Enac and hypertension

Science, Biology

Profiling Epithelial Sodium Channel (ENaC) Expression in the Human Kidney, a Possible Marker for Hypertension 0 Introduction 1 Hypertension Hypertension is the abnormal and repeated elevation of blood pressure with systolic pressure above 140 mmHg and diastolic pressure above 90 mmHg [1]. It is termed “ silent killer” because it has no warning signs or symptoms, and numerous individuals are not aware they have it [2]. Hypertension is one of the primary risks for a number of conditions that include stroke, heart attack, and kidney failure. One of the special prevalence of hypertension experienced in both males and females is an increase in blood pressure with the advancement of age. Males younger than 45 years old are more likely to have hypertension than females. However, for individuals past the age of 45, the incidences of hypertension rise and affect more women than men [1]. In the U. S. 25% of Americans suffer from hypertension. High blood pressure varies amongst ethnic groups where African Americans have the highest risk for hypertension among Americans. Forty percent of African Americans have hypertension and it is more likely to be manifested at an earlier age than Caucasians. Hypertension is the primary cause of 25% of deaths in America today. Additionally, 47% of individuals with hypertension have blood pressure that is not under control. Most of hypertensives are resistant to common medications. They have to take more than one medication to render their blood pressure stable[3]. The pathogenesis of hypertension remains unknown. Blood pressure must be regulated to prevent damaging of vital organs. From this point, studying the molecular basis and human protein level of this disease is needed for early detection and for distinguishing markers and designing targeting therapy.

The kidneys play a major role in hypertension mainly in the maintenance of sodium balance within the body. They also maintain the balance of the volume of extracellular fluid that leads to the long term control of blood pressure. Therefore, any imbalance in sodium entry expands the blood volume leading to hormonal and vascular change in the kidneys and increasing blood pressure [2].

1. 2 Categories of Hypertension

In relation to the various mechanisms involved in hypertension's inheritance, two major categories exist. These are Mendelian hypertension and essential hypertension. The Mendelian form of hypertension occurs because of a single or small number of genes which are defective. These can also be either dominant or recessive [4]. In contrast, essential hypertension, which is a heterogeneous disorder of hypertension, is more complex and does not follow the kind of inheritance found in Mendelian genetics. This describes 95% of hypertensive individuals. Essential hypertension has a prevalence rate that is 3. 8 times higher in people who have a positive history of hypertension in their families [5]. A study of twins has shown that the correlation of high blood pressure is more common among homozygotic twins than the dizygotic twin [6, 7]. However, there is still a sufficiently larger variation in hypertension in monozygotic twins because of the complex genetic heritability and interaction with epigenetic factors. Some of the commonly known epigenetic factors include stress, obesity, insulin resistance, and different diets that affect the variation of blood pressure among individuals [8]. There is also a differential response of blood pressure with regard to the dietary intake of salt. Individuals termed " salt sensitive"

express their response to elevated sodium intake with an elevation in blood pressure and manifest a declined in blood pressure with the restriction of salt. Almost 53% of white hypertensives are salt sensitive, and this proportion rises to 73% among African Americans [9].

1. 3 Role of the Kidney in the Control of Hypertension

The kidneys play a key role in the long term control of blood pressure by regulating the reabsorption of electrolytes which create an osmotic gradient for the reabsorption of water leading to the maintenance of fluid balance. Dysregulation of kidney function has been associated with effects on blood pressure [10, 11]. The total fraction of filtered sodium reabsorbed determines the volume of the extracellular fluid, as well as blood pressure. Blood pressure can be influenced by many systems on a short term basis, but apart from vascular effects, the long term regulation of blood pressure is ultimately dependent on the manner by which renal sodium is handled [10]. The moving of sodium across the epithelia of the nephron is a transport mediated process and the main elements responsible for the reabsorption of sodium and the balance of fluids within the kidney are the sodium transporters and channels. The reabsorption of sodium is localized and specific within different segments of the nephron [12]. In many instances the apical membrane channel or transporter is rate limiting to transepithelial sodium absorption. The majority of reabsorption occurs in the proximal tubules. It utilizes various Na dependent co-transporters and exchangers. Sodium is reabsorbed in the thick ascending limb by Na/K/Cl transporter and in the distal convoluted tubules by Na/Cl co transporter. The collecting duct represents the final regulated renal pathway for sodium absorption and

control of body Na. In this segment activity is limited by the action of the apical epithelial sodium channel (ENaC) and is also regulated by aldosterone hormone [13].

Aldosterone is a steroid hormone having a chief role in regulating blood pressure. The production of the hormone occurs at the zona glomerulosa, which is the external section of the adrenal cortex situated in the adrenal gland. The main function of this hormone includes it is ability to increases the reabsorption of Na ions in the kidney. In turn, conservation of sodium leads to increased water retention, the secretion of potassium and an increase in blood pressure. Hyperactivity of aldosterone secretion is referred to as primary aldosteronism and the result of this process is the occurrence of arterial hypertension [14]. Aldosterone acts synergistically with antidiuretic hormone or ADH; and this hormone which is also known as arginine vasopressin acts by preventing the loss of water from the kidney. This transpires via an increase of aquaporin2 water channels in the apical membrane of the collecting ducts. ADH is stimulated when hypovolemia leads to decrease pressure. The kidney then conserves water leading to concentrated urine and reduced volume. At high concentration, ADH also increases blood pressure [15].

Over the past 20 years the regulation and the role of ENaC dysfunction in the maintenance and causation of high blood pressure has been examined and verified. The molecular organization and some of the regulation of this channel is discussed in the next section [16].

1. 4 The Activities of the Epithelial Sodium Channel within the Kidney

The epithelial sodium channel (ENaC) rate limits the transepithelial

absorption of sodium within the collecting duct of the kidney that serve a crucial role in the general control of blood pressure, and blood volume [13]. However, the subtle dysregulation of ENaC may also be significant in essential hypertension, which is one of the principle causes of mortality and morbidity owing to various cardiovascular related disorders. The epithelial sodium channel, which is sensitive to amiloride, consists of three subunits: α, β, γ. In addition there is a fourth subunit, so called the subunit. Each ENaC subunit has more than one isoform. and subunits have two isoforms: short alpha 669 and long alpha 728 for the α subunit [17]; and long delta and short delta for the subunit [18]. ENaC subunits have a sequence homology that is approximately 37% at the amino acid level. Each ENaC subunit has two transmembrane helices, a large extracellular loop, and short amino and carboxyl termini in the cytosol. However, studies are still determining the composition of the channel and the exact relationship that exists amongst the subunits. The α subunit is pore forming and has been found to be located predominantly in the kidney, colon, and lung epithelia where it is known to form channels that are functional with β and γ subunits. It controls the balance of Na transport in these epithelia and indirectly blood volume and pressure. Similarly, the δ subunit is also considered a pore forming subunit. When it is expressed together with the β and γ subunits, it produces Na currents that are also sensitive to amiloride. Reports also indicate that the δ subunit forms a current that is constitutive, has greater magnitude when combined with the β and γ subunits that the homomeric δ ENaC alone [19]. The expression of α and γ subunits produce a slight increase in the current magnitude as

compared to alpha alone [20].

Multiple Proteases are known to be associated with activation and proteolytic processing of ENaC subunits. Proteases contribute to ENaC regulation by cleaving specific sites in the extracellular loops of the ✔, ■ and ❤ subunits [21]. This activates ENaC by changing its conformation. In addition, ENaC is also activated by soluble serine proteases [22]. Total ENaC protein pool is under intracellular proteolytic maturation by furin mediated cleavage in the Golgi complex. Furin cleaves the ✔ subunit at two sites and the ■ subunit at one site. Then these subunits form activated channels at the plasma membrane [23]. In contrast, trypsin and chymotrypsin are common proteases used as an experimental tool to study proteolytic activation [24]. It is undetermined whether they have a role in ENaC activation in vivo. In the absence of proteolysis, ENaC activity is markedly reduced and this is thought to be mediated by external Na, a process known as Na ion self-inhibition [25].

There are a number of views related to the structural and functional features of ENaC in relation to hypertension. First, particular emphasis on the mechanisms and processes that occur in the management of sodium channel action at the biochemical and cellular level exists. In addition, mutations have been clinically identified that can affect the activity of ENaC and lead to changes in blood pressure [13].

One of the major ways to understand the genetics of hypertension is the known linkage between ENaC and a rare form of heritable human hypertension referred to as Liddle’s Syndrome [26]. It is an uncommon genetic disorder which is associated with increased activity of the sodium

channel. Mutations in and subunits are responsible for Liddle's Syndrome [27]. These mutations lead to increased channel density at the membrane and increased sodium absorption leading to hypertension [28]. This occurs through the disruption of the PY motif of the epithelial sodium channel. It is a mutation in a C terminal motif in the and subunits known as the PY motif (PXXY). This results in increased ENaC activity [29]. This motif is necessary for the binding of a ubiquitin ligase Nedd 4-2 that targets these subunits for internalization and degradation. This mutation therefore causes a gain of function due to decreased ENaC turnover leading to increased sodium reabsorption and subsequent volume expansion. It increases the expression of the ENaC subunits within the collecting duct. The main hormone responsible for sodium retention, aldosterone acts on the Nedd 4-2 pathway. Aldosterone stimulates Sgk and ENaC by mediating phosphorylation of serine residues on Nedd 4-2. Such phosphorylation decreases the interaction between Nedd4 and ENaC, leading to increases ENaC cell surface expression [30].

A number of discoveries have shown that the Nedd 4-2 is located in the collecting ducts of rats. It has also been established that Nedd 4-2 reduces the endogenous activity of ENaC if over expressed within a collecting cell line [31]. Also a genetic linkage exists examining this process and ENaC activity and hypertension [31]. It indicates that the β and γ subunits are closely linked to systolic blood pressure. Several polymorphisms in these subunits have been described. One which was found to elicit a great amount of interest is the β-T594M variant. It is the variant that is more common in African Americans [32]. On the other hand diseases causing hypotension

have been observed. One of these is 21 – Hydroxylase Deficiency which is caused by a mutation in the CYP21A2 gene which causes a decrease in 21-hydroxylase enzyme levels[33]. This enzyme is located in the adrenal gland and plays a major role in producing aldosterone. The lack of aldosterone production contributes to salt wasting and hypotension in these individuals [33]. Given the role of the channel, ENaC expression and regulation maybe a larger contributor to hypertension in humans than that predicated from identified mutations and polymorphisms. This among other reasons makes ENaC expression and regulation of special interest in the study of potential contributors to essential hypertension. The majority of work examining ENaC is limited to in vitro experiments or to animal models. Given differences to humans, it is may not appropriate to examine the regulation of this renal epithelial sodium channel in animal models [34]. Therefore, it is important to study ENaC expression and regulation in humans.

1. 5 Exosomes

One new method of recovering human proteins and DNA is exosome isolation. Exosomes are extracellular small vesicles within the size range of 30-100nm and are only seen under an electron microscope. They are found in most biological fluids, especially blood and urine [35, 36]. Exosomes are protected by a lipid bilayer and have transmembrane proteins as well as intravesicular soluble proteins, mRNAs and microRNAs. The composition of exosomes is based on the originating cell. Exosomes are released from the cell when multivesicular bodies (MVB) fuse with the plasma membrane or directly from the plasma membrane [37]. Exosomes released from MVB are regulated by vesicular and endosomal trafficking proteins, calcium and

ceramide [38]. Cellular response and different types of stresses affect the release of exosomes [39]. Exosomes play a major role in intracellular communication [40]. They can transfer proteins and mRNAs from one cell to another by endocytosis, making the vesicle a good source of genetic information in different cells. In the kidney, urinary exosomes are generated in all segments of the nephron [41]. Exosomes are also taken and influenced by epithelial cells in the collecting duct to modify their content. At the end, urinary exosomes are derived from renal epithelial cells. This process allows exosomes to play a major role in regulating the co-function among different segments of the nephron mediated by uptake and secretion of its content leading to effects on the recipient cell. Therefore, exosomes are a good source of protein biomarkers for kidney disease. They contain proteins from glomerular, tubular, prostate and bladder cells. Exosomes account for 3% of the total urinary protein from a normal person’s urine[41]. Therefore, Exosomes can have a key role in expressing the kidney’s status without needing a biopsy. In addition to protein, exosomes have nucleic acid. The quality of mRNA from exosomes is similar to kidney tissues and more stable than mRNA from whole urine because the membrane protects against RNAses and degradation [41]. Exosomes also contain microRNA that regulates mRNA production. Therefore, urinary exosomes are an ideal way to isolate mRNA to examine the expression of ENaC in the human kidney. There is no standard way to isolate exosomes from the urine, but a common method is ultracentrifugation [42]. However, there are some concerns during the exosome isolation process that can affect the purity and quantity of exosomes. It is important for example to eliminate other urine rich proteins

such as Tamm-Horsfall protein which can agglutinate and affect sample yield. This can be partially avoided by using fresh samples [43, 44] and by optimizing the processing protocol.

1. 6 The Aim of the Study

The aim of our research study was to characterize the expression of ENaC subunits using quantitative PCR (q-PCR). Differences in the expression of ENaC subunits between individuals or ethnic groups may underlie the etiology of hypertension or salt sensitivity. We are aware that over expression of one of these subunits will lead individuals to hypertension. In addition, we are able to study ❤ ENaC, which is expressed in human kidney and possess important regulatory functions. Most researches have demonstrated the expression of ENaC subunits in the human eye and nasal polyps by using q-PCR [45, 46]. However, there is a lack of investigation in the human kidney. Our study focuses on finding a possible marker for hypertension by examining the expression of ENaC subunits in the human kidney. To this end we examine the profile of ENaC subunit expression in human kidney as well as the changes of expression and especially the expression of the structural ✔ and ❤ subunits relevant to non-structural subunits. Further, we also characterize the profile of expression in a family with 21- Hydroxylase Deficiency which causes salt wasting owing to the absence of aldosterone expression.

2. 0 MATERIALS AND METHODS

2. 1 Sample collection

2. 1. 1 Epithelial cells from the cheek

When we started this study we were looking for a method to isolate epithelial

cells from humans without the need of a biopsy. We used a lengthwise cut micropipette tip to collect epithelial cells from the cheek. However, the resulting RNA was degraded and with low purity and with low yield and insufficient for q-PCR. We then focused on isolation from urine.

2. 1. 2 Exosomes from urine

In this study, we were looking for the expression of ENaC in the kidney. We decided to collect urine from individuals and isolate exosomes from it. The structure of exosomes saves their content from degradation. The quantity and quality of mRNA that we can isolate from exosomes is the best way to see the expression of ENaC in the kidney [47]. Up to 75 ml whole stream early morning urine samples were collected from two groups of consenting adults. The First group donors had no prior acute or chronic illnesses. The second group of donors was a family that has 21-hydroxylase deficiency. Each group collected their urine by urinating directly into sterile polypropylene centrifuge tubes. The samples were labeled appropriately and brought to the laboratory for processing within 30 minutes of collection. All the urine samples were cooled to 4°C to aggregate and eliminate Tamm Horsfall protein (THP).

2. 3 Isolation of exosomes

2. 3. 1 Initial protocols

There is no standard protocol to isolate exosomes from urine. We used different protocols in this study until we found an optimized one. Initially, we used the total exosomes isolation kit (Invitrogen, Carlsbad, CA). The protocol of this kit was based on using 1 volume of urine and 1 volume of the total exosomes kit followed by incubation for 1 hour at room temperature. After

incubation, the sample was centrifuged at 10, 000g for 1 hour at 4 °C. The supernatant was discarding without disturbing the pellet. We added 1X phosphate buffered saline (PBS) to the pellet followed by RNA extraction. However, there was no sufficient success from this protocol to allow it is continued use.

The second protocol used utilized filtering the urine from large debris and dead cells using a 0. 22 ¦M filter, followed by centrifugation in a 30 kDa ultraconcentrator (Corning, (Lowell, MA) for 30 min, followed by isolation of the supernatant for RNA processing. In addition, we tried microconcentrated urine followed by the ultracentrifugation method to isolate the exosomes. These protocols were also unsuccessful.

2. 3. 2 Optimized protocol

We resorted to optimizing the original centrifugation protocol historically used to isolate exosomes [47]. First, urine samples were centrifuged at 300g for 10 minutes to remove urinary sediment (whole cells, large membrane fragments and THP). The supernatants were then transferred to a new set of polyallomer tubes appropriate for the ultracentrifugation rotor that was used.

In order to ensure that none of the pellet was collected and contaminated the supernatant a pipette was used rather than pouring off the supernatant [48]. The supernatants were centrifuged again at 17, 000g for 20 minutes, and the pellets were discarded. The resultant supernatants were transferred to new set of polyallomer tubes, appropriate for the ultracentrifugation rotor that was used with the help of a pipette. The final supernatants were then ultracentrifuged for 90 minutes, at 200, 000g resulting in a pellet. It is

important to note that exosomes are contained in pellets, which in some cases may not be easily visible at the bottom of the tubes. With this knowledge, the pellets in each tube were resuspended by adding a small volume 1 ml 1x PBS using a micropipette. This was done by pipetting up and down the tube and by vortexing. The resuspended pellets were then stored at -20°C in microcentrifuge tubes until proceeding with the RNA purification. The general scheme of exosomes isolation by ultracentrifugation is illustrated in Figure1.

Figure1. Flowchart for the adopted exosome isolation procedure using differential centrifugation.

2. 4 Purification of RNA

2. 4. 1 Initial protocol

The integrity of purified RNA is critical to all techniques of gene expression analysis. Therefore, the preparation of intact pure RNA is critical because it is the first marker in gene quantification [49]. First, we used the Trizol (Invitrogen , Carlsbad, CA). protocol to extract RNA from exosomes. We added different volumes of Trizol with the exosomes pellets followed by addition of 4 µl glycogen to increase RNA yield during extraction. Then, mixed and incubated for 5 min at room temperature followed by adding chloroform and centrifugation for 15 min at 4oC. The aqueous phase was transferred to a new tube, followed by addition of isopropanol and centrifugation for 20 min at 4oC. The supernatant was discarding and the pellets washed with 70% ethanol and left to dry for 5 min. The pellet was resuspended in nuclease free water. This protocol proved unpredictable and

likely, some Trizol carryover affected various stages of cDNA synthesis and q-PCR enzyme efficiency. We abandoned this approach and resorted to established methods for RNA isolation.

2. 4. 2 Optimized protocol

The protocol used was adapted from Current Protocols in Molecular Biology [50]. From Each of the previously isolated resuspended exosomes we transferred 500 µl by use of sterile pipettes into separate microcentrifuge tube followed by addition of 1 µl of RQ1 DNase to digest any genomic DNA. This was followed by addition of 5 µl triton X-100 as detergent to breakdown the exosomes. The resultant solutions were mixed thoroughly by use of a sonicator for 30 seconds. After proper mixing, 50 µl of 2 M sodium acetate (pH 5. 2), 500 µl phenol (pH 4. 3) and 100 µl chloroform were added to the solutions. The resultant solutions were mixed thoroughly and incubated for 15 minutes at 4°C. After the fifteen minutes incubation period, the solutions were centrifuged for 20 minutes at 10, 000g at 4 °C. Using sterile pipettes, the aqueous phases of the solutions were transferred to a new set of sterile microcentrifuge tubes with sequential addition of one volume of isopropanol, and incubation for 30 minutes at -20°C. The solutions were again centrifuged for 10 minutes at 10, 000g at 4°C after which the supernatants were removed from the tubes leaving only the pellets. The pellets were washed with 70% ethanol. The RNA pellets were air-dried for 5 minutes and resuspended in 30 µl H2O. The solutions were incubated for 10 minutes at 60°C. The RNA was stored at -20°C ready for reverse transcription [48].

2. 5 Reverse-transcription reaction

In this study, q-PCR was used to quantify RNA purified from the exosomes

isolated from the urine samples. This required the production of complementary DNA (cDNA) copies of the RNA through reverse transcrioption. One µg RNA in nuclease-free water (variable volume) was obtained from each of the refrigerated samples by use of sterile filter micropipettes. The reaction components including 4 µl 5x iScript select reaction mix (Bio-Rad Foster City, CA). Two µl random primer, 1 µl iScript reverse transcriptase and nuclease free H2O were added to the solution to a final volume of 20 µl. The reaction components were then incubated at 42oC for 30 minutes. In order to inactivate the transcriptase, the components were heated at 85oC for 5 min. Of primary consideration, are the properties of the q-PCR assay, including, its dynamic quantification range, sensitivity, reproducibility and specificity [49]. This was tested as shown below using standardized solutions containing fixed copy number of genes of interest.

2. 6 Primers design

The primers were designed and sequences checked using the Primer-Blast tool at the National Center for Biotechnology Information (NCBI) (www. ncbi. nlm. nih. gov). Initial gradient q-PCR experiments were performed for each primer at temperatures between 60°C and 70°C. The best temperatures for the five primer pairs were picked based on the melt curve and reaction efficiency. The primer sequences are summarized in Table1. We designed different primers for ❤638, however they did not provide specific amplification from exosomes although all were capable of amplification and detection in solutions with purified fixed copies of detecting ❤638. Those are summarized in Table2.

Table 1. Sequences of Primers and Optimized Annealing Temperatures Used

in this Study

Gene

Accession Number

Sequences

(5’-3’)

Annealing Temperature

Amplicon Size (bp)

✔669

NM_001038. 5

F: CTCCCTCTGTCACGATGGTC

R: GCCATCTCCACCACAGACAA

64°C

94

✔⏮🗗⏭

NM_00159576. 1

F: TTGACCCTGACCCTTGCTCT

R: TGGACTGTGGAGGGCTAGA

66. 4°C

150

🚲

NM_000336. 2

F: ACTACCGGAACTTCACGTCCA

R: CTTCTCTGTCATGCCCCAGTT

64°C

77

■

NM_001039. 3

F: ACAACCCATTCCTCGTGTCC

R: GACTCTGTCAGGTGCATTCCT

68. 4°C

136

❤802

NM_001130413. 3

F: ACAGAAGACAACACCGCTCC

R: ATGACCAGGTGAGCCTCCTT

60°C

74

Table 2. Attempted Primers for ❤638

Gene

Accession Number

Sequences

(5’-3’)

Annealing Temperature

Ampilcon Size (bp)

❤638

EU_489064. 1

F1: GGACCGGGAGATCCGTCT

R1: CGTGCTGTTGCACAGTCTGAA

F2: TCAGCATCCGAGAGGACGA

R2: TGCCTGGTGTAGGAGGTGTT

F3: GCAGGGAGTCTGCATTCAAG

R3: AGGCTGCTCCTCTGTCTGTG

60°C

64°C

62°C

79

112

130

2. 6 Reaction set up

In order to set up and run a hot start q-PCR reaction, the protocol described in the IQ™ SYBR® Green Supermix instruction manual was followed. IQ™ SYBR® Green supermix, cDNA (template) and primers were thawed. The components of each tube were carefully mixed to ensure thorough resuspension prior to use. The components were added into each of the reaction tubes in the order shown in Table 3. To eliminate variability, a supermix containing a mixture of the components shown in table 3, except for cDNA, was utilized.

Table 3. Mixture Components Used to Set up a 20 µl Reaction

Components

Volume

IQ™ SYBR® Green supermix

Primers (500 nmole)

cDNA(10ng)

Nuclease-free water

10 µl

2 µl

2 µl

6 µl

Total reaction volume

20 µl

Reaction duplicates were loaded into 96 well PCR microplates and the reaction vessels were sealed. The thermal cycler was programmed with the recommended 2-step q-PCR protocol followed by a melt-curve assessment (Table 4). The sealed reaction vessels were placed in the thermal cycler block, and the PCR protocol was started. After the run, the q-PCR products were stored at 4oC.

Table 4. A Typical 2-Step q- PCR Protocol

Cycling step

Temperature

Hold time

Number of cycles

Initial denaturation and enzyme activation

95 oC

3 min

1

Denaturing

95 oC

10 sec

40

Annealing and extension

60 – 68. 4oC *

30 sec

40

Melt curve

65-95 oC

5 sec/point

1

*Based on primer temperature.

2. 7 Agarose gel electrophoresis

To futher assess the quality and size of the PCR products we used gel electrophoresis. Electrophoresis is a method of separating DNA based on the rate of movement while under an electric field. DNA is negatively charged and will move to the positive electrode and away from the negative electrode. DNA is then visualized by the use of a dye that binds to DNA. The q-PCR products were loaded onto 2. 5% agarose gel containing ethidium bromide and run with 1X TAE buffer for 30 min at 100 V. Imaging was performed using the Versa Doc imaging system (Bio-Rad Foster City, CA).

2. 8 Standard curve

In order to accurately obtain RNA copy number we used linearized plasmid controls for each ENaC subunit to allow the production of a standard curve. Based on the DNA sequences, we used BamHI (New England Biolabs, MA) to cut a single plasmid containing ENaC subunit cDNA. The reactions were incubated overnight at 37 oC. We made a serial dilution with known quantity from 106 copies to 102 copies. Most studies use reference genes to normalize their unknown genes and to calculate the copy numbers. However,

mRNA expression of genes varies separately between individuals rendering normalization to so-called house keeping genes variable among individuals. Further, the mRNA expression of most reference genes is higher than hENaC genes making normalization to a high expressing gene very variable. To avoid these complications we decided to normalize the results using an ENaC subunit as a reference gene. We chose ENaC owing to the low individual variability.

3. 0 Results

Using q-PCR we demonstrated that , , and ENaC subunits are expressed in human kidney. Further, 728 which is a long form of subunit, was also expressed. Analysis of the data was based on two groups of individuals. These samples included healthy and 21-Hydroxylase Deficiency individuals. We used urine samples from these two groups of individuals.

3. 1 Primers Design

To study the mRNA expression for all the ENaC subunits, we designed primers for each ENaC subunit. The primers were designed to span exon - exon junctions to avoid amplifications of genomic DNA. However, 728 primers did not span an exon – exon junction because the difference between 669 and 728 subunits is contained within exon 1. The 669 primers consist of forward and reverse primers on exons 11 and 12 respectively. 728 primers have the reverse and forward primers on exon 1. The two primers are shown in Figure 2.

Figure 2. Genomic organization of 669 and 728 hENaC . 669 Primers were designed between exons 11 and 12 and are indicated by arrows. 728 Primers were designed to span the exon 1 and are indicated by arrows.

Numbers in boxes refer to exons. Those at start and end indicate genomic size.

Most mutations of the subunit are found in the C- terminus and have the potential to cause Liddle’s Syndrome. We avoided exons where mutations were discovered . Therefore, the forward primer was designed between exons 4 and 5 while the reverse primer was designed in exon 5 as shown in Figure 3. Mutations in the ■ subunit were also found to exist and to contribute to pseudohypoaldosteronism [51]. In addition, we designed primers for ■ subunit and the forward and reverse primers were spanned exons 5 and 6 as seen in Figure 4, also avoiding the C- terminus. We designed three primers for subunit as we mentioned in table 2, however there were unsuccessful results. For subunit, the forward and reverse primers were designed between exons 1 and 2 as shown in Figure 5.

Figure 3. Genomic organization of hENac. Primers were designed between exons 4 and 5 and are indicated by arrows. Numbers in boxes refer to exons number. Those at start and end indicate genomic size.

Figure 4. Genomic organization of ■ hENaC. Primers were designed between exon 5 and 6 and are indicated by arrows. Numbers in boxes refer to exons number. Those at start and end indicate genomic size.

Figure 5. Genomic organizations of 802 and 638 hENaC . 802 Primers were designed between exons 1 and 2 and are indicated by arrows. Numbers in boxes refer to exons number. Those at start and end indicate genomic size.

3. 2 Amplification of cDNA

One microgram of exosome isolated RNA was used in first strand cDNA synthesis. From that completed reaction 100-200 ng of cDNA was used in a qPCR reaction. Data were normalized to the equivalent yield from 1 ml of urine. For the accuracy of the primers we designed, standard curves were made by making a serial dilution for each ENaC subunit. The efficiencies of all q-PCR reactions were in range of 90% - 110%. There were no amplifications from the no template control (NTC) for some reactions. Amplifications of the NTC were however observer from some reactions. These did not affect our results because they were 1-3 orders of magnitude below the lowest copy number standard used. Results are shown in Figures 6A– 10A . Standard curves for each ENaC subunit were shown in Figures 6B - 0 10B.

3. 3 Melt Curve

Melt curve analyses were used as tools for q-PCR to confirm whether PCR assays produced one specific product. A single peak was observed in each primer we designed and it was compatible with the standard curve of each primer. In addition, this analysis allowed us to determine there were some primer-dimers or non-specific amplifications of some product. Figures 6C-10C shows the purity of the melt curve of each primer and melt temperature.

✔669 hENaC

A

B

C

Figure 6. Charactarization of ✔669hENaC primers. (A) Amplification of ✔669 DNA using a serial dilution spanning 106 to 102 copies. (B) Standard curve

generated from above serial dilution (C) Melting temperature analysis generated from above standard curve.

✔728 hENaC

A

B

C

Figure 7. Charactarization of ✔728 hENaC primers. (A) Amplification of ✔728 DNA using a serial dilution spanning 106 to 102 copies. (B) Standard curve generated from above serial dilution. (C) Melting temperature analysis generated from above standard curve. NTC amplification indicates by purple.

🚲 hENaC

A

B

C

Figure 8. Charactarization of 🚲 hENaC primers. (A) Amplification of 🚲 DNA using a serial dilution spanning 106 to 102 copies. (B) Standard curve generated from above serial dilution. (C) Melting temperature analysis generated from above standard curve.

■ hENaC

A

B

C

Figure 9. Charactarization of ■ hENaC primers. (A) Amplification of ■ DNA using a serial dilution spanning 106 to 102 copies. (B) Standard curve generated from above serial dilution. (C) Melting temperature analysis generated from above standard curve. NTC amplification indicates by purple.

802 hENaC

A

B

C

Figure 10. Charactarization of hENaC primers. (A)Amplification of 802 DNA using a serial dilution spanning 106 to 102 copies. (B) Standard curve generated from above serial dilution. (C) Melting temperature analysis generated from above standard curve.

3. 4 DNA gel electrophoresis

Agarose gel was used to determine the quality and the size of PCR product for each ENaC subunit primers. The results showed a single band of the predicted size, based on the molecular weight marker. In addition, we confirmed q-PCR products had no contamination from genomic DNA and there was no primer dimer as shown in Figures 11-15.

Figure 11. Verification of single fragment of 94bp for 669 by using 2. 5% agarose gel.

Figure 12. Verification of single fragment of 150bp for 728 by using 2. 5% agarose gel.

Figure 13. Verification of single fragment of 77bp for by using 2. 5% agarose gel.

Figure 14. Verification of single fragment of 136 bp for by using 2. 5% agarose gel.

Figure 15. Verification of single fragment of 74 bp for by using 2. 5% agarose gel.

3. 5 Expression of ENaC subunits in human kidney

We demonstrated the mRNA expression level of all 4 ENaC subunits and in human kidney. We then examined the expression in a family with 21-Hydroxylase Deficiency. This monogenic disease affects a small fraction of the population with only about 0. 1% of the population exhibiting a severe phenotype [52]. More frequent and less severe mutations are often from single nucleotide polymorphisms (SNPs) and have an allelic frequency of 1%-2%. It can be asserted that hypertension development depends on the interaction between epigenetic and genetic factors [52], therefore we were uncertain if the chosen family with mixed mild and severe phenotype would display marked changes to ENaC subunit copy number. Before assessing any copy number in individuals we wanted to determine the reproducibility of our results. We measured the mRNA copy number of one individual four times to see the accuracy of our results. The results are shown in Figure 16.

We measured the copy numbers of each ENaC subunit of both healthy and 21- Hydroxylase Deficiency individuals. 669 Subunit showed the highest expression levels in human kidney as seen in Figure17. Much lower expression level was observed in 728 subunit and there were no

expression from some subjects as seen in Figure 18. 🚲 subunit showed moderate levels of expression as shown in Figures 19. For ■ subunit mRNA levels were slightly higher than 🚲 subunit as seen in Figurer 19. There were less variable among individuals for ❤802 subunit as seen in Figure 20.

Figure 16. Repeatability quantification of hENaC mRNA copy number from one individual. Numbers indicate mean ± SE.

Figure 17. Quantification of ✔669 hENaC mRNA copy number in human urine. Numbers indicate mean ± SE. ND indicates not detected.

Figure 18. Quantification of ✔728 hENaC mRNA copy number in human urine. Numbers indicate mean ± SE. ND indicates not detected.

Figure 19. Quantification of 🚲 hENaC mRNA copy number in human urine. Numbers indicate mean ± SE.

Figure 20. Quantification of ■ hENaC mRNA copy number in human urine. Numbers indicate mean ± SE.

Figure 21. Quantification of ❤802 hENaC mRNA copy number in human urine. Numbers indicate mean ± SE.

3. 6 Comparison of hENaC subunits expression in healthy and 21-hydroxylase deficiency individuals

We demonstrated the expression of ENaC subunits in 21-hydroxylase deficiency individuals and compared that with the expression in healthy individuals. The mRNA levels in ✔669 subunit were variable in healthy and 21- hydroxylase Deficiency individuals. The mRNA levels of ■ were higher in healthy individuals than in 21- Hydroxylase Deficiency individuals. . The

mRNA level of subunit were similar in healthy and 21-hydroxylase deficiency individuals. There was weaker expression of 728 subunit in 21-Hydroxylase Deficiency individuals than in healthy individuals. The expression of 802 subunit was slightly higher in 21- Hydroxylase Deficiency individuals than in healthy individuals as shown in Figures 22 and 23.

Figure22. Quantification of mRNA expression levels of ENaC subunits in healthy individuals by using RT-PCR, and the results show the high variability in copy number in 669. The mRNA levels of and 802 are reasonably similar. Numbers indicate mean ± SE.

Figure23. Quantification of mRNA expression levels of ENaC subunits in 21 hydroxylase deficiency individuals by using RT-PCR, and the results show the high variability in copy number of 669. Slightly increase mRNA expression level in 802. The mRNA expression of and are almost similar. Numbers indicate mean ± SE.

3. 7 Normalized ENaC subunits to ENaC

People with 21-hydroxylase deficiency have a mutation in CYP21A2 gene, which cause a deficiency in their aldosterone production. Some studies showed that aldosterone is increases the abundance of ENaC subunit, but not the or subunits [53]. If true then we expect major changes to subunit expression in 21-hydroxylase deficiency patients. However, the effects on subunit were mineral also accompanied by small decrease of and subunits expression. To better examine these potential subtle differences we normalized the results to subunits because it has the lowest variable expression among other subunits. In healthy and 21-hydroxylase deficiency individuals, we can see the ratio of 669/ was

almost similar. The ratio of ♥802/🚲 was higher in 21- hydroxylase deficiency than in healthy individuals. The ratio of ■/🚲 was higher in healthy individuals than in 21 -Hydroxylase Deficiency individuals. The ratio of ✔728/🚲 was slightly higher in healthy individuals than in 21 -Hydroxylase Deficiency individuals as shown in Figures 24 and 25.

Figure 24. Variability in the expression of hENaC subunits as normalized to 🚲 hENaC. Numbers indicate mean ± SE.

Figure 25. Variability in the expression of hENaC subunits as normalized to 🚲 hENaC. Numbers indicate mean ± SE.

4. 0 Discussion

Several problems arising from changes in kidney function have been associated with variable effects on blood pressure [10, 11]. The current study examined how the expression of four ENaC subunits varies among individuals. We also examined ENaC expression in aldosterone deficiency. We optimized a protocol to extract exosomes from human urine followed by mRNA extraction and reverse transcription. Using q-PCR, we demonstrated the presence of all four ENaC subunits (✔,🚲,■ and ♥) in human kidney. We also demonstrated two isoforms for ✔ subunit 🗨✔⏷⏷⏮ and ✔728). The results showed variable expression of ENaC subunits between healthy and 21 – Hydroxylase Deficiency individuals and indicate that the in vivo effects of aldosterone are limited to small 2. 3 fold change of ENaC mRNA levels.

4. 1 The use of exosome

The major objective of characterizing the expression of ENaC subunits using q-PCR was to determine variability present that could lead to hypertension. In this case, it has been found that genetic aspects account for 30% of blood

pressure variation in human populations [52]. This fact is supported by our data which showed variations in the ENaC subunits. However, this cannot affirm if the polymorphism or variation can cause serious phenotypic impact. In order to determine a linkage with a specific gene we must examine the mRNA levels. However, this has not been possible without obtaing renal biopsies. To circumvent this we utilized the renal exosome preparation. Exosomes were subject to consider in the isolation of mRNA because the quantity of mRNA, which can be isolated from exosome is adequate. The biological structure of exosomes saves their contents from degradation. The copy numbers calculation obtained are also reproducible making this preparation an ideal diagnostic tool.

4. 2 Relative expression of ENaC subunits

The α669 ENaC subunit result showed the highest expression level as shown in Figure 17, with a mean of 710±134. 38. However, the α728 ENaC data indicated the lowest expression level with a mean of 103±28. 41 as shown in Figure 18. This may indicate that most channels are made by α669 subunit. The β ENaC result indicated a slight greater level than α728 ENaC subunit at a mean of 199. 07±22. 74 as shown in Figure 19. In addition, β ENaC result showed the lowest variable results among individuals. The ■ ENaC result showed a mean of 247±39. 13 as indicated in Figure 20. The δ802 ENaC was able to indicate a moderate level of mRNA expression at a mean of 291±26. 79 as shown in Figures 21. Further, after designing different primers for ❤638, it was possible to observe that there was no detection of any expression in the human kidney. It is possible that the level of ❤638 is too low for the q-PCR to amplify any expression or technical problems. These

results also indicate that and subunits are likely limiting for full channel expression.

4. 3 High vs. low activity channels 669, 728 and 802

It is indicated that since the level of expression α669 is almost similar in healthy and 21- Hydroxylase Deficiency individuals. It is may means that α669 is not associated with the interaction of aldosterone. It was also observed that mRNA levels of and 802 were reasonably different in healthy and unhealthy individuals. The mRNA levels of β are similar in 21-hydroxylase deficiency individuals. Thus, in human with 21- Hydroxylase Deficiency, we could confirm a transcriptional down regulation of subunit in the absence or in low aldosterone. In terms of function, it could mean that subunit interacts highly with aldosterone and plays a big role in sodium exchange hence it highly related to induction of hypertension [54].

In healthy individuals, it was observed that the ratio of 669/ had a high variability in this group, due to the high mRNA level of 669. The ratios of 728, and 802 compared to were almost similar. In 21-hydroxylase deficiency people, ratio of 669/ is slightly high. The ratios of other subunits were low. In this study, we demonstrated that aldosterone increases the level of subunit but not or 802 subunits.

The q-PCR experiments showed an enhanced mRNA level of δ802 subunit in urine of 21 – Hydroxylase Deficiency individuals. This indicates that aldosterone may not have effect on δ subunit expression and the increase may simply be a compensatory mechanism to Na wasting. The δ epithelial sodium channel is known to be selective to sodium ion channel as well as sensitive to amiloride ion channel in the ENaC subunits responsible for blood

pressure regulation and may cause hypertension. It is also regulated similar to other ENaC subunits which are subjected to an ubiquitin modification causing internalization from the epithelial cell surface as well as degradation of the channel [55].

4. 4 In vivo vs. In vitro induction of ENaC by aldosterone

Our research also aimed at determining how the four ENaC subunits are affected by the deficiency of aldosterone and their role in human kidney. It is well known that over expression of one of these subunits lead to hypertension. The key goal was also to investigate whether the δ subunit is expressed in the epithelium of human kidney and whether it has any active functional role in human beings. It has been reported δ ENaC is expressed in the brain, heart pancreas and other organs. It has high activity in some human nonepithelial tissues, however, the physiological impact of δ ENaC is still unknown. The δ subunit has an extensive distribution in different urine samples and hence, is frequently co-expressed with other subunits of ENaC. When linked to the brain, it was revealed that δ subunit of ENaC might be stimulated by protons. Therefore, it may play a role in the sensitivity of renal functions to urine pH.

Some studies from rat kidney showed aldosterone is associated with increase in ✔ ENaC protein abundance. Thus, ✔ ENaC appears to be an aldosterone induced protein. However, increase in protein abundance does not necessarily imply for increase transcription of ✔ ENaC gene. Further, it is does have to be the same in humans. Activation of Na transport by aldosterone is related with an increase in ENaC subunits gene expression in the collecting duct is still questionable. Some showed no change in renal

mRNA levels of ENaC subunits in response to aldosterone. However, other investigators found slight increase in ✔ ENaC mRNA level in response to increased aldosterone concentrations [53].

According to May et al (1), “ ENaC is the major target for the natriferic action of aldosterone.” The effect of aldosterone on the abundance of the ENaC mRNA as well as the rate of protein synthesis for α and ■ subunits is significant. However, the presence of aldosterone did not affect the synthesis of β subunits in any way (Acton 4). According to May et al findings, aldosterone enhanced a large as well as quick increase in abundance of ■ subunits, but a low increase in alpha subunit mRNA abundance. In connection with this finding, a study carried out in the University of Buenos Aires showed that aldosterone stimulates protein synthesis (Acton 4). This finding is in connection with May et al. findings that showed aldosterone leads to the increase in protein synthesis by inducing accretion of α, β, and ■ transcripts at the last phase of its actions. Aldosterone also affects the stability of the mRNA (Alpern 1169). This is evidenced by the fact that it has classical transcription effect in addition to having the ability of controlling the gene expression by many mechanisms.

“ Aldosterone also induces translation for the protein serum glucocorticoid kinase type 1 (sgk-1)” (Biaggioni and Robertson 117). The effect of the translation is to prevent the removal of the ENaC from the top surface of the cell. According to Robertson et al (117), this increases the number of the ENaCs on the apical surface that maintains the re-absorption of the required level of Na as well as K homeostasis.

According to the current research, over expression of ENaC subunit leads to

excessive reabsorption of Na in the kidney and it is coupled with the rise in Na transport. In this case, some individuals exhibit low aldosterone levels as well as associated arterial hypertension [57]. The mRNA level of ■ ENaC is 3 folds higher in healthy individuals than 21 – Hydroxylase Deficiency individuals. This may show that aldosterone act on synthesis of ■ ENaC subunit. It came in the same line with another study did with CCD cells which showed under aldosterone action a translational activation of ■ ENaC mRNA happened. The stability of 🚲 ENaC subunit in both groups indicates no hormonal effect. However, mRNA could be effects by protein stability or cleavage of the channel. ❤802 ENaC results were higher in 21-Hydroxylase Deficiency Individuals; this may indicate high activity of ❤ subunit in human kidney and may play a functional role by intermolecular crosstalk with other ENaC subunits. This is supported by a finding from other study that ❤ ENaC contribute to 50% of amiloride sensitive salt transport across primary human nasal epithelial cells. The direct anti-natriuretic influence of aldosterone appears to be specifically important in situations of shrinkage of extracellular fluid volume and dietary sodium restriction. Regulation of sodium transport in the renal tubule has been subject to examine in comparatively short-term experiments. Nevertheless, there was enough evidence that a range of intermediaries of transport regulation within the kidney, such as aldosterone, functioned in both long-term and short-term actions.

4. 5 Conclusion

ENaC subunits are major regulators for reabsorption of salt and water in various epithelial tissues. Any irregularity in ENaC function directly causes several human diseases, such as Liddle’s Syndrome and potentially leads to

salt-sensitive hypertension in humans. In this study, the research was aimed at determining how the four ENaC subunits are affected by the deficiency of aldosterone and their profile of expression in the human kidney. The key goal was also to investigate whether the δ subunit is expressed in the epithelium of human kidney and whether it has any an active functional role in humans. From the results of the study, it has been able to prove that the δ subunit is expressed in the epithelium of human kidney and that it has active functional role in humans. The ■ ENaC in 21 Hydroxylase Deficiency is lower than in healthy people. It thus indicates that aldosterone is important for ■ subunit. In addition, δ802 subunit is high in both groups. Indicating that aldosterone is not related to δ802 and ✔669 Subunits. It is tempting to speculate that modifying ENaC functions may contribute to the pathopysiology of steroid induced kidney diseases. Increase understanding of how aldosterone affects mRNA stability of ENaC subunits may yield to therapeutic control of expression of ENaC genes. Therefore, future functional studies are needed for significant advances in this field.

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