Expression, Transcription, and Possible Antagonistic Interaction of the Human Nedd4L Gene Variant
Implications for Essential Hypertension

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Abstract—Net sodium balances in humans are maintained through various ion transporters expressed along the entire nephron. Among these ion transporters, epithelial sodium channels (ENaC) located along the aldosterone-sensitive distal nephron (ASDN) play a pivotal role in the homeostasis of sodium balance. This is supported by analyses of inherited hypertensive disorders, showing that genes encoding ENaC and other modulatory proteins cause hereditary hypertension, such as Liddle syndrome. Among various modulating proteins, E3 ubiquitin ligase, Nedd4L, binds the PY motif of ENaC COOH terminals and catalyzes ubiquitination of the NH2 terminus of the protein for subsequent degradation. Both evolutionarily conserved and evolutionarily new C2 domains of human Nedd4L, a cryptic splice variant resulting in a disrupted isoform product formed by a frame-shift mutation, were reported previously. We focused on one of the isoforms, isoform I, generated by SNP (rs4149601), and studied its expression and interactions with other isoforms by molecular biological, immunohistochemical, and electrophysiological methods. We found that isoform I may interact with other human isoforms in a dominant-negative fashion. Such interactions might abnormally increase sodium reabsorption. Taken together, our analyses suggest that the human Nedd4L gene, especially the evolutionarily new isoform I, is a candidate gene for hypertension. (Hypertension. 2008;51:773-777.)

Key Words: tubular transport ■ epithelial sodium channel ■ Nedd4L ■ essential hypertension

A body of evidence has suggested that the epithelial sodium channel (ENaC)-Nedd4L-proteasome system has pivotal roles in the regulation of sodium reabsorption along the entire terminal nephron.1–3 Its potential involvement in human hypertensive disorders has thus attracted considerable interest. An improved understanding of this system might provide important clues to the pathogenesis of human essential hypertension and the development of new therapeutic strategies. A previous study has demonstrated novel C2 encoding exons in the human NEDD4L gene by intensive resequencing and bioinformatics.4 One of the new exons, “exon 1a” encodes an evolutionarily new isoform in humans, “isoform I”, with a cryptic splice variant named “variant 13(G/A) (rs4149601)” (the frequencies of these Japanese alleles are as follows: G allele, 87.6%–79.0%, A allele, 12.4%–21.0%),8 a disrupted isoform product resulting from a frame-shift mutation. The other exon, named “exon 1a,” encodes an ancestral isoform, designated “isoform II”, with an evolutionarily conserved C2 domain, found in species ranging from Xenopus laevis to higher vertebrates. In individuals with the G allele, 3 different isoforms of human Nedd4L are thought to be expressed in the same tissues. Because several studies besides our population-based genetic study have shown that variant 13(G/A) is significantly associated with the phenotypes of hypertensive disorders,5–8 we attempted to elucidate the role of human Nedd4L isoform I in the development of hypertension. The genome sequence of the human Nedd4L gene suggests the possibility of diverse translation products. Studies of single-nucleotide polymorphisms (SNPs) done by us and other investigators have provided evidence of an association between the Nedd4L gene and hypertension in humans. However, previous studies have examined genome sequences alone or the relation between the Nedd4L gene and clinical phenotypes. Clarification of the relation between the Nedd4L gene and hypertension requires the examination of protein levels of the gene. However, previous studies have assessed only sequence information or SNPs: whether translation products are expressed at the protein level remains unknown. First, we endeavored to clone the 3 human isoforms (isoform I, isoform II, and the C2-less isoform III). Next, we examined translation and transcription in vitro with the use of a cultured cell line. Quantitative polymerase chain reaction (PCR) analyses were then performed to evaluate tissue-specific expression of
the 2 C2 encoding isoforms in human kidney, lung, brain, liver, and colon. Immunohistochemical analyses of human kidneys were performed using anticluster C2 domain polyclonal rabbit antibodies. Finally, we performed electrophysiological experiments with the voltage clamp technique using heterologous expression systems in Xenopus oocytes.

Materials and Methods

Cloning of Human Nedd4L Isoforms

A full-length clone encoding the human Nedd4L isoforms was obtained as follows. The PCR products were amplified by high fidelity Taq polymerase (KOD plus, TOYOBO, Osaka, Japan), and the 3 isoforms were cloned into the TOPO TA Cloning Vector (Invitrogen). Subsequently, the coding regions of all 3 human Nedd4L isoforms were transferred into pcDNA3.1/myc-His(+) (Invitrogen) in frame with the COOH-terminal His tag from the TA cloning vectors. The entire sequences of the 3 isoforms were confirmed by sequencing using fluorescence technology (Applied Biosystems). The vectors with the 3 isoforms or an empty vector were transiently transfected into HEK293T cells using FuGENE 6 (Roche). After 48 h, whole cellular extracts (25 μg per lane) from cultured cells were loaded on 7.5% SDS-polyacrylamide electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were incubated with anti-His(C-term)-antibody (Invitrogen) in a 1:500 dilution in TBS-T-T with 5% skim milk. Sites of antibody-antigen reactions were visualized by enhanced chemiluminescence (GE Healthcare Life Science) and recorded on film.

Quantitative Analyses of mRNA for Various Human Tissues

Human kidney, brain, lung, liver, and colon total RNA (BD Biosciences, Bedford, Mass) were prepared for quantitative analysis of Nedd4L mRNA isoforms I and II. Each first-strand cDNA was synthesized from 1 μg total RNA. Quantitative real-time PCR was performed in triplicate using a SYBR RT-PCR Kit (TAKARA), following the manufacturer’s instructions. Expression of Nedd4L mRNA was normalized to that of GAPDH, used as an endogenous reference.

Electrophysiological Measurement of Amiloride Sensitive Sodium Present by the Voltage Clamp Technique

Human epithelial sodium channel cDNAs (αENaC:TC119542, βENaC:TC119979, and γENaC:TC119545) were purchased (OriGene Technologies Inc, Rockville, Md). Linearized plasmids were subjected to in vitro transcription using a Message Machine (Ambion) Kit to produce capped cRNA for each construct. The integrity of the cRNAs was evaluated by agarose gel electrophoresis. The cRNAs were diluted in water so that 50-nL injections with a Drummond Nanoject oocyte injector carried 1 ng of each cRNA (estimated molar amount of each isoform: isoform I=1.1×10−3 pmol, isoform II=1.1×10−3 pmol, isoform III=1.3×10−3 pmol). Mature female Xenopus laevis were purchased (HSK) and housed in dechlorinated tap water at 18 to 20°C. Stage V and VI oocytes were removed from toads anesthetized in an ice-cold water bath. The oocytes were defolliculated under an inverted stereo microscope and stored overnight at 18°C in Barth’s solution (see the data supplement available online at http://hyper.ahajournals.org for further details). After 12 to 24 h, the cRNA was injected into healthy oocytes. Whole cell hENaC currents were measured in voltage-clamp buffer (see the data supplement) 48 h after the cRNA injections, as described previously. All recordings were made for at least 5 oocytes at room temperature. Amiloride-sensitive currents were derived by subtracting the currents recorded for 10 μmol/L amiloride from the preamiloride currents.

Immunohistochemistry for Human Kidney Using Rabbit Polyclonal Antibodies Against the Common C2 Domain

A 14-aa synthetic peptide corresponding to amino acids 57 to 70 of the C-terminal tail of human Nedd4L was produced by standard solid-phase peptide synthesis techniques. Analysis with BLAST search computer programs showed no significant overlap of the immunizing peptide with any known eukaryotic protein. The peptide was purified, conjugated, and injected 4 times intradermally into rabbits at 2-week intervals to produce polyclonal antiserum. The rabbits had ELISA titers of >1:128 000 before exsanguinations. The selectivity of the antiserum was validated by the recognition of pcDNA3.1-isoform I/pcDNA3.1-isoform II-transfected HEK293T cells by Western blot analysis. Anti-Nedd4L polyclonal antibodies were affinity-purified and used in the present study. Immunohistochemical analysis of the human kidney sections was performed with biotinylated goat antirabbit IgG (Aion Laboratories, San Diego, CA) and injected 4 times intradermally into rabbits at 2-week intervals to produce polyclonal antiserum. The rabbits had ELISA titers of >1:128 000 before exsanguinations. The selectivity of the antiserum was validated by the recognition of pcDNA3.1-isoform I/pcDNA3.1-isoform II-transfected HEK293T cells by Western blot analysis. Anti-Nedd4L polyclonal antibodies were affinity-purified and used in the present study. Immunohistochemical analysis of the human kidney sections was performed with biotinylated goat antirabbit IgG (Aion Laboratories, San Diego, CA) and injected 4 times intradermally into rabbits at 2-week intervals to produce polyclonal antiserum.

Cellular Localization of Human Nedd4L Isoform I by Fluorescent Confocal Microscopy During Calcium Stimulation

mLMD-3 cells were seeded on 24-well plates the day before gene transfection. The His-tagged Nedd4L isoform I in pcDNA3.1 was transiently transfected in mLMD-3 cells, using FuGEN6 (Roche Applied Science). Forty-eight hours after transfection, the cells were divided into 2 groups and exposed to different conditions. One group of cells was treated with CaCl2 (1 mmol/L) plus ionomycin (1 mmol/L) for 10 min, and the other was untreated to serve as a control. Subsequently, the cells were fixed and permeabilized with 2% paraformaldehyde and 0.1% Triton X-100. Nedd4L isoform I was detected with mouse anti-His antibody and Alexa Fluora 488-labeled anti-rabbit IgG (1:128 000) and Alexa Fluora 594-labeled anti-mouse IgG (1:128 000) before exsanguinations. The selectivity of the antiserum was validated by the recognition of pcDNA3.1-isoform I/pcDNA3.1-isoform II-transfected HEK293T cells by Western blot analysis. Anti-Nedd4L polyclonal antibodies were affinity-purified and used in the present study. Immunohistochemical analysis of the human kidney sections was performed with biotinylated goat antirabbit IgG (Aion Laboratories, San Diego, CA) and injected 4 times intradermally into rabbits at 2-week intervals to produce polyclonal antiserum.

Statistical Analysis

Values are expressed as means±SE in both the text and figures. The data were analyzed by analysis of variance. If a statistically significant effect was found, a post hoc analysis (Tukey HSD post hoc test) was performed to detect differences between the groups. Values of P<0.05 were considered to indicate statistical significance.

Results

Cloning of Human Nedd4L Isoforms

Figure 1 shows representative findings of immunoblotting for the human Nedd4L isoforms I, II, and III, respectively. The molecular weight of each protein product was exactly as expected.

Figure 1. Western blot analysis of the Nedd4L isoforms. Representative immunoblots of the 3 isoforms of human Nedd4L expressed in HEK293T cells stained with anti-His(C-term) antibody (Invitrogen). The estimated sizes of the products were consistent with those of the 3 isoforms, as shown in the picture. (The expected molecular weights are 94 kDa for both isoforms I and II and 84 kDa for isoform III.)

Cell line: HEK293T
Antibody: anti-His(C-term)-antibody (Invitrogen, San Diego, CA)
Isoform I Is Expressed Predominantly in the Kidney and Isoform II Predominantly in the Lung

Figure 2 shows the results of quantitative analyses of the expression of the mRNA of human Nedd4L isoforms I and II in the kidney, lung, brain, liver, and colon. Human isoform I was significantly more abundantly expressed in the kidney ($P<0.001$), whereas human isoform II was significantly more abundantly expressed in the lung ($P<0.001$).

Dominant Negative Interactions of Human Nedd4L Isoform I Protein With the Two Other Isoforms

To study the effect of the 3 human Nedd4L isoforms on Na transport, we expressed ENaC with or without each Nedd4L isoform in xenopus oocytes followed by whole cell voltage clamp and amiloride-sensitive current measurements. We found that Nedd4L isoform II and isoform III both robustly reduced the ENaC present (bar graphs 3 and 5). In contrast, Nedd4L isoform I did not reduce the ENaC present (bar graph 2), but suppressed Nedd4L isoform II and III activities for downregulating ENaC, because the ENaC current for both isoforms II and III was restored by coinjection of isoform I (bar graphs 4 and 6). These results show that both human Nedd4L isoforms II and III can interact with and downregulate ENaC activities in a reconstituted system, whereas human Nedd4L isoform I has significant antagonistic activity against the 2 other isoforms (Figure 3).

Human Nedd4L Protein With C2 Domain Is Expressed Along the Aldosterone-Sensitive Distal Nephron

Panels A to D of Figure 4 show representative findings of the immunohistochemical analyses of the human kidney, using polyclonal antibodies against the common C2 domain. Positive staining was observed in the connecting tubules and cortical collecting ducts, identified as clusters of tubular sections near radial veins in the cortical labyrinths both the
inner-medullary and outer-medullary collecting ducts were simultaneously stained. We also performed nonimmune IgG staining of the human kidney but obtained no specific staining with this method (data not shown).

**Human Nedd4L Isoform I Was Targeted to the Cell Surface Membrane by Calcium Overload**

Figure 5 shows representative findings for human Nedd4L isoform I on confocal fluorescence microscopy after calcium overload. Cytosomal expression of human Nedd4L isoform I without any stimulation is shown in panel A. After calcium overload stimulation, cellular membrane staining was enhanced (panel B), suggesting membrane targeted binding of the protein.

**Discussion**

Our study yielded 5 main findings and observations. (1) Three human Nedd4L isoforms were successfully cloned, and transcriptions of the gene were also successfully confirmed in vitro in a cultured cell line. (2) Although isoforms I and II both had the C2-calcium–dependent membrane binding domain, they were expressed in different manners in various human tissues. (3) Using a Xenopus oocyte heterologous gene expression system, we found an antagonistic effect of the human Nedd4L isoform I product over the 2 other isoform products for reducing the ENaC current. (4) In the kidney, C2-containing isoforms were expressed along the aldosterone-sensitive distal nephron (ASDN), in which ENaCs are thought to be expressed. (5) Using a confocal fluorescence microscopic technique, we found that the novel C2-containing isoform I was mobilized to the cell surface membrane after intracellular calcium overload.

The possibility that Na reabsorption in the distal tubule contributes to high blood pressure was first suggested by an analysis of inherited high blood pressure by Lifton et al.\(^{11-14}\) It is generally accepted that blood pressure rises because of increased peripheral vascular resistance attributable to an excess of body fluid, which takes place against the background of accelerated sodium reabsorption by the ion transporter for which is distributed in the distal tubule. Such a model may represent the mechanism underlying sodium sensitivity in essential hypertension.

As for the ENaC-related type, which exhibits amiloride sensitivity, the mechanism for developing high blood pressure is clear from the excessive appearance of ENaC on the apical side of the urinary tubule, as is especially evident in Liddle syndrome.\(^{15-17}\) Ubiquitination of the Lys residues on the N terminus of ENaC, mediated by the WW domain of Nedd4L, binding to the PY motif in the C terminus, has an important role in the accumulation and retrieval of ENaC from the cell surface,\(^{15-17}\) and a gene mutation has been discovered in the PY motif in Liddle syndrome.\(^{13,14,18}\) Ubiquitination was abolished by the presence of a gene mutation in the PY motif, and ENaCs continuously appeared on the apical side of the urinary tubule. The dominance of Nedd4L as a ubiquitination enzyme was initially based on the discovery of interactions between the PY motif and Nedd4.\(^{15}\) Nedd4L is now considered the main ubiquitination enzyme for ENaC.\(^{19-22}\)

Dunn and Ishigami et al previously demonstrated the existence of the C2 domain of human Nedd4L by applying resequencing and bioinformatic methods to the genomic DNA of the human Nedd4L gene.\(^{4}\) There is a peculiar isoform of Nedd4L in humans, resulting from a single-nucleotide polymorphism (SNP) at the end of exon 1. A significant relation between this SNP and blood pressure was recently demonstrated by 24-hour ambulatory blood pressure monitoring,\(^{5}\) and we have since independently shown a similar relation in a Japanese man.\(^{8}\) Available evidence thus suggests that one of the isoforms of the human Nedd4L gene is related to the development of essential hypertension. This relation is supported by findings obtained by our laboratory and others,\(^{23}\) showing that the Nedd4L gene and Nedd4L protein are involved in the appearance of high blood pressure with sodium sensitivity in a rodent model of hypertension. Therefore, our study analyzed the expression, appearance, and protein interactions of the human Nedd4L gene isoform I in detail. Quantitative PCR showed that isoform I was abundantly expressed in the kidney, whereas isoform II was mainly expressed in the lung. Because ENaC is expressed in lung, kidney, and colon epithelial cells, the control of ENaC in these organs might be differentially regulated by different isoforms. The ENaC current was also measured using a heterologous gene expression system in Xenopus oocytes, and interactions among the different isoforms of Nedd4L were examined. Immunohistochemical analysis of kidney sections showed that human Nedd4L protein was expressed in the ASDN, where ENaC is thought to be the major ion transporter for sodium reabsorption. This finding suggested that ENaC and Nedd4L protein might be to be colocalized functionally as well as anatomically. We performed immunohistochemical analysis in humans for the first time, although we had previously studied the organization of the Nedd4L gene/protein by means of in situ hybridization/immunohistochemistry analyses in rodents and by in situ hybridization in the human kidney.\(^{19}\) The protein was found only in the ASDN with fair staining, similar to our previous observations for transcripts of the Nedd4L gene in humans.

Phylogenetic analysis has demonstrated that the Nedd4L isoform II has the ancestral C2 domain.\(^{4}\) This conserved C2
domain can reportedly mobilize to the cell surface membrane, increasing the density of calcium in the cell.24,25 In this study, mobilization of human Nedd4L isoform I to the cell surface was confirmed to take place in a calcium-dependent manner, although the N-terminal amino acid sequence differs between isoforms I and II. Whether this conserved cellular membrane-targeting property influenced the withdrawal of ENaC from the cell surface remains unclear. This issue needs to be addressed in a different manner to clarify the effects of calcium in vitro as well as in vivo.

The possibility that retrieval or downregulation of ENaC by isoform II/III protein is controlled by Nedd4L isoform I was suggested by the results of experiments in xenopus oocytes. However, our experiments were limited, and our results in this experimental system cannot be directly extrapolated to humans.

Taken together, our findings provide possible evidence that the human Nedd4L gene is a candidate gene for hypertension. Isoform I protein appears to be an evolutionarily new peptide caused by a uniquely human SNP, variant 13(G/A) (rs4149601). These proteins have a potential property to move to cellular membrane by calcium stimulation as isoform II have, and is expressed in the ASDN of kidneys. The expression of ENaC might be relatively increased by interactions with isoforms II, isoform III, or both, appearing in the same cells in association with the high blood pressure syndrome.

**Perspectives**

Future detailed analyses of the pathophysiological relevance of the Nedd4L gene in essential hypertension are expected to shed light on the mechanism underlying the activation of human Nedd4L isoform I, leading to the development of medicinal tools capable of modifying the ENaC-Nedd4L-proteasome axis in tubular epithelium. Therapeutic interventions targeting the ENaC-Nedd4L-proteasome system in the high blood pressure syndrome are based on the premise that a functional abnormality is present at the molecular level. Such new treatments might hopefully improve the outcomes of patients with cardiovascular events associated with essential hypertension.

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**Disclosures**

None.

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