on the manufacturer.
[2] Volumes of dilution medium in Step2a&2b of DNA

Appendix M: Real time PCR (Light cycler)

Products needed

LightCycler FastStart DNA Master SYBR Green I (Roche, cat #2 239 264)
LightCycler Capillaries (20ul) (Roche, 11 909 339 001)
Cold block of the light cycler

1 prepare the following mix

Reagent	Volume	Final concentration
cDNA	2ml	
SybiGreen	2ml	
Primers R+F (20mM)	0 6ml	0 6mM
MgCl2	1ml	1 25mM
Water	14 5ml	
Total	20 ml	

2 Load the cDNA in the capillaries and pour the master mix (18ul) (I prefer doing it like that but it depends on the user)
3 Put the cap
4 Softly spin the capillaries (5-10sec at 1000rpm), using the cold metal tube in the cold block provided with the light cycler
5 Load the capillaries in the light cycler and run the program

Appendix N: Western Blot Protocol

1 Transfer proteins from gel to membrane (nitrocellulose of PVDF) Gloves should be worn when handling gels or blot membranes

A Pour enough transfer buffer into a container so that half of the assembled cassette will be 0 5 cm below the surface of the buffer

B After the gel has finished running, carefully remove the front glass plate and remove the stacking gel Remove a small portion of the upper left-hand corner of the running gel to mark its orientation during the transfer process

C Wet the membrane with transfer buffer and carefully lay it across the exposed running gel, making sure that air bubbles between the gel and membrane are excluded In the same fashion, add two layers of pre-wetted filter paper to the membrane Pre-wet one of the cassette sponges and add to the stack Top with the outer half of the cassette itself and carefully turn the entire stack of glass plate, gel, membrane, filter paper, sponge and cassette over Place the stack in the container with transfer buffer

D Carefully remove the second glass plate from the gel Using the same process, make sure that no air bubbles have formed between the gel and the membrane

E Keeping the stack generously wet, top the gel with two layers of pre-wet filter paper, wet sponge and the remaining half of the cassette unit Snap the cassette unit together firmly

F Place the cassette into the transfer unit so that the current will flow through the gel onto the membrane This would be with the gel closer to the black (-) cathode and the membrane closer to the red (+) anode

G Add transfer buffer to the transfer unit according to manufacturer1s directions Transfer over night with a setting of 20V / 40 mA or for 3 hours at 70V/160 mA The transfer process needs to take place under cold temperatures to prevent the gel from sticking to the membrane This can be accomplished either by using a water cooling core in the transfer unit or placing the entire unit at 4°C Please follow the manufacturer1s recommendations

H At the end of the transfer period, disconnect the power connections and disassemble the cassette All of the kaleidoscope dye (from Bio-rad) bands should now be on the membrane leaving the gel fairly clear

2 Staining the Blot

A Briefly with water, rinse any bits of gel off the blot then incubate it in ponceau S solution for 1 minute Rinse in water until the background is reduced

B Place the membrane on a transparency sheet and scan or photocopy the blot for a permanent transfer record

C Rinse the blot several times in PBS-Tween to remove the stain

3 Blocking the Blot

A Gently shake the blot for 2 hours in 5% non-fat dry milk/PBS/Tween at room temperature (RT) or at 4°C on a rocker
B Either pour off blocking buffer or transfer the blot to a sealing bag or an appropriate container (small empty pipette tip's box will do the job) for probing

4 Probing the Blot

A Dilute the primary antibody in 5% non-fat dry milk/PBS Add to the blot, seal the container and gently rock for 2h at RT or OVN at 4°C If using a bag for the incubation, eliminate all air bubbles before sealing
B Remove the primary antibody and wash the blot 3 x 5 minutes in PBS/Tween The primary antibody can be saved for additional blot procedures by adding Na azide (1/1000)
C Dilute the secondary antibody in 5% non-fat dry milk/TBS Add to the blot, seal and incubate for 2h at room temperature
D Remove the secondary antibody and wash the blot 3 x 10 minutes in PBS/Tween

5 Detection / Chemiluminescence

A Cut two transparency sheets to fit into the film cassette
B Mix 5ml of oxidizing and luminol reagent (from Amersham) in a 1 1 ratio
C Pour onto a piece of parafilm and lay the blot on the pool of solution for 1 minute
D Blot off residue by holding te edge of membrane against kimwipes
E Insert the blot into the cassette and top with the remaining sheet of transparency, making sure that all bubbles are eliminated
E Insert film and close cassette Start with a 1 minute exposure and adjust from there The signal will decay over time If no bands are found, the blot can be rinsed and stored in PBS/Tween overnight for repeated blocking and probing

6 Stripping and Reprobing Blots

A Briefly rinse the blot in water
B Wash the blot 2 x 10 minutes in large volume of PBS/Tween at RT
C Re-block in 5% milk/PBS/Tween for one hour at RT
D Add stripping buffer to the blot, seal the container and incubate for 30 minutes at 50°C occasionally agitate the container for best results
E Wash the blot 2 x 30 minutes followed by 2 x 10 minutes in PBS/Tween

F Block the blot for 2 hours in 5% milk/PBS/Tween at room temperature before probing with the desired primary antibody again

Tripping Buffer

4 925 g	Tris-HCL (pH to 6 7 in 200 ml of dH_2O)
3 52 ml	2-Bmercaptoethanol (in fume hood WEAR gloves, carcinogenic)
10 g	SDS

Fill to 500 ml with dH_2O

Appendix O: ENaC α, β, γ (*SCNN1A, B and C*) Transcripts

Legend
Blue= coding regions of cDNA
Red= UTR cDNA
Light blue= coding boundaries
Orange= UTR boundaries

α ENaC SCNN1A

cDNA α ENaC NM_031548

```
TGAGGCAGG ATCAGAGAGC CTGGCACAGA AAGGGA[illegible]  50
CGGGAGTCAG CTGGGCCAAC ACCCCCTCAA ACCTCCACCC ACTCAAACAC  100
TCCGGGAGGA [illegible]  150
IGCTGIGGGG ACC[illegible]  200
CTGTGAAGTC CAGTGGCCAC TCCAGACAAC CTCAAIACTC CTTCCCTCCC  250
[illegible]  300
GGGAGAGTGA ACTCAGCCTG GGAICC[illegible]  350
TCTGCCTTCA CGCTAATCAT GCTGGACCAC ACCAGAGCCC CTGAGCTCAA  400
CATTGACCTA GACCTTCACG CCTCCAACTC GCCTAAGGGG TCCATGAAGG  450
GCAACCAATT CAAGGAGCAA GACCCTTGTC CTCCTCAGCC CATGCAAGGA  500
CTGGCCGAAGG GCGACAAACG TGAAGAGCAG GGCCTGGGCC CGGAACCCTC  550
AGCACCCCGG CAGCCCACCG AGGAGGAGGA GGCACTGATT GAGTTCCACC  600
GCTCCTACCG GGAGCTCTTC CAGTTCTTCT GCAACAACAC CACCATCCAC  650
GGGGCCATCC GCCTGGTGTG CTCCAAACAC AACCGCATGA AGACGGCCTT  700
CTGGGCGGTG CTGTGGCTGT GCACCTTCGG CATGATGTAC TGGCAGTTCG  750
CCTTGCTGTT CGAGGAGTAC CTCAGCTACC CAGTGAGCCT CAACATCAAC  800
CTCAATTCAG ACAAGCTGGT CTTCCCTGCC GTCACTGTCT GCACCCTTAA  850
TCCTTACA  TACACTGAAA TTAAAGAGGA GCTGGAAGAG CTGGACCGCA  900
TCACGGAGCA GACGCTTTTT GACTTGTACA AATACAACTC TTCCTACACT  950
CGCCAGGCTG GGGCCCGACG CCGCAGCTCC CGCGACCTCC TGGGTGCTTT  1000
CCCGCACCCC CTGCAGCGCC TGCGCACTCC ACCTCCGCCC TACTCCGGCC  1050
GCACGGCGCG CAGCGGGTCT TCCAGCGTAC GCGACAACAA TCCCCAAGTG  1100
GACCGGAAGG ACTGGAAGAT CGGCTTCCAA CT  GCAACC AGAACAAATC  1150
AGACTGTTTC TACCAGACAT ACTCCTCTGG GGTGGATGCA GTGAGGGAGT  1200
GGTACCGCTT CCATTACATC AACATTCTGT CCAGGCTGTC GGACACCTCG  1250
CCCGCTCTAG AGGAAGAAGC CCTGGGCAAC TTCATCTTCA CCTGTCGCTT  1300
CAACCAGGCC CCCTGCAACC AGG  AATTA TTCCAAGTTC CACCACCCCA  1350
TGTACGGGAA CTGCTACACT TTCAATGACA AGAACAACTC CAATCTCTGG  1400
ATGTCCTCCA TGCCTGGAGT CAACAAT  T TTGTCCCTGA CACTGCGCAC  1450
AGAGCAGAAT GACTTCATCC CCCTGCTGTC CACAGTGACG GGGGCCAGGG  1500
TGATGGTGCA TGGTCAGGAT GAGCCTGCCT TTATGGATGA TGGTGGCTTC  1550
AACTTGAGGC CTGGCGTGGA GACCTCCATC AGTATGAGAA A  AAGCCCT  1600
GGACAGCCTC GGAGGAAATT ACGGCGACTG TACTGAGAAT GGTAGCGATG  1650
TCCCGGTCAA GAACCTTTAC CCTTCCAAGT ATACACAGCA  TGTGCATT  1700
CACTCCTGCT TCCAGGAGAA CATGATCAAG AAGTGTGGCT GTGCCTACAT  1750
CTTCTACCCT AAGCCCAAGG GAGTTGAGTT CTGTGACTAC CGAAAGCAGA  1800
GCTCCTGG  CTATTGCTAT TATAAACTGC AGGGCGCCTT CTCCTTGGAC  1850
AGCCTGGGCT GTTTCTCCAA GTGTCGGAAG CCTTGTA  G TGATCAACTA  1900
CAAACTCTCT GCCGGCTACT CACGGTGGCC ATCTGTGAAG TCCCA  ATT  1950
GGATCTTCGA GATGCTGTCC TTGCAGAACA ATTACACTAT TAACAACAAA  2000
A  AACGGAG TTGCAAAGCT CAACATCTTC TTCAAGGAGC TGAACTATAA  2050
AACTAATTCG GAGTCTCCTT CTGTCAC  T GGTCAGCCTC CTGTCCAACC  2100
```

```
TGGGCAGCCA GTGGAGCCTG TGGTTTGGCT CGTCCGTGCT CTCTGTGGTG  2150
GAGATGGCGG AGCTCATCTT CGACCTCCTG GTCATCACAC TTCTCATGCT  2200
GCTACGCCGG TTCCGGAGCC GGTACTGGTC TCCAGGACGA GGGGCCAGGG  2250
GTGCCAGGGA GGTGGCCTCC ACTCCAGCTT CCTCCTTCCC GTCCCGTTTC  2300
TGTCCTCACC CTACATCCCC ACCACCTTCT TTGCCCCAGC AGGGCATGAC  2350
CCCTCCCCTG GCCCTGACAG CCCCTCCACC TGCCTATGCT ACTCTAGGCC  2400
CCAGTGCCCC TCCACTGGAC TCTGCGGCGC CTGACTGTTC TGCCTGTGCC  2450
CTGGCGGCGC TCTGAGAGAG GAGAAGGAGC CTCTCAGCCA GCCCCAGAGC  2500
CCCCCGAGCC CTCTGTCGTC TCCCTCCCTC ACCCAAGACC CTCACTAAAG  2600
GGCCCAGGAA GCCGTCCAGA GGACTAGGGGC TAACGAGCCC CCAGCAGCGG  2650
CCTCTTCCCT CCGATCAGCC CACCCACACT TCCTCCTCTC ACAACCAACT  2750
TTCCTGGGAG AGGACCAAGG AACAAATAGA AGAGCCAGCC CATCCCAGCC  2800
CCCGTGTACC AGAGCTGGCC TCGCCT ACT G CTTCAGCGC AGACTCATGT  2850
CTGCTCCTCT CCTTCAACTT CCCTCCCCAA CCCCACCCAA AACCCCCCTT  2900
GTTAGTTCTT TGGCAATTCC CCTCCCCTCA CTCCTCAGGG CCCCCACCAC  2950
ACTAAGACAG ACCAGGAGTC G CTCGTGTCT  TCTCT CCT  C AAGAG C  3000
TCTCCTTTGA TTCCCCATAC CCCACTCCCT CTCTCCTCTT CTCTCCTTCT  3050
TTCTCTTTCC TCAAGCTTGG AGCTTCTTTC ACTGTCCCTC ACAACCACAA  3100
GTCTATGCAT CCCTTAGAAA CTCCCTAAA GCACCCTTT CTTTCCTCAA  3150
CTCAGTTACC CTGTTATGTC TCCCCCACAA TTCTCCCCCT CTCCCCTCAC  3200
CTCTCTCTGG TTCTCTGTTT TTCTTTCTCT TTCTTTCTCC TCTCCCTCCC  3250
TGCCCTAAAA TGCCTCTTTC TGTCCCCACC CATGACAATT CCTCTCTTTT  3300
CTATCCTTCC CCTCTATCCC ACCTCTCCCC TTCCCACCCC TACCCACCTA  3350
CT AATTAAAG GTTGTTCCAT T aaaaaaaa aaaaaaaaaa aaaaa
```

β ENaC *SCNN1B*

cDNA β ENaC NM_012648

```
CCACCTTAG CTGCCATCAC TCCACATTGG ACCACCTTTC TAAACA  TC   50
CCTCCATGCC AGTGAAGAAG TACCTGCTGA AGTGCCTGCA CAGGCTGCAG  100
AAGGGCCCAG GCTACACCTA CAAGGAGCTG CTAGTGTGGT ACTGCAACAA  150
CACCAACACA CACGGCCCCA AACGCATCAT CTGCGAGGGG CCCAAGAAGA  200
AGGCCATGTG GTTCCTGCTC ACGCTGCTCT TCGCCTGCCT GGTGTGCTGG  250
CAGTGGGGCG TCTTCATCCA GACCTACCTG AGCTGGGAGG TCAGCGTCTC  300
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attccagccc cttcca TAC TCCAAGGTCA AGCACTTGCT GAAGGACTTG  400
tACAAGCTGA TGGAGGCTGT CCTGGACAAG ATTCTGGCTC CGaAGTCCAG  450
CCACACCAAC ACCACCAGTA CCCTGAACTT TACCATCTGG AACCACACGC  500
CCCTGGTCCT TATTGATGAG CGGAACCCTG ACCATCCAGT GGTCCTCAAC  550
TTGTTTGGGG ACAGCCACAA CAGCAGCAAC CCAGCCCCAG GAAGCACCTG  600
TAATGCCCAA GGGTGCAAAG TGGCCATGAG GCT  GCAGT GCCAATGGGA  650
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TGGTACATCC TGCAGGCCAC CAACATCTTC TCACAAGTGC TCCCCCAGGA  750
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CCACACCAAC TgTGtCTTCC AGCCTGACAC AACCAGCTGC AGGCCCAATG 1850
CCGAGGTCTA CCCTGACCAA CAGACTCTGC CCATTCCGGG CACTCCACCT 1900
CCCAACTATG ACTCCCTGAG GCTGCAGCCG CTGGACACCA TGGAGTCTGA 1950
CAGCGAGGTG GAGGCCATCT AGATCCCCAT CCCCACCCCC gGAGCTACTG 2000
ACTCAAAACT GAGCAGTCAC AACCATTCTC ACTGCCTCAT TTCATTACCC 2050
CTTGTTCAAA GAGCCAGGGC ACAGAGCCCA AGTCCCTCCC GACACCCCA 2100
GGCCoACGGG TTCATAGGGT CAAGATGCTG GTACCACGAA ATTCAA CCC 2150
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CCCCTAGTTG CCATTCGCTC AACCCTGTTA ATACACCTTC CCCTCTCCAC 2350
ACCCTCAGGGA GAGAGA AT A CGTTTGATGG CTTGTCAGGG GTACCATCCC 2400
TTTGTTACAA AATAAGAGAA AAAAAAAAAA aaaaa

γ ENaC *SCNN1G*

cDNA γ ENaC - NM_017046

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GGCGCCTGGA GAGAAGATCA AAGCCAAAAT CAAAAAGAAT CTGCCGGTTC 150
GAGGCCCCCA GGCACCAACC ATTAAGGACC TGATGCATTG GTACTGCATG 200
AACACCAACA CCCACGGgTG CCGCCGCATC GTGGTGTCCC GAGGCCGCCT 250
TCGGCctCTG CTGTGGATCG CGTTCACGCT AACTGCAGTG GCCCTCATTA 300
TCTGGCAGTG CGCCCTCCTC GTCTTCTCTT TCTACACCGT CTCTGTCTCC 350
ATCAAAGTCC ACTTCCAGAA ACTGGATTTC CCTGCCGTCA CTATCTGCAA 400
TATCAACCCT TACA TACA GTGCTGTGAG TGACCTTCTG ACTGACTTGG 450
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TACATGAATA TCATGGCACA GGTGCCTCTA GAGAAGAAAA TCAACATGAG 850
CTATTCTGCC gAAGAACTGC TGGTGACCTG CTTCTTCGAT GGGATGTCCT 900
GTGATGCCA AACTTCACG CTTTTCCACC ATCCAATGTA TGGGAACTGC 950
TACACATTTA ACAACAAAGA AAATGCCACC ATCCTCAGCA CCTCCATGGG 1000
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CTCCAGCAGC CAATTACTGC AACTACCAGC AACACCCCAA CTGG GTAT 1400

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ACTCCCTCCA GCCGGCAAGG CCAGGATAAT CCAGCTCTGG ATACGGACGA 1900
TGACCTGCCC ACTTTTACCT CTGCTATGCG CCTGCCTCCA GCCCCGGGGT 1950
CCACGGTGCC TGGCACACCA CCTCCCAGAT ACAATACCTT GCGCTTGGAT 2000
AGAGCCTTTT CATCCCAGCT CACAGACACT CAGTTGACCA ATGAGTTGTA 2050
AGACAGGATT GGGAAACAAT CATGGTTTGA GGAGGCTCTC TTCCTGACAC 2100
AGATGGCCCT CCTATCCTTG CTCTGTCTTT CAATACATGA CTAACAGCCT 2150
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TGCCCCAACA CTCATAACTT CCAGAATAAG CTATATGCTA CAACCTGGAC 2250
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TCAGCTCAAG TGGTATACTT TGAGTTGTAC CA g CACTT ACATAAGTTA 2750
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TATGTCTGTA TGTGTTTATA ATTTTTATAT AGCTAATATA TACATTTATC 2900
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Appendix P: α ENaC - *SCNN1A* Transcripts

cDNA α ENaC - CK479583

```
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TTTCTCCAAG TGTCGGAAGC CTTGTA  GT GATCAACTAC AAACTCTCTG  250
CCGGCTACTC ACGGTGGCCA TCTGTGAAGT CCCA  ATTG GATCTTCGAG  300
ATGCTGTCCT TGCAGAACAA TTACACTATT AACAACAAAA   AACGGAGT  350
TGCAAAGCTC AACATCTTCT TCAAGGAGCT GAACTATAAA ACTAATTCGG  400
AGTCTCCTTC TGTCAC  TG GTCAGCCTCC TGTCCAACCT GGGCAGCCAG  450
TGGAGCCTGT GGTTTGGCTC GTCCGTGCTC TCTGTGGTGG AGATGGCGGA  500
GCTCATCTTC GACCTCCTGG TCATCACACT TCTCATGCTG CTACGCCGGT  550
TCCGGAGCCG GTACTGGTCT CCAGGACGAG GGGCCAGGGG TGCCAGGGAG  600
GTGGCCTCCA CTCCAGCTTC CTCCTTCCCG TCCCGTTTCT GTCCTCACCC  650
TACATCCCCA CCACCTTCTT   CCCCAGTG CCCCTCCACT GGACTCTGCG  700
GCGCCTGACT GTTCTGCCTG TGCCCTGGCG GCGCTCTGAG AGA  GAACG  750
ATCCTCTCAC CC tcct
```

cDNA α ENaC - CK475461

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tccagtatac acag AGGTG TGCATTCACT CCTGCTTCCA GGAGAACATG  50
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AACTGCAGGG CGCCTTCTCC TTGGACAGCC TGGGCTGTTT CTCCAAGTGT  200
CGGAAGCCTT GTA  GTGAT CAACTACAAA CTCTCTGCCG GCTACTCACG  250
GTGGCCATCT GTGAAGTCCC A  ATTGGAT CTTCGAGATG CTGTCCTTGC  300
AGAACAATTA CACTATTAAC AACAAAA  A ACGGAGTTGC AAAGCTCAAC  350
ATCTTCTTCA AGGAGCTGAA CTATAAAACT AATTCGGAGT CTCCTTCTGT  400
CAC  TGGTC AGCCTCCTGT CCAACCTGGG CAGCCAGTGG AGCCTGTGGT  450
TTGGCTCGTC CGTGCTCTCT GTGGTGGAGA TGGCGGAGCT CATCTTCGAC  500
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CCTTCTTTGC CCCAGCAGGG CATGACtCCT CCCCTGGCCC TGACAngCCC  700
TC cactg T ATGCTACTCT AGGCC  GTG CCCCT  CTG G  CTGncGC  750
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gcctgactcc tgta
```

cDNA α ENaC - CK364785

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GGCTGTTTCT CCAAGTGTCG GAAGCCTTGT A  GTGATCA ACTACAAACT  100
CTCTGCCGGC TACTCACGGT GGCCATCTGT GAAGTCCCA   ATTGGATCT  150
TCGAGATGCT GTCCTTGCAG AACAATTACA CTATTAACAA CAAAA  AAC  200
GGAGTTGCAA AGCTCAACAT CTTCTTCAAG GAGCTGAACT ATAAAACTAA  250
TTCGGAGTCT CCTTCTGTCA C  TGGTCAG CCTCCTGTCC AACCTGGGCA  300
GCCAGTGGAG CCTGTGGTTT GGCTCGTCCG TGCTCTCTGT GGTGGAGATG  350
GCGGAGCTCA TCTTCGACCT CCTGGTCATC ACACTTCTCA TGCTGCTACG  400
CCGGTTCCGG AGCCGGTACT GGTCTCCAGG ACGAGGGGCC AGGGGTGCCA  450
GGGAGGTGGC CTCCACTCCA GCTTCCTCCT TCCCGTCCCG TTTCTGTCCT  500
CACCCTACAT CCCCACCACC TTCTTTGCCC CAGCAGGGCA TGACCCCTCC  550
CCTGGCCCTG ACAGCCCCTC CACCTGCCTA TGCTACTCTA GGCCCCAGTG  600
CCCCTCCACT GGACTCTGCG GCGCCTGACT GTTCTGCCTG TGCCCTGGCG  650
GCGCTCTGAG AGAGGAGAAG GATCCTCTCA CCCAGCCCTG AGCTCCCTTG  700
TAAACTGATG GATATCTCAC TTCAGCACAT CT t `CCCAG CG  CCGCCG  750
```

AGCCCTCTGT tgg

cDNA α ENaC - CK475819

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ACGGTGGCCA TCTGTGAAGT CCCA  ATTG GATCTTCGAG ATGCTGTCCT  150
TGCAGAACAA TTACACTATT AACAACAAAA   AACGGAGT TGCAAAGCTC  200
AACATCTTCT TCAAGGAGCT GAACTATAAA ACTAATTCGG AGTCTCCTTC  250
TGTCAC  TG GTCAGCCTCC TGTCCAACCT GGGCAGCCAG TGGAGCCTGT  300
GGTTTGGCTC GTCCGTGCTC TCTGTGGTGG AGATGGCGGA GCTCATCTTC  350
GACCTCCTGG TCATCACACT TCTCATGCTG CTACGCCGGT TCCGGAGCCG  400
GTACTGGTCT CCAGGACGAG GGGCCAGGGG TGCCAGGGAG GTGGCCTCCA  450
CTCCAGCTTC CTCCTTCCCG TCCCGTTTCT GTCCTCACCC TACATCCCCA  500
CCACCTTCTT TGCCCCAGCA GGGCATGACC CCTCCCCTGG CCCTGACAGC  550
CCCTCCACCT GCCTATGCTA CTCTAGGCCC CAGTGCCCCT CCACTGGACT  600
CTGCGGCGCC TGACTGTTCT GCCTGTGCCC TGGCGGCGCT CTGAGAGAGG  650
AGAAGGATCC TCTCACCCAG CCCTGAGCTC CCTTGgtAAC TGATGGATAT  700
CTCACTTCAG CACATCTTCC CCAGCGGCCC CGCCG  CCT CTGTGGT
```

cDNA α ENaC - CB690980

```
ccacgcgtcc g AGAAACGG AGTTGCAAAG C  ACATCTT CT  AGGAGC  50
TGAACTATAA AACTAATTCG GAGTCTCCTT CTGTCAC  T GGTCAGCCTC  100
CTGTCCAACC TGGGCAGCCA GTGGAGCCTG TGGTTTGGCT CGTCCGTGCT  150
CTCTGTGGTG GAGATGGCGG AGCTCATCTT CGACCTCCTG GTCATCACAC  200
TTCTCATGCT GCTACGCCGG TTCCGGAGCC GGTACTGGTC TCCAGGACGA  250
GGGGCCAGGG GTGCCAGGGA GGTGGCCTCC ACTCCAGCTT CCTCCTTCCC  300
GTCCCGTTTC TGTCCTCACC CTACATCCCC ACCACCTTCT TTGCCCCAGC  350
AGGGCATGAC CCCTCCC ca cctgtt
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cDNA α ENaC-a

```
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ACCGGGAGGA AAAAAGGGCA AGAGCGGACA ACCCTAAGCC ACGCAGCCC  150
TGCTGTGGGG ACCCACGCAC CCCCTACCGG CCAAACCAAC CTCCCCCTCC  200
CTGTGAAGTC CAGTGGCCAC TCCAGAGCAG CTCCCTACCC CCCCCCCCAC  250
CCTGACTCCA GAAGGTCAGC TGCCTCCTCC CAAGGTCCAC CAGCCCCCCA  300
GGGAGAGTGA ACTCAGCCTC CCATCCCCCC ACCCTCCCCC ACACCCCCAT  350
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CATTGACCTA GACCTTCACG CCTCCAACTC GCCTAAGGGG TCCATGAAGG  450
GCAACCAATT CAAGGAGCAA GACCCTTGTC CTCCTCAGCC CATGCAAGGA  500
CTGGGGAAGG GGGACAAACG TGAAGAGCAG GGCCTGGGCC CGGAACCCTC  550
AGCACCCCGG CAGCCCACCG AGGAGGAGGA GGCACTGATT GAGTTCCACC  600
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GACCGGAAGG ACTGGAAGAT CGGCTTCCAA CT  GCAACC AGAACAAATC  1150
AGACTGTTTC TACCAGACAT ACTCCTCTGG GGTGGATGCA GTGAGGGAGT  1200
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TGATGGTGCA TGGTCAGGAT GAGCCTGCCT TTATGGATGA TGGTGGCTTC  1550
AACTTGAGGC CTGGCGTGGA GACCTCCATC AGTATGAGAA A  AAGCCCT  1600
GGACAGCCTC GGAGGAAATT ACGGCGACTG TACTGAGAAT GGTAGCGATG  1650
TCCCGGTCAA GAACCTTTAC CCTTCCAAGT ATACACAGCA   TGTGCATT  1700
CACTCCTGCT TCCAGGAGAA CATGATCAAG AAGTGTGGCT GTGCCTACAT  1750
CTTCTACCCT AAGCCCAAGG GAGTTGAGTT CTGTGACTAC CGAAAGCAGA  1800
GCTCCTGG  GCGCCTTCTC CTTGGACAGC CTGGGCTGTT TCTCCAAGTG 1850
TCGGAAGCCT TGTA GTGA TCAACTACAA ACTCTCTGCC GGCTACTCAC 1900
GGTGGCCCAA ACTCTCTGCC GGCTACTCAC GGTGGCCATC TGTGAAGTCC 1950
```

cDNA α ENaC-b

```
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CGGGAGTCTG CTGGACCTAG ACGGCGTGAA AGCTCCACCA ACCAAAAGAG  100
TCCGGGAGGA AAAAAGGGCA ACACCCACAC ACCCTAACCC ACCCACTCCC  150
TGCTGTGGGG ACCCAGGGAG GCGCTACCGG GCAAAGCAAG GTCCCCTTCC  200
CTGTGAAGTC CTGTGGCCAC TGCACAAGAA GCTCAATACC CCCCCCCCAC  250
CCCGACTCCA CAACGTCACC TCGCTCCTCC AAACCTCCAC CACCCTCCCA  300
GGGAGGAGTG ACTCAGCCTG GCATCCGGGC ACCGTCCAAA CCCCTCCCCT  350
TCTGCCTTCC CGCTTATGAT GCTGGACCAC ACCAGAGCCC CTGAGCTCAA  400
CATTGACCTA GACCTTCACG CCTCCAACTC GCCTAAGGGG TCCATGAAGG  450
GCAACCAATT CAAGGAGCAA GACCCTTGTC CTCCTCAGCC CATGCAAGGA  500
CTGGGGAAGG GGGACAAACG TGAAGAGCAG GGCCTGGGCC CGGAACCCTC  550
AGCACCCCGG CAGCCCACCG AGGAGGAGGA GGCACTGATT GAGTTCCACC  600
GCTCCTACCG GGAGCTCTTC CAGTTCTTCT GCAACAACAC CACCATCCAC  650
GGGGCCATCC GCCTGGTGTG CTCCAAACAC AACCGCATGA AGACGGCCTT  700
CTGGGCGGTG CTGTGGCTGT GCACCTTCGG CATGATGTAC TGGCAGTTCG  750
CCTTGCTGTT CGAGGAGTAC CTCAGCTACC CAGTGAGCCT CAACATCAAC  800
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TCCTTACA   TACACTGAAA TTAAAGAGGA GCTGGAAGAG CTGGACCGCA  900
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GCACGGCGCG CAGCGGGTCT TCCAGCGTAC GCGACAACAA TCCCCAAGTG 1100
GACCGGAAGG ACTGGAAGAT CGGCTTCCAA CT  GCAACC AGAACAAATC 1150
AGACTGTTTC TACCAGACAT ACTCCTCTGG GGTGGATGCA GTGAGGGAGT 1200
GGTACCGCTT CCATTACATC AACATTCTGT CCAGGCTGTC GGACACCTCG 1250
CCCGCTCTAG AGGAAGAAGC CCTGGGCAAC TTCATCTTCA CCTGTCGCTT 1300
CAACCAGGCC CCCTGCAACC AGG  AATTA TTCCAAGTTC CACCACCCCA 1350
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ATGTCCTCCA TGCCTGGAGT CAACAAT  T TTGTCCCTGA CACTGCGCAC 1450
AGAGCAGAAT GACTTCATCC CCCTGCTGTC CACAGTGACG GGGGCCAGGG 1500
TGATGGTGCA TGGTCAGGAT GAGCCTGCCT TTATGGATGA TGGTGGCTTC 1550
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GGACAGCCTC GGAGGAAATT ACGGCGACTG TACTGAGAAT GGTAGCGATG 1650
TCCCGGTCAA GAACCTTTAC CCTTCCAAGT ATACACAGCA   TGTGCATT 1700
CACTCCTGCT TCCAGGAGAA CATGATCAAG AAGTGTGGCT GTGCCTACAT 1750
CTTCTACCCT AAGCCCAAGG GAGTTGAGTT CTGTGACTAC CGAAAGCAGA 1800
GCTCCTGG TG TCGGAAGCCT TGTA  GTGA TCAACTACAA ACTCTCTGCC 1850
```

cDNA α ENaC-c

```
GCTCCTGGGT GTGATCAACT AAGGTGAAGG TCGGAGAAGA CATCCCCACC 50
ACTTCTTT
```

cDNA α ENaC-d

```
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AAGTCATAAA AGTTAATGCA ATGAAAGCAC ATCCCCACCA CCTTCTTTA  99
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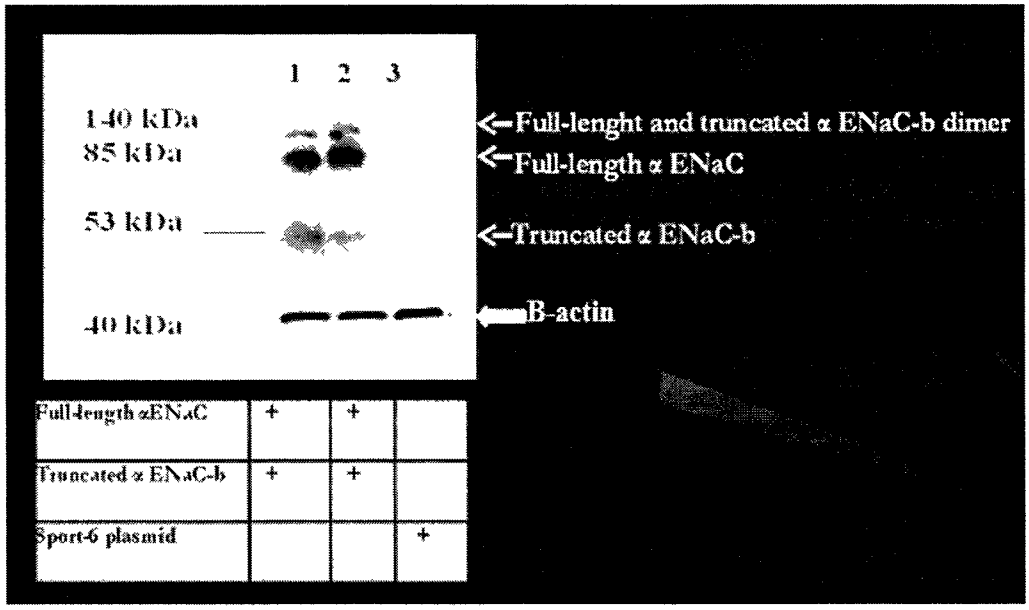
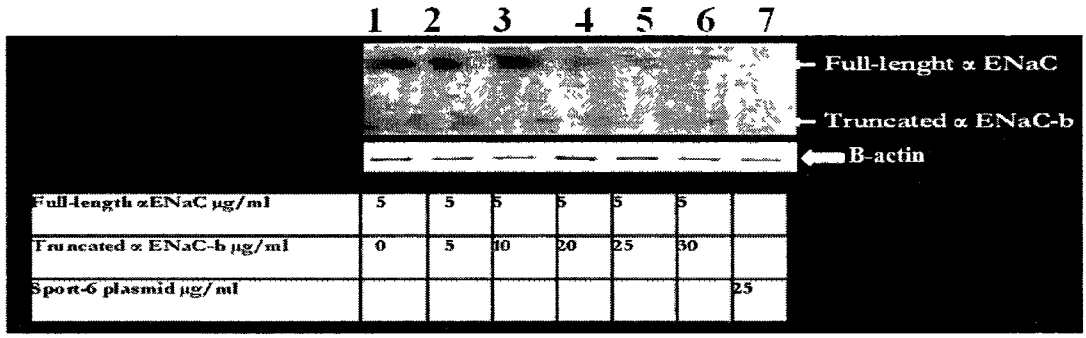
Appendix Q: Co-expression of full-length α ENaC and truncated αENaC-b in cotransfection experiments- Figure 4.3 Cont'd



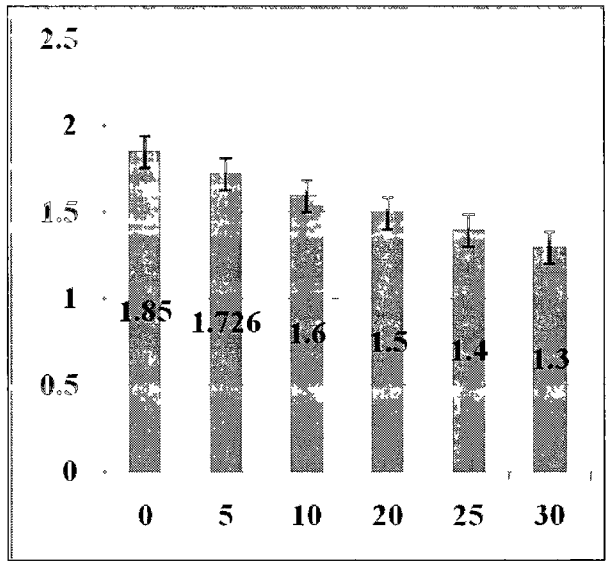
Analysis of α ENaC-wt and α ENaC-b expression by native Western analysis. αENaC-wt and α ENaC-b interacts in vivo by the formation of a dimer of a molecular mass of 140 kDa. Lanes 1 and 2 represent proteins from COS7 cells co-expressing α ENaC-wt and α ENaC-b in equal proportions (1:1), lane 3 represents proteins from COS7 cells transfected with the empty PCMV-sport 6 vector. Bands are normalized against the house keeping gene β-actin and the experiment was done in triplicate (Figure 4.3 on page 112 - Cont'd with β-actin). *[Shehata et al., 8th Annual Clinical Pharmacology and Therapeutics Congress 2007, Amsterdam].*

Appendix R: Dose-dependent suppression in αENaCwt by increasing levels of αENaC b- Figure 4.4 on page 114 Cont'd

A.



B.



Dose-dependent suppression of α ENaC-wt by increasing doses of α ENaC-b. The *upper panel* represent proteins of COS7 cells co-transfected by α ENaC-wt and α ENaC-b (lanes 1-6) in the ratios provided. Denaturing Western analysis demonstrate a dose-dependent reduction in full-length α ENaC expression. Lane 7 represents proteins from cells transfected with the empty PCMV-sport 6 vector. A weak signal of α ENaC-b can be detected at 53 kDa, possibly indicating that dimerization of α ENaC-wt with α ENaC-b proteins degrades α ENaC-wt primarily and eventually degrades α ENaC-b. Bands are normalized against the house keeping gene β-actin and the experiment was done in triplicate. The *lower panel* shows the corresponding densitometry analysis of the results using ImageJ method ®. Protein expression was quantified by measuring the optical density of the bands at medium exposure of X-ray film and then dividing the values by the corresponding β-actin values. The values represent the mean of 3 independent experiments. Using one-way ANOVA, a statistically significant difference was found between α ENaC -wt expression when increasing doses of α ENaC-b were co-transfected ($P=0.02$, $P<0.05$).

Appendix S: QRT-PCR analysis of α ENaC wt and alternatively spliced forms a, and b

Variant ID	Size	Amino acids	Dahl S (normal salt diet)	Dahl S (high salt diet)	Dahl R (normal salt diet)	Dahl R (high salt diet)
α ENaC wt	2094 bp	699	17.00×10^{-2} +/- 0.02	16.00×10^{-2}+/-0.02	24.00×10^{-2}+/-0.03	29.00×10^{-2}+/-0.07
α ENaC a	1497 bp	499	3.85×10^{-2}+/-0.09	4.02×10^{-2}+/-0.08	9.00×10^{-2}+/-0.09	9.50×10^{-2}+/-0.08
α ENaC b	1446 bp	482	5.20+/-1	5.70+/- 1	7.00+/-1	10.20+/-1.20

Relative amounts of cDNA as determined by quantitative real time polymerase chain reaction (PCR) for α ENaC wt and alternatively spliced forms a, and b normalized against the 3-phosphoglycerate kinase (Pgk) housekeeping gene that is constitutively expressed in rat tissues. The above table represents the results of 7 independent experiments. We can conclude the following:

a) α ENaC-a is a low abundance transcript (~23%- 37% lower than full-length α ENaC).
b) α ENaC-b mRNA levels are ~32±3-fold higher than full-length α ENaC in kidneys Dahl rats.
c) α ENaC-b mRNA concentrations are significantly higher in Dahl R *versus* S rats kidneys on regular salt diet,
d) α ENaC-b is a salt-sensitive transcript, because high *versus* normal salt diet caused a large increase in α ENaC b mRNA levels ($P<0.05$) in Dahl R rats.

Appendix T

The Epithelial Sodium Channel α subunit (α ENaC) Alternatively Spliced Form "b" in Dahl rats: What's next?

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Published manuscript

Int Arch Med. 2010 Jul 6;3:14.

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Abstract

Background: The amiloride-sensitive Epithelial Sodium Channel (ENaC) is critical in maintaining Na^+ balance, extracellular fluid volume and long term blood pressure control. ENaC is composed of three main subunits α, β, & γ. While α ENaC is critical for channel functionality, β & γ ENaC maximize channel function. To date, there are four alternatively spliced forms of the α subunit of ENaC (α ENaC-a, -b, -c, & -d) that have been published in rats, in addition to the major α ENaC transcript. While α ENaC-a, -c & -d transcripts are low abundance transcripts compared to full-length α ENaC, α ENaC-b is a higher abundance and salt-sensitive transcript compared to full-length α ENaC. **Presentation of the hypothesis:** α ENaC-b protein, which is preferentially produced in Dahl R rats, to a greater extent on high salt diet, exerts a dominant negative effect on full-length α ENaC subunit by physically binding to and trapping full-length α ENaC subunit in the endoplasmic reticulum, and finally accelerating full-length α ENaC proteolytic degradation in a dose-dependent manner. **Testing the hypothesis:** 1) To examine the mRNA and protein abundance of α ENaC-b relative to α ENaC full-length in kidney, lung, and taste tissues of Dahl rats. 2) To compare the expression (mRNA and protein) of α ENaC-b in kidneys of Dahl S and R rats on regular and high salt diet. 3) To examine the putative binding of α ENaC-b proteins to full-length α ENaC in vitro and to determine the impact of such binding on full-length α ENaC expression in vitro. **Implications of the hypothesis:** Our studies will be the first to demonstrate the over-expression of salt-sensitive α ENaC-b spliced form in kidney tissues of Dahl R rats at the expense of full-length α ENaC. The current proposal will provide highly novel insights into the putative mechanisms leading to ENaC hypoactivity in high-salt-fed Dahl R rats. Finally, findings from the present proposal will uncover a new mechanism by which alternative splicing may control the regulation of ENaC expression/function.

Background

The amiloride-sensitive Epithelial Sodium Channel (ENaC) constitutes the major route for transporting the sodium ion (Na^+) into the cell and hence, is critical in the maintenance of Na^+ balance, extracellular fluid volume and long term blood pressure control. ENaC is primarily composed of three subunits denoted by α, β, & γ. α ENaC subunit is critical for channel functionality, while β & γ subunits serve to maximize channel functionality. As such, being the most critical subunit in channel functionality, α ENaC is the focus of the present hypothesis. To date, there are four alternatively spliced forms of the α subunit of ENaC (α ENaC-a, -b, -c & -d) that have been published in rats, in addition to the major α ENaC transcript [1, 2]. α ENaC-a, -c & -d transcript are low abundance transcripts compared to full-length α ENaC and have been defined in terms of expression. Additionally, α ENaC-a has demonstrated non-functionality in oocytes as well as binding to ENaC blocker [1]. On the other hand, α ENaC-b is yet to be fully characterized, but appears to play a role in modulating ENaC, for the following reasons: a) α ENaC-b mRNA expression is significantly higher in Dahl salt-resistant (R) rats (with suppressed Na^+ transport related to ENaC [3, 4]) *versus* Dahl salt-sensitive (S) rats [2] (with enhanced Na^+ transport related to ENaC [3, 4]); b) α ENaC-b is a salt-sensitive transcript, because high *versus* normal salt diet caused a large increase in α ENaC b mRNA ($P<0.05$) levels in Dahl R rats [2]; c) unlike α ENaC-a, -c &-d, α ENaC-b mRNA levels are ~32±3-fold higher than full-length α ENaC; and finally d) the splice site generating α ENaC-b is highly conserved across species. As such, our results demonstrate a clear shift from constitutive to alternative splicing of α ENaC pre-mRNA in favor of α ENaC-b formation, indicating an increased stability and/or preferential synthesis of the latter.

These facts, combined with the realization that large structural defects in an encoded

protein (such as α ENaC-b) are usually associated with considerable effects on protein function that may extend to a drastic switch-off effect [5], in turn, emphasize the need for investment in basic research with a view to identifying novel targets for diagnosis, prevention and treatment of disorders related to ENaC dysfunction. The overall objective of these studies is to continue to examine the differential expression of α ENaC-b relative to full-length α ENaC in an array of tissues where ENaC plays a significant role. Subsequently, we will investigate whether α ENaC-b suppresses α ENaC expression/activity by binding to and/or accelerating proteolytic degradation of the latter in Dahl R rats (a model of suppressed ENaC). We will pursue the following three specific aims:

A. **What is the comparative expression profile of α ENaC-b in kidney, lung and taste tissues of Dahl S and R rats fed regular salt diet?** We will continue to define the existence and later examine the mRNA and protein expression profiles of α ENaC-b compared to full-length α ENaC in Dahl rat kidney (already reported in our previous contribution [2]), lung and taste tissues where ENaC plays a significant role. Cell-specific expression of α ENaC-b might allow particular cell types to individually regulate ENaC expression/function.

B. **Is α ENaC-b over-expressed in response to salt loading in kidneys of Dahl R rats with suppressed ENaC activity compared to Dahl S rats with overly active ENaC?** We will narrow our studies to studying the impact of high dietary sodium on α ENaC-b expression in Dahl rat kidney tissues. We will continue our QRT-PCR experiments that demonstrated an enhanced mRNA[2] and will follow by Western analysis to examine protein expression of α ENaC-b in kidneys of Dahl R compared to S rats on high *versus* regular salt diet. We will then proceed to quantify α ENaC-b expression relative to full-length α ENaC in Dahl R *versus* S rats. These studies will show whether suppressed full-length α ENaC expression in

Dahl R rats is consistent with an enhanced α ENaC-b mRNA and protein levels.

C. **Does α ENaC-b protein interact with and/or directly bind to full-length α ENaC and subsequently alter full-length α ENaC expression**? We will proceed to examine if α ENaC-b protein sequesters full-length α ENaC (by co-immunoprecipitation assays). The impact of α ENaC-b on full-length α ENaC expression will be elucidated (dose-dependent co-expression experiments) in cellular models (as recently reported in our studies [6]. These studies will demonstrate the mechanism by which α ENaC-b contributes to a suppressed overall Na^+ transport related to ENaC in Dahl R rats on high Na^+ (e.g., by enhanced binding of α ENaC-b to full-length α ENaC, followed by proteolytic degradation of the latter).

Presentation of the hypothesis

General Hypothesis:

α ENaC-b protein, which is preferentially produced in Dahl R rats, to a greater extent on high salt diet, exerts a dominant negative effect on full-length α ENaC subunit by physically binding to and trapping full-length α ENaC subunit in the endoplasmic reticulum, and finally accelerating full-length α ENaC proteolytic degradation in a dose-dependent manner.

Specific Hypotheses:

- α ENaC-b is differentially expressed in the kidneys, lungs and taste tissues of Dahl rats to individually regulate ENaC expression/function in these tissues.
- At the expense of full-length α ENaC expression levels, α ENaC-b expression (mRNA and protein) is enhanced in response to salt, in kidneys of Dahl R, but not S rats to suppress α ENaC expression/activity.
- α ENaC-b proteins trap full-length α ENaC in the endoplasmic reticulum and enhance

full-length α ENaC proteolytic degradation in a dose-dependent manner and hence serve as dominant negatives on α ENaC expression/activity.

Objectives:

1. To examine the abundance of α ENaC-b relative to α ENaC full-length in kidney, lung, and taste tissues of Dahl rats.
2. To compare the expression (mRNA and protein) of α ENaC-b in kidneys of Dahl S and R rats on regular and high salt diet.
3. To examine the putative binding of α ENaC-b proteins to full-length α ENaC in vitro and to determine the impact of such binding on full-length α ENaC expression in vitro.

Rationale:

Owing to the established body of evidence pointing out the crucial role of alternatively spliced forms (particularly those lacking important functional domains) as dominant negative variants on full-length proteins [7-9], we chose to examine the putative role of alternatively spliced form α ENaC-b in ENaC regulation for the following **six** reasons: a) α ENaC-b mRNA levels are ~32±3-fold higher than full-length α ENaC in kidneys of Dahl rats [2], indicating increased stability and/or synthesis of the former, b) α ENaC-b mRNA concentrations are significantly higher in Dahl R *versus* S rat kidneys on regular salt diet [2], c) At the expense of full-length α ENaC protein, Dahl R rats kidney tissues may demonstrate enhanced expression of α ENaC-b compared to the full-length α ENaC; d) α ENaC-b is a salt-sensitive transcript, because high *versus* normal salt diet caused a large increase in α ENaC-b mRNA levels ($P<0.05$) and protein levels in Dahl R rats[2][2,

10], e) the splice site used to generate α ENaC-b is conserved in humans, f) α ENaC-b is translatable [2, 6] (Translate tool ® http://ca.expasy.org/tools/dna.html). Consistent with the putative role of α ENaC-b in ENaC dysregulation, our previously reported results are consistent with a dominant negative effect imposed by α ENaC-b on full-length α ENaC [6, 11]. Dahl R and S rats serve as attractive models to study the contribution of α ENaC-b on differential ENaC expression and activity. This is because on high salt diet, Dahl R, but not S rat kidneys have a suppressed Na^+ transport related to ENaC [3, 4]. Our findings could provide novel targets for regulating α ENaC congener levels by manipulating the expression of the alternatively spliced form-b or by expressing an exogenous α ENaC-b.

Our current proposal examines three potential mechanisms by which α ENaC-b may regulate the renal full-length α ENaC by a dominant negative effect. The **first mechanism** is through suppressing α ENaC **transcription** via a shift from constitutive to alternative splicing of α ENaC pre-mRNA in favor of α ENaC-b formation. The **second mechanism** is through suppressing **translation** of full-length α ENaC more prominently in Dahl R ***versus*** S rats. The **third mechanism** is via an enhanced binding of α ENaC-b protein to the full-length α ENaC and accelerated degradation of the latter as a result of direct binding to α ENaC-b, a phenomenon that has been reported previously in several other channels and membrane proteins [7-9, 12-14]. Enhanced binding of α ENaC-b to the full-length α ENaC might trap α ENaC in the endoplasmic reticulum and/or inhibit proper ENaC assembly and trafficking to the plasma membrane, resulting in the formation of non functional channels. On the other hand, suppressing α ENaC full-length protein expression will, in turn, disturb the proportions of the ENaC αβγ proteins to be translocated to the plasma membrane and ultimately hinder overall channel cell surface expression and activity.

Knowledge of the mechanism by which α ENaC-b regulates full-length α ENaC and possibly reduces expression/activity of ENaC in Dahl R rats and the subsequent genesis of salt-dependent hypertension [a disease that comprises a large subgroup (over 50%) of Canadian adults] would enhance the understanding of the basic regulation of ENaC and the pathophysiology of ENaC-associated disorders such as salt-sensitive hypertension. It may also create one or more specific targets for the development of novel anti-hypertensive drug or gene therapy.

Testing the hypothesis:

Animal models

Dahl S rats are used as a genetic model for enhanced ENaC activity. ENaC activity is twice in Dahl S kidney *versus* Dahl R kidney [3, 4]. In contrast, Dahl R rats are the primary control strain with suppressed ENaC function. Usually, 8-10 rats per group are sufficient for statistical significance. Our research plan can be summarized as follows:

- We plan to examine the expression (mRNA and protein) of α ENaC-b relative to α ENaC wt in lung, kidney and taste tissues of Dahl S and R rats.
- We will subsequently proceed to investigate the effect of salt loading on the expression (mRNA and protein) of α ENaC-b in kidneys of Dahl rats. We already published the mRNA levels of α ENaC-b in kidney cortex of Dahl S *versus* R rats [2]. We will continue by studying the mRNA levels and protein levels in kidney medulla.
- Finally, we will examine α ENaC-b heterodimerization with full-length α ENaC, and the impact of such heterodimerization on full-length α ENaC expression (using COS7 cells, antibodies, co-immunoprecipitation assays and dose-dependent expression studies [6]).

Experiments will be performed on male Dahl S and R rats (n=24), 3-4 weeks of age,

obtained from Harlan Sprague Dawley (Indianapolis, IN) and handled as previously described [2, 6, 15, 16]. The rats will be divided into 4 groups (6 rats/group) by placing them on either regular (normal) (120μmol Na^+/g) or high-salt (8% NaCl or 1,370 μmol Na^+/g, Teklad; Madison, WI) diet for four weeks. After 4 wks, blood pressure (BP) will be measured invasively by intra-arterial catheter and the average mean arterial pressure will be estimated. The animals will then killed by decapitation and kidney, lung and tongue tissues will be removed and placed in cold methylbutane and then on dry ice. Tissues will be preserved at -80 °C for later protein and RNA isolation. All experiments will be carried out in accordance with the guidelines of the University of Ottawa Animal Care Committee for the care and use of laboratory animals.

Specific Methodology:

Aim 1: What is the expression profile of α ENaC-b in kidney, lung and taste tissues of Dahl S and R rats? To determine the mRNA and protein expression levels of α ENaC-b *versus* full-length α ENaC, we will isolate protein and total RNA from kidney, lung and taste tissues. Then we will proceed by performing western analyses and QRT-PCR to examine and later quantify α ENaC proteins and mRNAs. Detailed protocols are as follows:

Protein and RNA isolation

Whole kidney, lung and tongue tissues are homogenized with polytron. Total protein and total RNA are isolated using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Isolated RNA is subjected to DNAse treatment for removing potential genomic DNA contamination using DNase treatment kit (Ambion). The availability of an antiserum specific for α ENaC proteins (directed against the N-terminus amino acid residues 46-68 of the rat α ENaC) will allow us to probe for these proteins in kidney, lung

and tongue cell homogenates.

Reverse transcription

2 μg mRNA is reverse transcribed to first strand cDNA by superscript II RNase H- Reverse transcriptase (Invitrogen, CA, USA). To examine the co-existence of α ENaC wt and -b form, we will utilize wt primers that are common between α ENaC wt and -b form (no C-terminus sequence is shared among the two forms together). For QRT-PCR, we will use primers that are designed specifically to amplify the α ENaC -b form by including the nucleotide deletions (79 bp in α ENaC-b reverse primer). For α ENaC wt sequence, we will use the GeneBank accession number NM_031548, and for α ENaC -b, we will use the sequences previously reported by us [2].

Cloning

Full-length α ENaC and alternatively spliced form -b are cloned using DH5 α competent cells, and TOPO TA cloning kit ® (Invitrogen, ON, Canada). The resulting clones are sequenced using ABI prism 3100 (PE Applied Biosystems, Foster City, CA). Sequencing is performed using the DYEnamic ET Terminator kit according to the instructions provided by the manufacturer (PE Applied Biosystems, Foster City, CA). Sequencing products are purified (DyeEx 2.0 spin kit columns; Qiagen Canada, Mississauga, ON, Canada).

Quantitative real-time PCR

cDNAs are amplified using the previously described primers for wt α ENaC and α ENaC-b form. The relative amount of full-length α ENaC and alternatively spliced forms mRNA are measured by quantitative real time RT-PCR using Roche light cycler and Fast Start DNA master SYBR Green I dye. Known plasmid concentration is used as a calibrator and water is used as negative control for each reaction. Experiments are done in triplicates for each form (full-length and –b form). Melting curve analysis is performed for each reaction to assess

product specificity. Products are visualized on gel using gel electrophoresis assay to confirm the correct product sizes. Normalization is done using 3-phosphoglycerate-kinase (PGK) as a housekeeping gene.

Electrphoresis Analysis (Western Blot Analyses)

Concentrations for whole kidney, lung and tongue proteins (and proteins harvested from cultured cells under **aim 3**) are measured by Bradford. Proteins are then separated by SDS-PAGE resolving gels containing 10 % acrylamide, and 4 % of the same solution for stacking gels (NuPAGE Bis-Tris gels, Invitrogen). Briefly, 20 μg of proteins are mixed with 100 μl 2X SDS-PAGE sample buffer containing 0.1% SDS (sodium dodecyl-sulphate (w:v)) and supplemented with β- mercaptoethanol. Before loading, SDS samples are heated at 100 °C for 5 minutes. Samples are then transferred onto a nitrocellulose membrane (Invitrogen) and incubated with primary (directed against α ENaC N-terminus) at a 1:1000 dilution. Blots are washed thrice for 5 minutes each with PBST. For standard Western blotting detection, blots are incubated with either an anti-rabbit HRP-conjugated antibody (1:10,000, Amersham), for 1 h at 25 °C. For improved Western blotting detection to avoid HC and LC signals, blots are incubated (1 h at 25 °C) with a 1:5,000 dilution of Protein A-HRP (Amersham) or a 1:10,000 dilution of Protein G-HRP (Upstate Biotech.), prepared in blocking buffer. After washing three times at 25 °C with PBST (5 min each), blots are developed with ECL Plus (Amersham). The marker used is high range Biorad ladder catalogue 161-0309. Corresponding protein bands for α ENaC-b will be later sequenced for confirming the protein sequence of α ENaC-b. We will be using Cambridge Peptides ® for sequencing α ENaC proteins (http://www.cambridgepeptides.com/index.html).

Aim 2: Is α ENaC -b over-expressed in Dahl R rats with suppressed ENaC activity

compared to Dahl S rats with overly active ENaC in response to salt loading?

Protein, total RNA extraction, QRT-PCR and western blot analyses will be performed as described under Aim 1. Dahl rats fed either regular or high salt diet will be examined for α ENaC-b expression *versus* full-length α ENaC in kidney tissues.

Statistical Analysis

Differences between the expression levels of full-length α ENaC and alternatively spliced form -b will be analyzed in Dahl S *versus* R rats on normal and high salt diet using two way ANOVA (SigmaSTAT ®). The statistical test is two sided to identify strain and dietary differences, the data will be expressed as means and ranges, and differences of P values of less than 0.05 will be considered as significant.

Aim 3: Does α ENaC-b protein directly bind to full-length α ENaC and subsequently alter full-length α ENaC expression?

RNA extraction, RT-PCR and PCR

To amplify full-length α ENaC and α ENaC-b for expression studies, specific primers will be designed to flank the open reading frames of full-length α ENaC and b forms. High fidelity Expand Long Range, dNTPack (Roche Applied Science®, Quebec, Canada) will be employed to amplify full-length α ENaC wt and -b spliced form. Touchdown PCR method will be employed to improve the efficiency of gene amplification as follows (start Tm at 68°C and the annealing temp reduced 2°C per cycle for the next cycles up to Tm=58, then the remaining cycles at a 58°C annealing temperature). After amplification, the specific fragments of full-length α ENaC and α ENaC-b will be visualized in 1% agarose gels.

Plasmids and Generation of Constructs

PCR products for α ENaC wt and b form will be ligated with with pCR®2.1-TOPO® vector

using the TOPO TA® cloning kit (Invitrogen, CA, USA), with blue/white screening of DH5α competent cells on ampicillin selective plates. Positive clones will be cultured in LB broth. DNA extracted from these cultures using Qiagen DNA miniprep kit, will be sequenced using the M13 universal forward and reverse primers, in addition to embedded primers within α ENaC wt and b forms to verify their full-lengths. Sequencing will be performed using the DYEnamic ET Terminator kit according to the instructions provided by the manufacturer (PE Applied Biosystems, Foster City, CA). The sequence of the cloned α ENaC-b form is compared to the one previously reported [1], and the sequence of the full-length α ENaC is compared to the one previously reported for Dahl rats [17].
Ligated pCR®2.1-TOPO® - α ENaC wt clone will be digested using EcoR1, while that of α ENaC-b with EcoRV and HindIII and then subcloned overnight at 16 °C into PCMV-sport6 vector (Invitrogen, CA, USA) downstream the T7 promotor using the EcoRI site for α ENaC wt and the EcoRV and HindIII sites for α ENaC-b.

Cell culture and transfection

COS-African green monkey kidney cells will be maintained in culture at 37 °C/5% CO2 using Dulbecco's Modified Eagle's Medium (DMEM, Hyclone ® Thermo Fisher Scientific Laboratories, Logan, UT) supplemented with glutamine and 10% heat-inactivated foetal bovine serum (FBS, PAA Laboratories). Transfections will be performed using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and up to 50 μg DNA per 10 cm plate. Immediately before transfection the culture medium will be replaced with 5 ml of DMEM supplemented with 10% (v/v) fetal bovine serum. PCMV-sport6 /α ENaC wt and b forms will be transfected into COS-7 cells separately at a concentration of 24 μg DNA /plate, as well as co-transfected together in a 1:1 ratio of α ENaC wt: α ENaC-b. For dose-dependent expression experiments, α ENaC and α ENaC-b form will be transfected into

COS-7 cells separately at a concentration of 24 μg DNA /plate, as well as co-transfected together in a dose dependant manner as follows (full-length α ENaC: α ENaC-b is (8: 1 μg, 8:3 μg, 8:6 μg, 8: 12 μg, 8: 18 μg, 8: 24 μg, 8: 30 μg, 8: 42 μg). The empty PCMV-sport6 vector at a concentration of 24 μg DNA /plate will be transfected as a control. Then the cells will be harvested for >2 h at 4°C in RIPA lysis buffer (10 mM $NaPO_4$, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, and 0.1% SDS, pH 7.2) supplemented with the protease inhibitor cocktail (Sigma, St Louis, MO). Proteins will be handled and separated by electrophoresis as described under Aim 1.

Co-immunoprecipitation Assay

Co-immunoprecipitation (Co-IP) will be employed to identify interaction between α ENaC-b with full-length α ENaC. Co-IP is conducted in essentially the same manner as a western blot (see aims 1 and 2 above). However, in a co-IP, α ENaC target antigen precipitated by the antibody against α ENaC "co-precipitates" a binding α ENaC-b complex from a lysate, i.e., the interacting protein is bound to the target antigen, which becomes bound by the antibody that becomes captured on a Protein A or G gel support (Pierce kit ®).

Implications of the hypothesis

Depending on the results obtained from the proposed studies, we will proceed to examine the sub-cellular localization of α ENaC-b (if localized in the cytosolic and/or particulate fractions) using immunohistochemistry and the same antibodies used in the present proposal. Then, we will again employ immunohistochemistry to investigate if the co-localization of α ENaC-b with α ENaC wt is perinuclear (such as in endoplasmic reticulum) or closer to the plasma membrane. Results from these studies will demonstrate if heteromerization of α ENaC-b with α ENaC wt traps the latter in the endoplasmic reticulum,

or it still allows α ENaC wt migration to the plasma membrane. Then, it would be interesting to examine the impact of α ENaC-b on ENaC cell surface expression. We will use the same procedure employed by Firsov and colleagues in [18], where α ENaC-b together with, β, and γ rat ENaC subunits will be tagged with the FLAG reporter octapeptide (DYKDDDDK) that is recognized by the anti-FLAG M_2 (M_2Ab) mouse monoclonal antibody (Kodak). Then, expression in Xenopus oocytes and binding assays (immunoprecipitation) will follow. Results from this set of experiments will reveal if α ENaC-b suppresses ENaC cell surface expression.

To this end, we will proceed and assess the overexpression of α ENaC-b in Dahl S rats where α ENaC-b expression is suppressed compared to Dahl R rats. A replication-defective adenovirus (AV) will be used as the vector for delivery of over-expression sequences, as previously described [19]. Injecting AV in the CNS maintains the high levels of AV for at least 28 days post i.c.v. because of a diminished immune response in the CNS *versus* the periphery. There are at least two areas of potential clinical importance for these studies. First, identification of spliced forms that impair the hyperactivity of ENaC to sodium in the kidney of Dahl S rats would identify specific targets for the development of novel antihypertensive drug therapy for salt-dependent hypertension. Second, as gene therapy delivery systems continue to evolve, we will be examining in vivo delivery of adenoviruses carrying α ENaC-b and then assessing the resultant blood pressure effects. This may eventually gain relevance as a possible "proof-of-concept" gene therapy.

Conclusion

Our studies will be the first to demonstrate the over-expression of salt-sensitive α ENaC-b spliced form in kidney tissues of Dahl R rats at the expense of full-length α ENaC. The

current proposal will provide highly novel insights into the putative mechanisms leading to ENaC hypoactivity in high-salt-fed Dahl R rats. Finally, findings from the present proposal will uncover a new mechanism by which alternative splicing may control the regulation of ENaC expression/function.

Competing interests

The author declares no competing interests.

Author's contributions

The author conceptualized the hypothesis, wrote and reviewed the manuscript.

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