The sodium-activated sodium channel is expressed in the rat kidney thick ascending limb and collecting duct cells and is upregulated during high salt intake

Lucienne S. Lara,1,2,3 Ryousuke Satou,1,2 Camille R. T. Bourgeois,1 Alexis A. Gonzalez,1,2 Andrea Zsombok,3 Minolfa C. Prieto,1,2 and L. Gabriel Navar1,2

1Department of Physiology, Tulane University School of Medicine and 2Hypertension and Renal Center of Excellence, Tulane University School of Medicine, New Orleans, Louisiana; and 3Instituto de Ciencias Biomedicas, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Submitted 26 August 2011; accepted in final form 20 March 2012

Address for reprint requests and other correspondence: L. S. Lara, Tulane Univ. School of Medicine, 1430 Tulane Ave., SL39, New Orleans, LA 70112 (e-mail: lara@ich.ufrj.br).

http://www.ajprenal.org

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were euthanized by conscious decapitation on day 7. Kidneys from NS and HS rats were removed and sectioned into cortices and medullas for protein, total membrane, and RNA extractions. The brain stem, containing the area postrema, nucleus tractus solitarius, and dorsal motor vagus of NS rats was also harvested and used as a positive control for Western blot and RT-PCR analyses. A separate group of rats subjected to the NS of NS rats was also harvested and used as a positive control for Western blotting and RT-PCR analyses. A separate group of rats subjected to the NS diet (n = 3), was anesthetized with pentobarbital sodium (50 mg/kg) and kidneys were perfused with 4% paraformaldehyde; the two kidneys were then removed and bisected with a razor blade for immunofluorescence as described below.

Brain and kidney cortex homogenate and total membrane fraction preparation. The total membrane fraction was obtained as described previously (16). Briefly, the kidneys were removed, maintained in cold solution containing (in mmol/l) 250 sucrose, 10 HEPES-Tris (pH 7.6), 2 EDTA, and 1 PMSF, dissected into cortex and medulla, and homogenized in the same cold solution. The final pellet after sequential centrifugations containing the total membrane fraction was resuspended and homogenized in 250 mmol/l sucrose and stored at −80°C until used for Western blotting.

RT-PCR and cDNA sequencing. First-strand cDNA synthesis was performed using 1 μg of total RNA and a SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). The primers used were forward 5′-TATATACGGAATTCCACA-3′ and reverse: 5′-CAACAAATATTGCTTATTG-3′. The cDNA sequencing was performed to identify the PCR product. After DNA sequencing, a basic local alignment search (BLAST) was performed to confirm the goal identity. cDNA sequencing was performed using 1 μg of total RNA and a SuperScript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). The primers used were forward 5′-H9262 and reverse 3′-H11001 anti-rat Tamm-Horsfall antibody, a marker for TAL cells; 2) anti-rat AQP1 antibody, a marker of proximal tubule cells; 3) anti-rat AQP2 antibody, a marker of the principal cells of connecting tubules and collecting ducts; and 4) markers for intercalated cells: anti-rat anion exchanger 1 (AE-1), anti-rat Rh B glycoprotein (Rhbg), and anti-rat Rh C glycoprotein (Rhcg) antibodies. The specificity of immunostaining was determined by the omission of the primary antibody and substitution with normal horse serum.

RESULTS

Localization of the Na sensor in kidneys of normal Sprague-Dawley rats. To determine the presence of the Na sensor in the kidney, we examined the presence of the Na sensor gene transcript and protein in the renal cortex and medulla of normal male Sprague-Dawley rats. Figure 1, A and B, shows the PCR product extract from brain stem was used for positive controls. Specific binding was determined by the preadsorption of the antibody for 72 h with 5× excess of the recombinant protein NAV2. Densitometric determinations, using Image J software (National Institutes of Health), were calculated as the ratio between the Na sensor bands (190 kDa) and β-actin expression. Values were expressed as fold-increase compared to the t-test analysis and considered different at P < 0.05.

Na sensor immunofluorescence in the normal rat kidney. Kidneys perfused and fixed with 4% paraformaldehyde and zinc-saturated formalin were used for immunohistochemical studies. Kidney sections (3 μm) were processed by an immunoperoxidase technique as previously described (7, 15) and incubated overnight at 4°C with rabbit polyclonal anti-rat Scn7a (1:100) followed by incubation of the specific immunofluorescent secondary antibody (1:1,000, Alexa Fluor, Invitrogen). For the colocalization studies, the sections were sequentially incubated at room temperature for another 30 min with blocking serum and 1 h with different immunomarkers: 1) anti-rat aquaporin-1 (AQP1) antibody, a marker of proximal tubule cells; 2) anti-rat Tamm-Horsfall antibody, a marker for TAL cells; 3) anti-rat AQP2 antibody, a marker of the principal cells of connecting tubules and collecting ducts; and 4) markers for intercalated cells: anti-rat anion exchanger 1 (AE-1), anti-rat Rh B glycoprotein (Rhbg), and anti-rat Rh C glycoprotein (Rhcg) antibodies. The specificity of immunostaining was determined by the omission of the primary antibody and substitution with normal horse serum.

Fig. 1. Na sensor expression in normal rat kidney. A and B: representative ethidium bromide-stained gel showing RT-PCR product (448 bp) obtained from total RNA of normal whole rat kidney (WK), brain stem (BS), positive control), kidney cortex (KC), and kidney medulla (KM). NTC, non-template control. C: Na sensor Western blot analysis in the normal rat kidney. Sixty micrograms of protein from kidney cortex and medulla samples were electrophoretically separated in a NOVEX 8% bis-Tris precast gel (Invitrogen) and were transferred to a nitrocellulose membrane to detect the presence of the Na sensor by using rabbit polyclonal anti-rat Scn7a (1:500, Abcam, Cambridge, MA). The membranes were incubated with an infrared dye-labeled secondary antibody in accordance with previous protocols (LI-COR Biosciences, Lincoln, NE). The fluorescent-tagged anti-IgG antibody (1:20,000) was used to identify the primary antibody (IRDye-labeled antibodies, LI-COR Biosciences, Lincoln, NE). Fluorescent intensity was detected using the infrared Odyssey System (LI-COR Biosciences).
products from reverse transcripted RNA from kidney cortex and medulla, as well as brain stem, using specific primers for Scn7a, the Na sensor gene, according to the BLAST searches. A specific single band of the expected product (448 bp) demonstrates Na sensor mRNA expression in the normal rat kidney cortex and medulla. The absence of the band in the nontemplate control lane, in which the cDNA was omitted, ruled out the possibility of a contamination of the RT-PCR reagents. DNA sequencing of the Na sensor PCR product from the kidney indicate 100% identity to the reported cDNA sequence. Protein expression in the rat kidney was confirmed using two different primary antibodies, rabbit polyclonal anti-rat Scn7a (Abcam) (Fig. 1C, top) and mouse polyclonal anti-human NAV2 (Abnova, Taipei, Taiwan) (data not shown). We detected a prominent band with the estimated molecular mass of 190 kDa consistent with the molecular weight determined by the antibody company. The specific binding is supported by our observations that both antibodies recognized a specific band in the brain stem and by showing that the preabsorption of the antibody with the specific recombinant Na sensor protein prevented the appearance of the band (Fig. 1C, bottom). Cross-reactivity with another Na⁺ channel, such as the epithelium Na⁺ channel (ENaC) is ruled out because the hybridization with its specific antibody demonstrated a band of 85 kDa (12). As shown in Fig. 1D, the increase in Na sensor protein expression in the total membrane preparation compared with kidney homogenates from the cortex and medulla clearly demonstrate enrichment of its expression in the cell membranes.

Immunofluorescence and confocal microscopy detection of the Na sensor was performed to determine the specific intrarenal localization of the Na sensor and to assist in cell type immunoexpression (Fig. 2). In the cortex and outer medulla,
the Na sensor was colocalized with AQP2, a marker for principal cells of the connecting tubule and collecting duct, and with Tamm-Horsfall glycoprotein, a marker of the TAL (Fig. 2, A and B). As shown, the Na sensor did not colocalize with AQP1, indicating proximal tubule cells (Fig. 2A). Na sensor expression was not detected in the glomeruli (Fig. 2D). In the inner medulla, the Na sensor was present only in the principal cells of the collecting duct (Fig. 2, C and D), since there was no colocalization with AE-1 and the glycoproteins Rhcg and Rhbg, all immunomarkers of intercalated cells (9) (Fig. 2C).

**Na sensor expression is increased by a HSD.** To determine the effect of a HSD on Na sensor gene expression, we quantified its levels by qRT-PCR and Western blotting from control rats fed a normal-salt diet (NS; n = 5) and rats subjected to 8% NaCl diet for 7 days (HS; n = 5). If the Na sensor in the kidney has similar properties as reported in the brain (10, 15), so that it would detect high [Na\(^+\)] above 150 mM, it is reasonable to use a significant high NaCl content diet that leads to high intratubular [Na\(^+\)], as observed in a 8% HSD. In this group of rats, Na\(^+\) excretion increased from 0.6 ± 0.05 in NS rats to 15.2 ± 1.7 meq·100 g body weight\(^{-1}\)·24 h\(^{-1}\) in the HS group, and, more importantly, urinary [Na\(^+\)] markedly increased from 136 ± 8 in the NS rats to 339 ± 32 mM in the HS group, suggesting that [Na\(^+\)] in the distal tube reaches the threshold to activate the Na sensor. HSD increased Na sensor mRNA (from 1.2 ± 0.2 to 5.1 ± 1.3 au; P < 0.05) and protein (from 0.98 ± 0.15 to 1.74 ± 0.28 au; P < 0.05) levels in the renal medulla, but not in the cortex (Fig. 3). These data indicate that the Na sensor in the collecting duct and TAL is upregulated by HSD and suggest a role in monitoring changes in tubular fluid [Na\(^+\)].

**DISCUSSION**

This study demonstrates the presence of Na sensor transcript and protein in normal Sprague-Dawley rat kidneys and shows clear evidence of specific localization in collecting ducts and ascending loop of Henle. The DNA sequencing of the Na sensor PCR product from the rat kidney indicates 100% identity to the Na sensor described according to the ENTREZ Gene Technology Information. In addition, by using two different primary antibodies, we demonstrated that the enriched total membrane fraction from the kidney cortex and medulla exhibit enriched protein levels of the prominent band of 190 kDa, as reported in studies using lung and glial cells (17, 18). The cross-reactivity with another Na\(^+\) channel, ENaC, can be ruled out since its molecular mass is ~85 kDa (12).

To date, the Na sensor-expressing cells have been identified in 1) specialized central neurons; 2) sensory neurons in the peripheral nervous system; 3) specialized ependymal and glial cells in the central nervous system; 4) nonmyelinating Schwann cells; and 5) epithelial cells which include the alveolar type II cells in the lung (17). Here, we present novel findings establishing the presence of the Na sensor in rat kidney epithelial cells specifically localized to the apical side of the TAL and collecting duct. These results provide a new perspective of the Na\(^+\)-sensing capabilities of the kidney and suggest another mechanism allowing renal regulation of Na\(^+\) balance.

Our findings of immunolocalization of the Na sensor in kidney TAL epithelial cells and in the principal cells of the collecting duct differ from the original report on the Na sensor, which demonstrated its presence only in the nonmyelinating Schwann cells of the kidney nerve endings (17). Watanabe et al. (17) reported the distribution of the Na sensor in visceral organs from gene-targeted mice in which the Scn7a gene was substituted by a Lac-Z using bromo-chloro-indolyl-galactopyranoside (X-gal) staining of tissues. This technique identified X-gal staining as a blue-labeled fibrous structure running on the surface of the bladder. A similar staining pattern was observed surrounding major blood vessels in the kidney, which was attributed to the nonmyelinating Schwann cells of sympathetic and/or parasympathetic nerve endings (17). However, staining in kidney epithelium cells was not reported. In the present study, the direct visualization of the Na sensor using specific antibodies for Na sensor protein with high-resolution immunofluorescence and confocal microscopy along with the detection of its transcript in renal cortical and medullary tissues confirm the presence of the Na sensor in the rat kidney nephron segments.

The presence of the Na sensor in the luminal membrane of kidney epithelial cells of the TAL and principal cells of the collecting duct suggests the involvement of this channel in the vectorial Na\(^+\) reabsorption activated by the Na\(^+\)-K\(^+\)-ATPase.

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Fig. 3. High-salt (HS) diet alters Na sensor expression in the rat kidney medulla. NS, normal-salt diet; LS, low-salt diet; A and C: representative images of Na sensor detection using rabbit polyclonal anti-Scn7a (1:500, Abcam) and mouse polyclonal β-actin (1:5,000, Santa Cruz Biotechnology, Santa Cruz, CA). B and D: densitometric analysis of the immunoreactive band (190 kDa) correlated by the β-actin expression. Fluorescent-tagged anti-IgG antibody (1:20,000) was used to identify the primary antibody (IRDye-labeled antibodies from LI-COR Biosciences). Fluorescent intensity was detected using an infrared Odyssey System (LI-COR Biosciences). Left: kidney cortex. Right: kidney medulla.
Although only a small fraction of filtered Na\(^+\) is normally reabsorbed in distal nephron segments, the demonstration of augmented distal tubule Na\(^+\) reabsorption in animal models of hypertension and hypertensive patients supports the relevance that this part of the nephron has in the development of hypertension (8, 19). The demonstration that the principal cells of the collecting duct express the Na-sensor suggests that the sensor detects increases in luminal fluid [Na\(^+\)] and may also contribute, together with ENaC, to distal nephron Na\(^+\) reabsorption. Watanabe et al. (17) reported the perinatal induction of the Na sensor in the lungs and proposed its role, in association with ENaC, to mediate Na\(^+\) absorption and maintain the alveolar spaces free of fluid.

We also demonstrated the increase in Na sensor expression during 7 days of HS intake. In contrast to our observation, Ergonul et al. (5), using the same rat model as in this study, salt-resistant Sprague Dawley rats, showed that Na\(^+\) current is increased in rats subjected to a LS diet for 7 days and is downregulated by salt repletion through saline drinking water. This is likely due to a downregulation of ENaC and opposite to the increased expression of the Na sensor. This differential regulation suggests that Na sensor function may be distinct from that of ENaC. Also, the use of salt in the drinking water leads to an increase in arginine vasopressin, increasing thirst, which may contribute to the Na\(^+\) current downregulation.

In summary, our study provides novel evidence demonstrating the presence of the Na sensor in rat kidney epithelial cells and indicates that its expression is augmented in the renal medulla during chronic HS diet intake. The Na sensor may play an important role in assessing tubular fluid [Na\(^+\)] and may also contribute to Na\(^+\) reabsorption along with ENaC and other Na transporters, as proposed for the lung by Watanabe et al. (17). These findings may open perspectives for new investigations regarding the stimulation, regulation, and cell-signaling pathways of the Na sensor in the kidney.

ACKNOWLEDGMENTS

We thank Omar Acres for technical assistance. Digital images of histological specimens were obtained at the Imaging Core Facility of Hypertension and Renal Center of Excellence at Tulane University Health Sciences Center and DNA sequencing at the Tulane Center for Stem Cell Research and Regenerative Medicine. Dr. I. David Weiner provided the rabbit polyclonal anti-rat Rh B glycoprotein (Rhbg) and rabbit polyclonal anti-rat Rh C glycoprotein (Rhcg).

GRANTS

This work was supported by grants from the National Heart, Lung, and Blood Institute (HL26731) and from a COBRE grant (P20RR017659) from the National Center for Research Resources. M. C. Prieto and A. Zsombar are BIRCWH scholars supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (K12HD043451), and M. C. Prieto is supported by the American Heart Association (09BGIA2280440). L. S. Lara is a recipient of a CNpq Post-Doctoral Fellowship from Brazil.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: L.S.L. and M.C.P. drafted manuscript; L.S.L., M.C.P., and L.G.N. edited and revised manuscript; L.S.L., R.S., C.R.B., A.A.G., A.Z., M.C.P., and L.G.N. approved final version of manuscript.

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