Original Article

Dietary salt regulates ubiquitination and urinary excretion of Na-Cl cotransporter

Muhammad Zakir Hossain Khan[1], Eisei Sohara[2], Shinichi Uchida[3]

Abstract

Background: *Membranous localization of NCC is mandatory for its function at distal convoluted tubule. Post-translational modification like phosphorylation and ubiquitination are important for various membrane proteins for their membranous localization. It has previously been reported that phosphorylation of NCC increased its membranous localization. We previously reported, low salt diet increased and high salt diet decreased expression and phosphorylation of NCC in kidney.* ***Objectives:*** *In this study, we investigated whether ubiquitination is involved in the mechanisms of NCC regulation by dietary salt.* ***Materials & Methods:*** *We examined ubiquitination and urinary excretion of NCC from mice fed with low-, regular- and high-salt diet. Study was done at Tokyo Medical and Dental University,during the period of January, 2013 to March, 2013.* ***Results:*** *We found that, high salt diet increased and low salt diet decreased ubiquitination of NCC, correlated well with their total NCC abundance in kidney. Urinary excretion of exosomal NCC was increased both under low and high salt diet.* ***Conclusion:*** *These results clearly indicated that dietary salt regulates ubiquitination of Na-Cl cotransporter and its urinary excretion.*

Keywords: *Dietary salt, Exosomes, Na-Cl co-transporter, Ubiquitination.*

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Introduction

Maintaining BP is essential for the adequate perfusion of organs. The ability of the kidney to adjust NaCl excretion plays a critical role in long-term blood pressure control.[1] The currently available genetic data in humans strongly reinforce the concept that regulation of extra-cellular fluid volume by the kidneys is essential in the pathogenesis of PHT as well as salt sensitivity (SS) and stress the crucial role of tubular sodium transport in this process.[2] The sodium-chloride cotransporter (NCC) is the key regulator of salt reabsorbtion in the mammalian distal convoluted tubule (DCT) which is a target for the thiazide diuretic class of antihypertensive drugs. It reabsorbs about 5-10% of total filtered sodium and is the final regulator of sodium delivery to the collecting duct where secretion of potassium by ROMK is depended on tubular Na concentration.[1] Although ~80% of filtered Na+ is reabsorbed in the proximal tubules (60%) and the thick ascending limb of the loop of Henle (20%), fine-tuning of the body's Na+ balance take place in the distal part of the nephron. Abnormal function of NCC is responsible for dysregulation of blood pressure that associated with its abnormal membranous abundance. Genetic disorder like Gordon's syndrome (associated with hypertension, hyperkalaemia and metabolic acidosis) caused by mutations in one of two serine-threonine kinases (WNK1 and WNK4). Constitutive activation of the WNK kinase-OSR1/SPAK kinase-NCC phosphorylation cascade is the molecular pathogenesis of salt-sensitive hypertension in Gordon's syndrome (pseudohypoaldosteronism type II) that associated with increased membranous abundance of NCC.[3] The abundance of NCC was also found to be significantly increased in kidneys of obese Zucker rat a model of metabolic syndrome and hypertension.[4,5] Various recent studies, suggesting the importance of proper functioning of NCC in body's ion balance regulation and blood pressure control. Dietary salt intake regulates phosphorylation and membranous expression of NCC in mouse kidney.[6] Post-translational modification like phosphorylation and ubiquitination are important for cell surface abundance of a membrane protein. Ubiquitination of a membrane protein is associated with its endocytosis and lysosomal degradation.[7] In current study, to get more inside of this regulatory effect of dietary salt intake, we investigated ubiquitination and urinary excretion of NCC under different dietary salt intake condition in mice.

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Materials and Methods

The study was conducted at Tokyo Medical and Dental University, during the period of Jan, 2013 to Mar, 2013. All of the animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University.

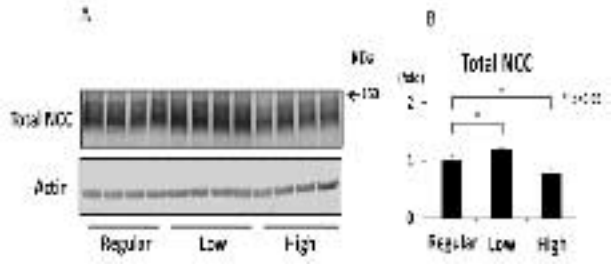

Figure 1: Membranous expression of NCC

Membranous expression of total NCC in mice kidney under different dietary Salt intake condition. A) Crude membrane fraction of kidney homogenates from three different dietary salt intake groups of mice was immunoblotted with antibody against total NCC. Representative immunoblot showing total NCC in mice kidney under low salt diet (LSD) is increased and under high salt diet (HSD) it decreased in comparison to under regular diet (NSD). B) Densitometry analysis of immunoblots showing significant difference in total and phosphorylated NCC of mice kidney under NSD, LSD and HSD. Values are normalized by ß actin and expressed as mean ± S.D. (n=5,**P <0.01, *P <0.05).

Urinary exosomal NCC

After C57BL6 mice were fed regular, low, and high sodium diet for 7 days, 24 hours urine was collected. Urinary exosomes was isolated by differential centrifugation[8] and the samples were immunoblotted[3] using anti-NCC antibody. After urine collection, mice were sacrificed and corresponding kidney homogenates were also immunoblotted using the same antibodies.

Ubiquination of NCC in urinary exosome and mouse kidney

After C57BL6 mice were fed regular, low, and high sodium diet for 7 days, urine was collected for 2 consecutive days and urinary exosomes were isolated by differential ultracentrifugation[8] and used for immunoprecipitation of NCC[9] by rabbit anti-NCC antibody. Immunoprecipitated samples were immunoblotted using guinea pig anti-NCC and anti-ubiquitin antibody. After urine collection, mice were sacrificed and kidney was homogenized and used for Immunoprecipitation of NCC. Immunoprecipitated samples were immunoblotted using guinea pig anti-NCC and anti-ubiquitin antibody.

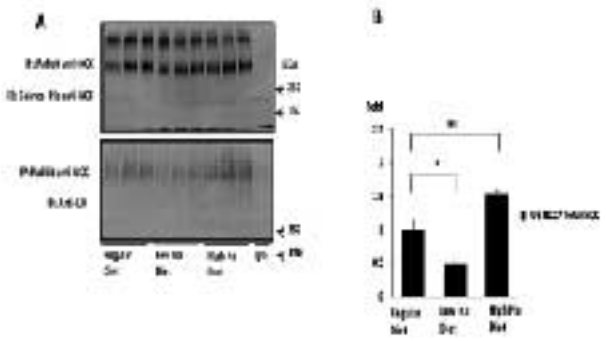

Figure 2: Ubiquitination of NCC

Ubiquitination of NCC, in mice kidney under different dietary salt intake condition. A) Immunoprecipitated NCC from crude membrane fraction of mice kidney homogenates were immunoblotted with antibody against total NCC (upper panel) and ubiquitin (lower panel). Representative immunoblot showing ubiquitination of NCC is lower under LSD and higher under HSD in comparison to under NSD. B) Densitometry analysis showing significant difference in ubiquitination (normalized by immunoprecipitated total NCC) of mice kidney NCC under three different dietary salt intake conditions. Values are expressed as mean±S.D. (n=3, **P <0.01, *P <0.05).

Results

High salt diet decreased and low salt diet increased membranous expression of NCC (Figure 1). On the contrary, High salt diet increased and low salt diet decreased ubiquitination of NCC in the kidney, respectively (Figure 2). The ratio of ubiquitinated urinary exosomal NCC to total urinary exosomal NCC was not significantly different by change of salt intake, suggesting that urinary excretion of exosomal NCC is mediated by ubiquitination (Figure 4).

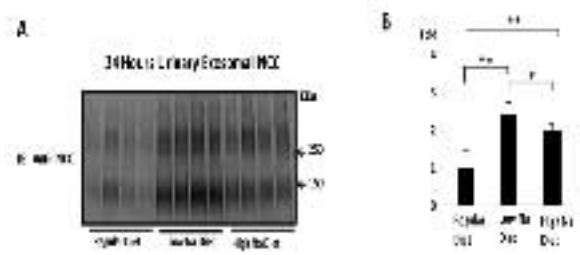

Figure 3: Urinary exosomal NCC

Mouse urinary exosomal NCC under different dietary salt intake condition. 24 hours urinary exosomes were collected from 3 different diets (regular, low and high salt) in taking mice. A) Western blot analysis showing increased 24 hours urinary exosomal NCC under low and high salt intake

condition in comparison to the regular diet intake condition. B) Densitometry analysis of 24 hours urinary exosomal NCC. All values are expressed as mean ± S.D. (n=4, **P <0.01, *P <0.05). Increased ubiquitination of kidney NCC under high salt diet correlated well with its decreased total NCC abundance in kidney and increased urinary excretion of NCC (Figure 3). Under low salt diet, although the ubiquitination of NCC in the kidney was decreased, there was an increment of exosomal NCC in urine (Figure 3). This may be due to increased expression of total NCC under low salt diet. In this study, we found that dietary salt intake regulates ubiquitination of NCC in the kidney, suggesting that membranous expression of NCC is not only mediated by phosphorylation but also by other mechanism (s) involving ubiquitination.

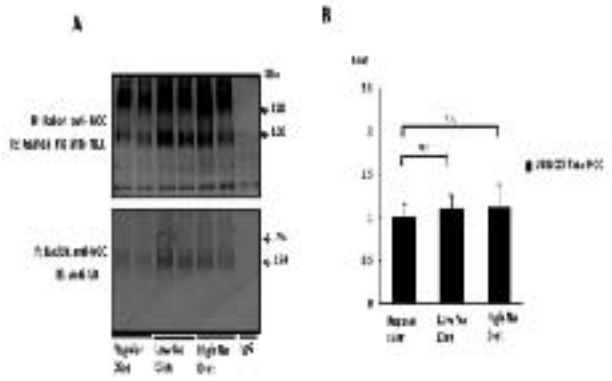

Figure 4: Urinary eaceretion of exosomal NCC mediated by ubiquitination

Ubiquitinated urinary exosomal NCC is proportional to the total excreted exosomal NCC in regular, low and high salt intake condition. A) 24 hours total urinary exosomal NCC from regular, low and high salt intaking mice, were immunoprecipitated and immunoblotted with anti NCC (upper panel) and anti ubiquitin antibody (lower panel). Representative immunoblot showing ubiquitinated exosomal NCC of mice under regular diet (NSD), low salt diet (LSD) and high salt diet (HSD). B) Densitometry analysis showing ratio between ubiquitinatd NCC and immunoprecipitated total NCC, as there is no significant difference among three dietary salt intake groups of mice.

Discussion

The kidney plays an important role in linking salt intake to BP as supported by renal cross-transplantation in humans.[1] Several hypertensive rat strains have been shown to retain significantly more sodium compared to the normotensive Wister-Kyoto rat.[10] However, this vital function of kidney is carried out by several ion transporters that expressed throughout the nephron. Till to date a good deal of knowledge is achieved about membranous abundance in different metabolic condition, secretory pathway, endocytosis, recycling and degradation process of ion transporters like NKCC, Na-K ATPase, ROMK, ENaC etc. But, despite a great importance in regulation of body's ion balance and blood pressure regulation, characterization of NCC remain in its infancy. In this study we have shown that regulation of NCC abundance in mice kidney under different dietary salt intake condition is mediated by its ubiquitination. For many of the substrates identified, ubiquitination occurs in a regulated manner, playing important roles in cellular processes for which regulation of protein levels are crucial. In a previous study it is shown that low salt diet increases phosphorylation and membranous abundance of NCC while high salt diet having the reverse affects.[6] In the present study, it revealed that NCC up regulation under low salt diet is associated with its less ubiquitination and down regulation under high salt diet is associated with its increased ubiquitination. This ubiquitination pattern of kidney NCC under different dietary salt intake condition was well correlated to the abundance of urinary exosomal NCC. As plasma membrane proteins usually appear into the urinary space through their ubiquitination and endocytosis.[11]

We found, increased urinary exosomal NCC both under low and high salt diet (Figure 3). Although in crude membrane fraction of kidney homogenate we found NCC gets less ubiquitinated under low salt diet (Figure 2), the total amount of membranous NCC was as high as to be exceeding the urinary excretional NCC under regular and high salt diet. This indicating a strong forward trafficking of NCC towards the cell membrane in addition to its less endocytosis under low salt diet. This increased forward trafficking of NCC may be due to increased NCC transcript and its phosphorylation under low salt diet.[12] Variation in the dietary salt intake induces change in multiple hormonal levels like aldosterone, renin, angiotensin, prostaglandin etc. Genomic and non-genomic actions of these hormones are responsible for up or down regulation of various ion transporters of the kidney via multiple intermediate kinase networks. NCC transcript increased under low salt diet and decreased under high salt diet is probably due to genomic action of aldosterone.[13] However, genomic up regulation of NCC is not sufficient for its membranous and functional up-regulation.[14] Aldosterone and angiotensin-II are individually involve in regulation of phosphorylation and membranous abundance of NCC,[6,15,16] which indicating a strong relation between phosphorylation and ubiquitination of NCC. Individual pathway that involve in ubiquitination process is yet to be evaluated in vivo although Benjamin Ko et. al. have noticed that stimulation of RasGRP1 enhaces NCC ubiquitination and endocytosis in cultured MDCK and mDCT cell.[17]

Conclusion

This study revealed that, dietary salt regulates ubiquitination and urinary excretion of NCC, ultimately regulating its' cell membrane abundance. This finding will help to go more inside of pathophysiology of salt sensitive hypertension. However, some previous study revealed dietary salt regulates NCC surface abundance by regulating its' phosphorylation. So, relation between phosphorylation and ubiquitination should be studied.

Acknowledgements

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References

1. Guyton AC. Physiologic regulation of arterial pressure. Am J Cardiol. 1961;8:401-407.
2. Wowern, F. Genetic Factors and Dietary Salt Intake as Determinants of Blood Pressure and Risk of Primary Hypertension Medical Faculty, Lund University, 2006.
3. Yang SS, Morimoto T, Rai T, Chiga M, Sohara E, Ohno M, et al. Molecular pathogenesis of pseudohypoaldosteronism type II: *Generation and analysis of a Wnk4 (D561A/+) knockin mouse model* Cell Metab. 2007;5:331-344.
4. Crystal AB, Joseph GV, Mark AK, Carolyn AE. Increased Renal Na-K-Atpase, Ncc, And ß-ENaC abundance in obese Zucker rats. Am J Physiol Renal Physiol. 2001;281:F639-648.
5. Bickel CA, Knepper MA, Verbalis JG, Ecelbarger CA. Dysregulation of renal salt and water transport proteins in diabetic Zucker rats. Kidney Int. 2002;61:2099-2110.
6. Chiga M, Rai T, Yang SS, Ohta A, Takizawa T, Sasaki S, Uchida S. Dietary salt regulates the phosphorylation of OSR1/SPAK kinases and the sodium chloride cotransporter through aldosterone. Kidney Int. 2008;74:1403-1409.
7. Juan SB, Allan MW, Ubiquitin and the Control of Protein Fate in the Secretory and Endocytic Pathways. Annu. Rev. Cell Dev. Biol. 1998;14:19-57.
8. Fernández-Llama P, Khositseth S, Gonzales PA, Star RA, Pisitkun T, Knepper MA. Tamm-Horsfall protein and urinary exosome isolation. Kidney Int. 2010;77:736-742.
9. De Jong JC, Willems PH, Mooren FJ, van den Heuvel LP, Knoers NV, Bindels RJ. The structural unit of the thiazide-sensitive NaClcotransporter is a homodimer. J Biol Chem. 2003;278:24302-24307.
10. Bernd AJF, Olaf G, Norman B, Siegfried W, Peter B, Rainer R. Sodium homeostasis in transplanted rats with a spontaneously hypertensive rat kidney. Am J Physiol Regulatory Integrative Comp Physiol. 2000;279:R1099-R1104.
11. Susan CS, Katherine ESM, Linda H. Monoubiquitin carries a novel internalization signal that is appended to activated receptors. EMBO J. 2000;19(2):187-198.
12. Volker V, Jana S, Florian L, Dietmar K, Shinichi U. Expression and phosphorylation of the Na+-Cl^- cotransporter NCC *in vivo* is regulated by dietary salt, potassium, and SGK1. Am J Physiol Renal Physiol. 2009;297:F704-F712.
13. Lai L, Feng X, Liu D, Chen J, Zhang Y, et al. Dietary salt modulates the sodium chloride cotransporter expression likely through an aldosterone-mediated WNK4-ERK1/2 signaling pathway. Pflugers Arch. 2012;463:477-485.
14. Mc Cormick JA, Nelson JH, Yang CL, Curry JN, Ellison DH. Overexpression of the sodium chloride cotransporter is not sufficient to cause familial hyperkalemic hypertension. Hypertension. 2011;58:888-894.
15. Talati G, Ohta A, Rai T, Sohara E, Naito S, et al. Effect of angiotensin II on the WNK-OSR1/SPAK-NCC phosphorylation cascade in cultured mpkDCT cells and in vivo mouse kidney. BiochemBiophys Res Commun. 2010;393:844-848.
16. Van der Lubbe N, Lim CH, Fenton RA, Meima ME, Jan Danser AH, et al. Angiotensin II induces phosphorylation of the thiazide-sensitive sodium chloride cotransporter independent of aldosterone. Kidney Int. 2011;79:66-76.
17. Ko B, Kamsteeg EJ, Cooke LL, Moddes LN, Deen PM, Hoover RS. RasGRP1 stimulation enhances ubiquitination and endocytosis of the sodium-chloride cotransporter. Am J Physiol Renal Physiol. 2010;299:F300-F309.