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Effect of Dietary Sodium on α , β , and γ Epithelial Sodium Channel (ENaC) Gene Expression in Kidney Tubules of Wistar Rats

Ronny Lesmana,^{1,3} Genta Syaifrin Laudza,² Trianing Tyas Kusuma,³ Hanna Goenawan,^{1,3} Yulia Sofiatin,⁴
Yuni Susanti Pratiwi,^{1,3} Nova Sylviana,^{1,3} Setiawan¹

¹Physiology Division, Departement of Biomedical Sciences Faculty of Medicine Universitas Padjadjaran, Indonesia, ²Faculty of Medicine Universitas Padjadjaran, Indonesia, ³Physiology Molecular Laboratory, Biological Activity Division, Central Laboratory, Universitas Padjadjaran, Bandung, Indonesia,

⁴Department of Public Health Faculty of Medicine Universitas Padjadjaran, Bandung, Indonesia

Abstract

Hypertension is a condition of persistently high blood pressure. It is currently a big health issue as its prevalence is high in Indonesia and its complications are numerous and deadly. Salt intake is one of the modifiable factors of hypertension. According to a study by Indonesian Ministry of Health, salt consumption in Indonesia is almost two times greater than the recommended salt diet from WHO. Sodium reabsorption in kidney plays a role in regulating blood pressure. Epithelial sodium channel (ENaC) is one of the structures that function in sodium reabsorption in kidney tubules. This study was conducted at the Central Laboratory and Animal Laboratory of the Faculty of Medicine Universitas Padjadjaran from June to Desember 2018. The aim of this study was to analyze the effect of high sodium diet on the expression of ENaC gene in kidney tubules of rats. Twelve *Rattus norvegicus* wistar rats were divided into two groups of control and treatment. Treatment group was given daily 2 mL NaCl solution treatment using gavage for 8 weeks. The expression of ENaC α , β , and γ was obtained by running tissue samples from kidney cortex and medulla in polymerase chain reaction and electrophoresis. The result showed that there was an insignificant decrease in ENaC α , β , and γ gene expressions in both kidney cortex and medulla of the treatment group when compared to control group. This study concludes that ENaC gene expression is not significantly affected by high sodium diet.

Key words: Cortex, dietary sodium, ENaC α , ENaC β , ENaC γ , kidney, medulla

Pengaruh Diet Tinggi Natrium terhadap Perubahan Ekspresi Gen *Epithelial Sodium Channel (ENaC)* pada Tubulus Ginjal Tikus

Abstrak

Hipertensi merupakan kondisi tekanan darah tinggi dalam waktu lama. Prevalensi dan komplikasi hipertensi menyebabkan hipertensi menjadi isu kesehatan yang cukup besar. Jumlah asupan garam merupakan faktor hipertensi yang dapat dimodifikasi. Berdasarkan studi Kementerian Kesehatan Indonesia, konsumsi garam di Indonesia rerata dua kali lipat lebih banyak daripada rekomendasi WHO. Reabsorpsi natrium pada ginjal berperan penting pada regulasi tekanan darah. Fungsi ini diperankan oleh *epithelial sodium channel (ENaC)* yang berfungsi untuk reabsorpsi natrium. Penelitian ini dilakukan di Laboratorium Sentral dan Laboratorium Hewan Fakultas Kedokteran pada Juni–Desember 2018. Tujuan penelitian ini menganalisis efek diet tinggi natrium pada ekspresi gen dari ENaC di tubulus ginjal pada tikus. Duabelas ekor tikus *Rattus norvegicus* dibagi menjadi dua kelompok, yaitu kontrol dan perlakuan. Kelompok perlakuan diberikan 2 mL larutan NaCl setiap hari selama 8 minggu. Larutan diberikan melalui paksa (*gavage*). Ekspresi gen ENaC α , β , γ dari korteks dan medula ginjal diamplifikasi dengan PCR dan dideteksi dengan elektroforesis. Pita protein dari gel elektroforesis dinilai intensitasnya dengan *software ImageJ*. Hasil dari elektroforesis menunjukkan penurunan ekspresi gen ENaC α , β , γ di korteks dan medulla pada kelompok perlakuan dibanding dengan kontrol. Studi ini menyimpulkan ekspresi gen ENaC pada korteks dan medulla ginjal tidak signifikan dipengaruhi oleh diet tinggi natrium.

Kata kunci: Diet tinggi natrium, ENaC α , ENaC β , ENaC γ , ginjal, korteks, medula

Corresponding Author: Ronny Lesmana, Physiology Division, Departement of Biomedical Sciences Faculty of Medicine Universitas Padjadjaran, Jalan Jatinangor Km 21.6 Sumedang, West Java., Indonesia. Email: ronny@unpad.ac.id

Introduction

Hypertension, a condition of persistently high blood pressure (systolic pressure and diastolic pressure of more than 140 and 90 mmHg, respectively), is currently a global health issue.¹ Hypertension is a health problem with a high prevalence in Indonesia. The Indonesian Basic Health Research (IBHR) 2013 had described that the prevalence of hypertension in Indonesia has reached 25.8%.² Without adequate treatment, persistent increase of blood pressure for a long period may lead to kidney failure, cardio-vascular disease, and stroke.³ Factors contributing to hypertension mainly consist of both environmental and genetic factors. Therefore, hypertension is classified as a complex and multifactorial disease⁴. Majority of hypertensive patients have modifiable environmental risk factors such as overweight, sedentary lifestyle, smoking habits, and salt intake.⁴

Salt intake is one of the modifiable environmental hypertension risk factors. Recommendation from World Health Organization stated that sodium intake reduction to less than 2 g/day salt will reduce blood pressure as well as cardiovascular disease, stroke, and coronary heart disease risks.⁵ However, a study of Indonesian diet by Indonesian Ministry of Health concludes that average salt consumption in Indonesia is 3.6 g/day.⁶ In addition, salt taste threshold is positively correlated with the measure of salt intake.⁷ Thus, Indonesian population may not realize the high consumption of salt in its society, making the high salt intake one of the most prominent contributor of hypertension in Indonesia.

Kidney is an organ that functions in regulating electrolyte balance in the circulatory system. Sodium is one of the essential minerals which concentration is maintained by the kidney. The homeostasis can be maintained by regulating the renal blood flow, reabsorption of sodium and water, and Renin Angiotensin Aldosterone System (RAAS). Abnormal increase of sodium reabsorption may occur in the pathophysiology of hypertension. Increase in sodium reabsorption will then increase the total extracellular volume. According to Ohm's law, the increase in extracellular volume causes an increase in blood pressure.⁸ Thus, sodium reabsorption plays a role in the mechanism of hypertension.

Epithelial sodium channel (ENaC), which is an amiloride-sensitive sodium channel, is a sodium reabsorption ion channel that is prominent in distal tubules and collecting duct of kidney

and in lungs, parotid glands, sweat glands, and brain.^{11,10} ENaC is one of the channels functioning in sodium reabsorption in kidney tubules. The importance of ENaC in the regulation of blood pressure is highlighted by several diseases associated with gain or loss of ENaC function.⁹ There is little evidence on the influence of the amount of salt consumption on ENaC expression which may determine the ENaC protein levels and function. Liddle's syndrome is one of the diseases caused by the mis-sense mutation on PY motif in ENaC β and γ sub units which increase in ENaC expression and eventually contribute to increased blood pressure.¹⁰ ENaC consists of α , β , and γ sub units.¹² Until now, there are only few literatures that explain the physiologic changes taking place in kidney sodium reabsorption during high salt diet. Therefore, the aim of this study was to analyze the effect of high sodium diet towards the expression of ENaC gene in kidney tubules of rats.

Methods

Two groups (Group C and T) of 12 weeks old *Rattus norvegicus* male wistar rats, with each group consisted of 6 rats, were given two different treatments. Group C rats were the control group and group T was the treatment group. Each group was provided a 50x47x45 cm cage with ad libitum access to food and water. Animals in the study were obtained from Eyckman Animal Experimental Laboratory. Previously, methods concerning ethical issues of this animal experimental study were approved by the Faculty of Medicine Research Ethics Committee with the issuance of ethical clearance Number 1308/UN6.KEP/EC/2018.

Repeatedly in 8 weeks, both group C and T rats were given 200 g ad libitum pellet per day and 500 mL ad libitum water per day. Additionally, Group C rats were given 2 mL distilled water per day while Group T rats were given 2 mL NaCl solution consisting 50 mg of NaCl and distilled water. The amount of NaCl is obtained by converting recommended NaCl consumption per day for human to rat dose using a conversion method from FDA 2005. Each treatment was administered by gavage. The 2 mL volume was decided according to the maximum capacity of 210–230 g rats. During the treatment, 4 rats died, consisting of 3 rats from the treatment group and 1 rat from the control group.

After 8 weeks of dietary sodium treatment, rats were sacrificed for sample collection. Before

being sacrificed, rats were given isoflurane gas for anesthesia. Rats were sacrificed by cervical dislocation. On paraffin board, rats were dissected in the abdominal region and the left and right kidney were removed and stored temporarily for transport in liquid nitrogen with a temperature of around -180°C . The samples were stored in -80°C freezer until extraction. The remaining body parts and organs were then collected for burial.

The medulla and cortex parts were extracted from each kidney sample and pulverized for homogenization. Pulverized medulla and cortex were homogenized after being added with 200 μL TRizol. Acid-phenol method was used in the RNA isolation of this study. The isolated RNA concentration in each sample were quantified with TECAN. Samples with inadequate RNA concentration and/or contamination were not used for PCR.

Three primers used in this study were primers of three subunits of ENaC, ENaC α , ENaC β , ENaC γ , and GAPDH. Primer sequences were obtained from NCBI (National Center for Biotechnology Information) Primer BLAST, while the PCR Kit used were from Gradient Sensoquest and Bioline. Prepared master mix composed of 4.35 μL DEPC treated water, 7.5 μL MyTaq reaction buffer, RNase Inhibitor, 0.6 μL Primers, and RT Enzyme was added to the template RNA and run in Gradient Sensoquest.

Then, 1.4 g Agarose gel mixed with 70 mL tris-acetate-ethylenediaminetetra acetic acid

(TAE) buffer were heated to boil for 1 minute and 7 μL fluorescence dye SYBR safe DNA gel stain were added to 2% agarose mixture before it was cooled into solid agar. Agarose gel soaked in TAE buffer and PCR products were run in 80 V for 40 minutes. Protein bands in gel were observed and photographed. Image J software was then used for quantification of photographed protein bands from BluPAD.

Data used for analysis was the ratio between the gene expression of ENaC- α , ENaC- β , ENaC- γ and GAPDH. Data were then tested for normality and homogeneity. Independent t test was used for analysis with a confidence interval of 95%. For statistical analysis, this study used SPSS version 25.

Results

In kidney cortex, the mean ENaC- α gene expression was 0.6096 ± 0.02289 in control group and 0.5970 ± 0.03048 in treatment group. The mean ENaC- β gene expression was 0.4976 ± 0.06551 in control group and 0.4512 ± 0.09875 in treatment group. Mean ENaC- γ gene expression was 0.7682 ± 0.11671 in control group and 0.6927 ± 0.07083 in treatment group. These can be observed in Figure 1 below.

In kidney medulla, the mean ENaC- α gene expression was 0.6952 ± 0.04636 in control group and 0.6696 ± 0.06839 in treatment group. The mean ENaC- β gene expression

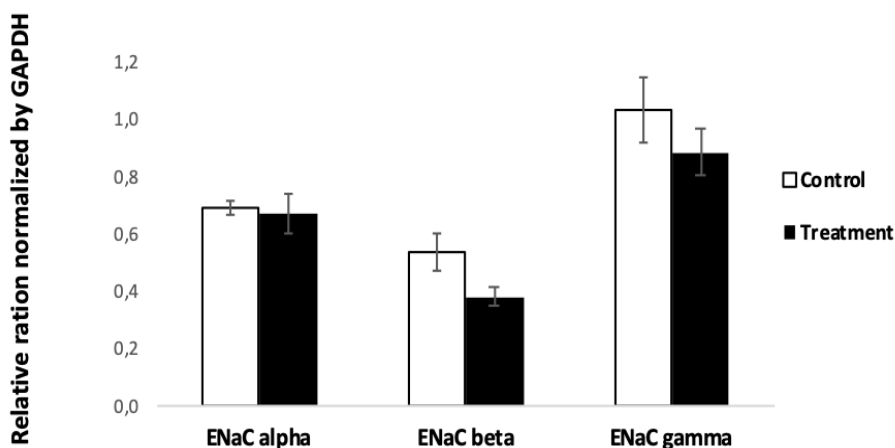


Figure 1 PCR Results of ENaC- α , - β , and - γ Gene Expressions in Kidney Cortex. Values are expressed as Mean \pm SEM

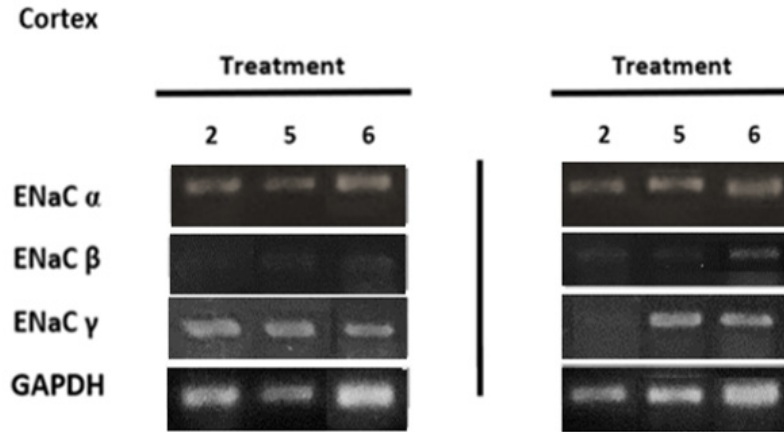


Figure 2 PCR Results of ENaC- α , - β , and - γ Gene Expressions in Kidney Medulla. Values are expressed as Mean \pm SEM

Table 1 Primers used for PCR

Primer Gene	Primer Sequence (5'-3')	Product Length (bp)	Tm (°C)
ENaC α Forward	TGGAGCCAGTCAAACAGTCC	189	60
ENaC α Reverse	CAGCTGACCTTCTGGAGTCG		
ENaC β Forward	TGAGCTGCCTTCTTGGGTTCTA	181	60
ENaC β Reverse	TTAGAGAGCAGCCACACGAT		
ENaC γ Forward	GTACGGGCTGCAAGTCATCT	204	60
ENaC γ Reverse	TGGCTGTAAGGTTTCGCTCAG		
GAPDH Forward	GTTACCAGGGCTGCCTTCTC	177	61
GAPDH Reverse	GATGGTGATGGGTTTCCCGT		

was 0.5395 \pm 0.1512 in control group and 0.3840 \pm 0.0290 in treatment group. Mean ENaC- γ gene expression as 1.0349 \pm 0.0780 in control group and 0.8875 \pm 0.0781 in treatment

group, as seen in Figure 2.

The result of this experimental study showed that there as a decrease in ENaC- α , - β , and - γ gene expression in both kidney cortex and medulla of

Table 2 Effects of High Salt Diet (50 mg/d) on ENaC- α , - β , and - γ Gene Expressions

Cortex and Medulla	ENaC- α	ENaC- β	ENaC- γ
Cortex			
Control	0.6096 \pm 0.02289	0.4976 \pm 0.06551	0.7682 \pm 0.11671
Treatment	0.5970 \pm 0.03048	0.4512 \pm 0.09875	0.6927 \pm 0.07083
Medulla			
Control	0.6952 \pm 0.04636	0.5395 \pm 0.1512	1.0349 \pm 0.0780
Treatment	0.6696 \pm 0.06839	0.3840 \pm 0.0290	0.8875 \pm 0.0781

Control (n=5), treatment (n=3), mean \pm SEM; (p>0.05) insignificantly different compared to control group

dietary sodium-treated rats when compared to control group. The decrease was not statistically significant as observed in Table 2 ($p>0.05$).

Discussion

Based on the result, it was observed that there was no increase of ENaC- α , - β and - γ gene expressions in treatment group when compared to the control group. There was an insignificant decrease of ENaC- α , - β , and - γ gene expressions ($p>0.05$). The explanation for this decrease of the ENaC gene expression is that during high salt diet, there are several factors regulating ENaC expression, such as hormones and ENaC regulatory proteins. Normally, ENaC regulatory proteins in ENaC regulatory complex (ERC) plays a role in ENaC degradation during high salt diet. According to Rossier¹³, in chronic high salt intake, the ERC binds to Raf-1 and Nedd-4-2 to phosphorylate and ubiquitylates the ENaC subunits.¹⁴ This phosphorylation and ENaC ubiquitylation cause degradation. On the contrary, during normal diet (low salt diet), several ENaC regulatory proteins are recruited by ERC, such as Sgk1 and GILZ. This leads to an increase of ENaC activity.¹⁵ Aldosterone also plays a role in ENaC activity.

In the condition of high salt diet, RAAS responds by decreasing renin, thus later decrease Angiotensin and Aldosterone. Aldosterone decrease causes a decrease of ENaC regulatory proteins inhibition of that then decreases the ENaC activity¹⁶. Other than aldosterone, several other hormones such as arginine vasopressin, atrial natriuretic peptide, insulin, and endothelin also play a role in regulating ENaC. Hormonal regulation of ENaC occurs through modulation of intracellular signaling pathways which involve kinase cascades, such as stimulation of Sgk1 or extracellular signal-regulated kinase (ERK) for aldosterone, protein kinase A for arginine vasopressin, and other different kinase for other hormones.^{17,1}

According to Rest et al.¹⁹, a significant increase of ENaC- α , - β , and - γ mRNA expressions was seen in Dahl-Salt-sensitive rats that received high salt diet. On the other hand, salt-resistant rats given treatment of high salt diet presented a significant decrease in expression of ENaC- α , - β , and - γ expressions. Similar to human, salt-sensitive and salt-resistant rats are normally distributed among normal rats.²⁰ In this study, the insignificant decrease of ENaC- α , - β , and - γ gene expressions may be caused by the unknown

distribution of salt-sensitive and salt-resistant rats among the total samples. As a result, one of the rats from the treatment group showed an increase in the expression of ENaC α , which is a characteristic of salt-sensitive rat.

This study concludes that wistar rats given dietary sodium treatment experiences insignificant decrease of ENaC- α , - β , and - γ gene expressions. This decrease is possibly caused by the activities of ENaC regulatory proteins through ENaC degradation and hormonal regulation. The insignificant decrease is possibly caused by the unknown distribution of salt-sensitive and salt-resistant rats. Increased ENaC gene expression during high salt diet plays a role in hypertension only in salt-sensitive rats.

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