**K⁺-dependent Na⁺/Ca²⁺ exchanger 3 is involved in renal active calcium transport and is differentially expressed in the mouse kidney**

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Submitted 17 October 2008; accepted in final form 25 May 2009


The kidney plays an important role in the maintenance of calcium balance in the body by regulating calcium reabsorption and excretion. Calcium ions are filtered daily by the glomeruli, and <2% are excreted into the urine (14). The majority of Ca²⁺ reabsorption along the nephron occurs via a passive paracellular Ca²⁺ transport mechanism in the distal part of the proximal tubules and the thick ascending limb of the loop of Henle. Approximately 20% of Ca²⁺ is reabsorbed via an active transcellular Ca²⁺ transport mechanism in the distal part of the nephron (3, 13–15). Paracellular Ca²⁺ transport is driven by the transepithelial electrochemical gradient that is generated by sodium and water reabsorption and is indirectly regulated by hormones (14). In contrast, the transcellular pathway allows the body to regulate Ca²⁺ reabsorption independently of Na⁺ balance. This pathway is specifically controlled by the calctropic hormones, including parathyroid hormone (PTH), calcitonin, and 1,25-dihydroxy vitamin D₃ [1,25(OH)₂D₃] (3, 13–15). Due to this active process, an organism can respond to fluctuations in dietary Ca²⁺ and adapt to changes in demand during certain processes, i.e., growth, pregnancy, lactation, and aging (14). Disturbances in active Ca²⁺ reabsorption are most likely to be accompanied by significant alterations in overall Ca²⁺ homeostasis (14).

At the cellular level, active calcium transport can be divided into three functional steps, Ca²⁺ influx into cells, transfer through the cytosol, and extrusion into the bloodstream, which are mediated by three types of proteins: 1) the calcium entry-channel proteins of the outer membrane; 2) cytosolic buffering or transfer proteins; and 3) excretory pump proteins (9, 11, 14, 44). Two highly selective calcium channels at the apical sides of cells, members of the transient receptor potential (TRP) superfamily of ion channels (TRPV6 and TRPV5), are the main calcium ion entry channels (14, 37). Calbindin-D₉k and -D₂₈k (CaBP-28k) are intracellular calcium ion-binding proteins that are thought to participate in shuttling calcium ions from the apical to the basolateral membrane, where the Na⁺/Ca²⁺ exchanger (NCX) and plasma membrane Ca²⁺-ATPase 1b (PMCA1b) mediate Ca²⁺ extrusion (9, 10).

There has been great progress in our understanding of the mechanism of active calcium transport and its role in calcium-related disorders, such as hypocalcemia, rickets, and osteomalacia, using vitamin D receptor-null mice and 1α-hydroxylase-deficient mice (43, 46). The role of several calcium-processing proteins of the active calcium transport system, including TRPV5/6, CaBP-9k/28k, PMCA1b, and NCX1, has been verified recently using gene knockout studies. Previously, we reported on the phenotype of CaBP-9k-null mice and the effect of compensatory gene induction of calcium-related genes in these mice (29). Interestingly, several renal calcium-processing genes were highly expressed in both wild-type and CaBP-9k-null female mice in the absence of any treatment. At the time of that study, we did not examine differential expression in male and female kidneys. In the current study, we examined whether calcium-processing genes are differentially expressed in male and female mice. We also examined whether the regulation of active calcium transport genes in male and female mice correlated with development and identified a potential mechanism of regulation of the gender-specific pattern of expression of these genes.

**MATERIALS AND METHODS**

Materials. DMSO was purchased from Amresco (Solon, OH). Flutamide (catalog no. F9397), an androgen receptor-specific antagonist, RU486 (catalog no. M8046, a progesterone and gluocorticoid receptor-specific antagonist), 17β-estradiol (catalog no. E8875), progesterone (catalog no. P3972), 1,25-dihydroxyvitamin D₃ (catalog no. C9756), PTH (catalog no. P3921), aldosterone (catalog no. A9477), and hydrocortisone...
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(catalog no. H4001) were obtained from the Sigma-Aldrich (St. Louis, MO). ICI 182,780 (catalog no. 1047, an estrogen receptor antagonist) was purchased from Tocris (Ellisville, MO).

Experimental animals and treatments. Male and female mice (C57BL/6 and ICR) were obtained from KOATECH (Pyeongtaek-si, Gyeonggi-do, Korea). All animals were housed in polycarbonate cages and used after acclimation to an environmentally controlled room (temperature: 23 ± 2°C, relative humidity: 50 ± 10%, frequent ventilation, and a 12:12 light-dark cycle).

Male and female mice (C57BL/6, 10 wk old, n = 6/group) were euthanized, and tissue samples from the kidney and whole brain were obtained for microarray analysis, RT-PCR, real-time PCR, and Western blot analysis. To determine the levels of expression of renal K+-dependent Na+/Ca2+ exchanger 3 (NCKX3) during development, male and female mice (ICR, n = 5/group) were divided into eight groups (1, 3, and 5 days and 1, 2, 4, 10, and 15 wk of age). On the indicated day, mice were euthanized and tissue samples from the kidney were isolated for real-time PCR analysis.

Where indicated, chemicals were dissolved in DMSO and then injected subcutaneously into 9.5-wk-old ICR mice of both genders daily for 3 days. Where indicated, groups of mice were subjected to a bilateral adrenalectomy (ADX) and/or an ovariectomy (OVX) at 8.5 wk of age and then allowed to heal for 1 wk before treatment (25). To determine the effect of steroid receptor antagonists on renal NCKX3 expression, flutamide (5 mg/kg), RU486 (10 mg/kg), and ICI 182,780 (catalog no. 1047, an estrogen receptor antagonist) was purchased from Tocris (Ellisville, MO). ICI 182,780 (catalog no. 1047, an estrogen receptor antagonist) was injected subcutaneously into 9.5-wk-old ICR mice of both genders daily for 3 days. Where indicated, groups of mice were subjected to a bilateral adrenalectomy (ADX) and/or an ovariectomy (OVX) at 8.5 wk of age and then allowed to heal for 1 wk before treatment (25). To determine the effect of steroid receptor antagonists on renal NCKX3 expressions, flutamide (5 mg/kg), RU486 (10 mg/kg), and ICI 182,780 (10 mg/kg) were injected into male and female ICR mice (17, 26, 28). To determine the effect of sex steroids on renal NCKX3 expression, estrogen (40 µg/kg) and progesterone (4 mg/kg) were injected into male and OVX female mice (17, 26, 28). The hormones 1,25(OH)2D3 (1 µg/kg) and PTH (0.1 mg/kg) were administered to male and female mice (5, 18, 42, 45), and aldosterone (1.25 mg/kg) and hydrocortisone (12 mg/kg) were injected into ADX and/or OVX mice (40). All mice were euthanized 24 h after the final injection.

To determine the effect of dietary calcium on renal NCKX3 expression, 10-wk-old male and female ICR mice were fed a normal diet (DYET 113295, AIN-76A purified rodent diet, Dyets, Bethlehem, PA) or a low-calcium diet (DYET 113294, AIN-76A purified rodent diet, Dyets, Bethlehem, PA) according to the manufacturer's instructions.

RNA extraction and PCR. Mice were euthanized and the kidneys were rapidly excised and washed in cold sterile saline (0.9% NaCl). Total RNA was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA), and the concentration of RNA was determined by absorbance at 260 nm. Isolated total RNA (10 µg) was subjected to electrophoresis on a 1% formaldehyde denaturing agarose gel for 1 h at 110 V. The 28S and 18S rRNAs were analyzed using the Quantity One program (Gel Doc EQ, Bio-Rad, Hercules, CA) and served as an indicator of the quantity of total RNA. Reverse transcription was performed as described previously (24, 27). In brief, total RNA (1 µg) was reverse-transcribed to first-strand cDNA using murine Moloney leukemia virus reverse transcriptase (Invitrogen) and random primers, according to the manufacturer's protocol.

To determine the parameters for logarithmic-phase PCR amplification of NCKX3 mRNA, aliquots (5 µl) of RNA were amplified using different numbers of cycles. The β-actin gene was also amplified as a control for RNA degradation and mRNA concentration. A linear relationship between amplified product and cycle number was observed for NCKX3 and β-actin mRNA using 30 and 20 cycles, respectively. The cDNA was amplified in a 20-µl reaction volume containing 1 U Taq polymerase (iNtRON Biotechnology, Sungnam, Kyungki-Do, Korea), 1.5 mM MgCl2, 2 mM deoxy-NTP, and 50 pmol of each primer. The following cycle parameters were used: denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s. The oligonucleotide sequences of the NCKX3-specific primers were 5'-TAT GGC TGT GTC CAA TTC CA-3' (sense) and 5'-ACA CAT GGG CAA GTT CAC AA-3' (antisense). The expected size of NCKX3 transcripts is 307 bp, and its PCR product was cloned into TA vector (TOPO TA Cloning kit, Invitrogen) for nucleotide sequencing (24). The cloned NCKX3 transcripts were sequenced by using M13 reverse and T7 primers (Genotech, Daejeon, Korea). The oligonucleotide sequences of β-actin-specific primers were 5'-AGC CAT GTG CTA CGC CAT CC-3' (sense) and 5'-TTT GAT GTC ACG CAC GAT TT-3' (antisense). Amplified products (10 µl) were fractionated on a 2% agarose gel, stained with ethidium bromide, and then photographed under UV illumination. The photograph was scanned, and DNA levels were quantified using the Quantity One program (Gel Doc Eq, Bio-Rad).

Real-time PCR was carried out in a 20-µl reaction volume containing 10 µl of SYBR Premix Ex Taq (TaKaRa Bio, Otsu, Shiga, Japan), 0.5 µl of 100 pM specific primers, and 5 µl of cDNA. Amplification was conducted using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) and the following parameters: denaturation at 95°C for 5 min; then 40 cycles of denaturation at 95°C for 30 s, followed by annealing and extension at 58°C for 30 s and 72°C for 45 s, respectively. The relative expression level of NCKX3 (normalized to that of β-actin) for each sample was determined using RQ software (Applied Biosystems).

Microarray assay. The microarray assay was performed by using a commercial microarray supporting service (E-Biogen, Seoul, Korea; http://www.e-biogen.com). For control and test RNAs, the synthesis of cRNA probes and hybridization was performed using Agilent's Low RNA Input Linear Amplification kit (Agilent Technology, Santa Clara, CA), according to the manufacturer's instructions. Briefly, 1 µg of total RNA and T7 promoter primer mix were incubated at 65°C for 10 min. The cDNA master mix (5× first-strand buffer, 0.1 M DTT, 10 mM dNTP mix, RNase-Out, and murine Moloney leukemia virus-RT) was prepared and then added to the RNA. The samples were incubated at 40°C for 2 h, and then RT and dsDNA synthesis were terminated by incubation at 65°C for 15 min.

The transcription master mix was prepared according to the manufacturer's protocol (4× transcription buffer, 0.1 M DTT, NTP mix, 50% PEG, RNase-Out, inorganic pyrophosphatase, T7-RNA polymerase, and cyanine 3-CTP). Transcription of dsDNA was initiated by the addition of the transcription master mix to the dsDNA reaction products, followed by incubation at 40°C for 2 h. The amplified and labeled cRNAs were purified on an RNase mimicolumn (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Labeled cRNA was quantified using an ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE).

After labeling efficiency was determined, cyanine 3-labeled cRNA was fragmented by the addition of 10× blocking agent and 25× fragmentation buffer, followed by incubation at 60°C for 30 min. The fragmented cRNA was resuspended in 2× hybridization buffer and then applied with a pipette directly onto an Agilent Whole Mouse Genome Oligo Microarray (Agilent Technology). The arrays were hybridized at 65°C for 17 h using an Agilent Hybridization oven (Agilent Technology). The hybridized microarrays were washed using the manufacturer's protocol (Agilent Technology).

The microarrays were analyzed using GenePix Pro 6.0 (Axon Instruments, Foster City, CA). The average fluorescence intensity for each spot was calculated, and local background was subtracted. All data normalization and the selection of fold-changes were performed using GeneSpring 7.3.1 (Agilent Technology). Intensity-dependent normalization (Lowess) was performed, where the ratio was reduced to the residual of the Lowess fit of the intensity-vs.-ratio curve. The averages of the normalized ratios were calculated by dividing the average normalized signal channel intensity by the average normalized control channel intensity.

Western blot analysis. Protein was extracted using Proprep (iNtRON Biotechnology), according to the manufacturer's instructions. Total protein (50 µg/lane) was resolved by 8% SDS-PAGE and then transferred to a nitrocellulose membrane using a Trans-Blot Cell (Bio-Rad), according to the manufacturer's protocol. The membrane was blocked overnight with PBS containing 0.05% Tween 20 (PBS-T)
and 5% skim milk. The membrane was then incubated with the indicated primary antibodies diluted in 1% BSA for 2 h at room temperature. Primary antibodies for NCKX3 (72-kDa; catalog no. sc-50129, diluted 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (1:1,000; Santa Cruz Biotechnology) were used. Horse-radish peroxidase-conjugated anti-goat IgG and anti-rabbit IgG (1:3,000, diluted in 1% BSA, Santa Cruz Biotechnology) were used as the secondary antibodies for NCKX3 and β-actin, respectively. The membrane was incubated with Western Lighting Chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA), according to the manufacturer’s protocol, to visualize the immunoreactive proteins. Signals were detected using Chemi Doc EQ (Bio-Rad).

Immunohistochemical staining. The localization of NCKX3 was examined by immunohistochemistry. Kidneys from 10-wk-old female ICR mice were embedded in paraffin. Sections (8 μm thick) were deparaffinized in xylene and then hydrated in descending grades of ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS-T for 20 min. Sections were incubated in 10% normal goat serum for 2 h at room temperature to block nonspecific binding. The sections were incubated with a polyclonal goat anti-NCKX3 antibody (diluted 1:50, Santa Cruz Biotechnology) in 10% normal goat serum at 4°C overnight. After washing with PBS-T, the sections were incubated with biotinylated secondary antibody (goat IgG, Vector Laboratories, Burlingame, CA) for 30 min at 37°C, and immunoreactive proteins were detected by incubation with ABC-Elite for 30 min at 37°C. Diaminobenzidine (DAB; Sigma-Aldrich) was used as a chromogen. Sections were counterstained with hematoxylin, followed by mounting with a coverslip.

Data analysis. Data were analyzed by nonparametric one-way ANOVA using the Kruskal-Wallis test, followed by Dunnett’s test for multiple comparisons with vehicle. Values were converted to ranks for tests. Statistical analysis was performed using SPSS for Windows Edition (SPSS Inc, Chicago, IL). A P value of <0.05 was considered statistically significant.

RESULTS

Sex-dependent differences in expression of renal calcium-processing genes. The renal expression of calcium-processing genes in mature male and female mice was compared using microarray analysis (Table 1). The related genes for calcium processing were briefly verified by general RT-PCR in the identical samples from the microarray (Fig. 1A). The expression of NCKX3/solute carrier family 24 member 3 (Slc24a3) was three times higher in the female kidney than in the male kidney. NCKX3 belongs to four ontological categories: calcium ion homeostasis, calcium ion transport, cellular calcium ion homeostasis, and cytoplasmic calcium ion homeostasis.

Expression of renal NCKX3 in male and female mice. The predicted structure of NCKX3 contains several integral membrane regions, and it is believed to be involved in the regulation of calcium ion transport and cellular calcium ion homeostasis. As shown in Fig. 1B, the protein levels of renal NCKX3 were differentially expressed in male and female mice. Quantitative analysis of renal NCKX3 transcription was carried out using real-time PCR. Renal NCKX3 transcripts in female mice were three times higher than in male mice (Fig. 1C). When we examined NCKX3 mRNA expression in the brain, where expression is the highest (19), there were no differences in mRNA levels between male and female mice (Fig. 1D). These results indicated that renal expression of NCKX3 is differentially regulated in male and female mice and that the expression of NCKX3 in the kidney is significantly lower than expression in the brain in both male and female mice.

While NCKX3 has been implicated in active calcium transport, its function in the kidney has not been fully elucidated. To begin to understand the role of NCKX3 in the kidney, tissue sections were subjected to immunohistochemistry using normal goat serum as a negative control (Fig. 2A) and an anti-NCKX3 antibody (Fig. 2B). NCKX3 localized to the basolateral layer of the distal convoluted tubules in the female kidney. Protein expression was not detected in the glomerulus or proximal convoluted tubules. The spatial distribution of renal NCKX3 in male mice was not significantly different than in females, but the density of anti-NCKX3–positive signals in the male kidney was significantly lower than in the female (data not shown). These results indicated that renal NCKX3 localizes to sites of active calcium transport in the kidney and may participate in calcium reabsorption along with other calcium-processing proteins such as TRPVs, CaBPs, PMCA1b, and NCX1 (25, 32, 37).

Developmental changes in renal NCKX3 gene expression. To determine whether renal NCKX3 expression was developmentally regulated, we measured mRNA levels in the kidneys of male and female mice ranging in age from 1 day to 15 wk. As seen in Fig. 3A, during infancy (1 day to 2 wk), NCKX3 mRNA levels were similar between genders and were six times higher than in the adult (10 wk of age) male mouse. Renal NCKX3 mRNA levels decreased between 2 and 4 wk of age in both sexes and then began to diverge. Expression continued to decrease in male mice with age, whereas in female mice the levels of renal NCKX3 mRNA were maintained. At 10 wk of age, renal NCKX3 mRNA expression was significantly down-regulated in males compared with females. In parallel with NCKX3 mRNA expression, its protein levels of renal NCKX3 were similarly expressed at 4 wk in females; however, its levels in males were quite lower than those in females at 10 wk (Fig. 3B). These results indicated that the differential expression of NCKX3 in males and females correlates with adult development, i.e., 10 wk of age and older.

Regulation of renal NCKX3 expression. Renal NCKX3 expression decreased in an age-dependent manner in both male and female mice (Fig. 3). NCKX3 mRNA in both genders

Table 1. Sex dependently regulated genes related to calcium processing

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>GenBank ID</th>
<th>Probe ID (Agilent)</th>
<th>Fold-Induction of Microarray (vs. Males)</th>
<th>Fold-Induction of RT-PCR (vs. Males)</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>Expected Size, bp</th>
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<tr>
<td>NCKX3 (Slc24a3)</td>
<td>NM_053195</td>
<td>A_51_P268496</td>
<td>2.84 ± 0.22</td>
<td>3.41 ± 1.13</td>
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<td>acacatgaggaatttc</td>
<td>307</td>
</tr>
<tr>
<td>Ibk</td>
<td>AK077387</td>
<td>A_52_P311491</td>
<td>2.56 ± 0.56</td>
<td>ND</td>
<td>aaaaaacctgctgcct</td>
<td>ttcatgagagcctt</td>
<td>260</td>
</tr>
<tr>
<td>Best2</td>
<td>NM_145388</td>
<td>A_51_P653578</td>
<td>0.18 ± 0.18</td>
<td>ND</td>
<td>gggtgtaagagacat</td>
<td>aagctaatctgctc</td>
<td>250</td>
</tr>
<tr>
<td>Atp2a3</td>
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<td>A_52_P262209</td>
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<tr>
<td>Prss2</td>
<td>NM_009430</td>
<td>A_51_P418901</td>
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<td>0.86 ± 0.23</td>
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<td>tcaacaactgaggagc</td>
<td>279</td>
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</tbody>
</table>

Values are means ± SD. Fold-induction of microarray results from 2 individual analyses is shown. Fold-induction of RT-PCR is attested by the same RNA sample of the microarray analyses. ND, nondetected by general RT-PCR.

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was downregulated during prepuberty (2–4 wk of age), and in male mice was further decreased during puberty (4–10 wk of age). These results suggested that hormonal factors, in particular, sex steroid hormones, might be involved in the differential regulation of renal NCKX3 expression during puberty in male and female mice and that other calcium factors, such as 1,25(OH)2D3, PTH, and dietary calcium, might be involved during the prepubertal stage.

To determine whether hormonal or other calcium factors were involved in the developmental regulation of NCKX3,

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Fig. 1. Expression of K⁺/H⁺-dependent Na⁺/Ca²⁺ exchanger 3 (NCKX3) mRNA and protein in the kidney and brain. Microarray data were confirmed by a general RT-PCR using the same RNA samples (307 bp of NCKX3 PCR products; A). NCKX3 expression was examined in male and female mice (10 wk of age, n = 6/group). The protein expressions of NCKX3 (72 kDa of NCKX3 protein; B) were attested to by Western blot analysis. The relative levels of NCKX3 mRNA expression in the kidney (C) and brain (D) were also analyzed by real-time PCR. NCKX3 expression was normalized to that of β-actin, and renal expression in male mice was set as 100%. Values are means ± SD of duplicate experiments. *P < 0.05 compared with renal expression in male mice.

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Fig. 2. Localization of renal NCKX3. The localization of renal NCKX3 was examined by immunohistochemistry [normal goat serum as a negative control (A); polyclonal goat anti-NCKX3 antibody (B)]. Positive signals are indicated in brown. Red asterisks, distal convoluted tubules; g, glomerulus.
Differential renal expression of NCKX3 gene in female mice

9.5-wk-old male and female mice were treated with steroids or steroid receptor antagonists. Androgen (fluamide), progesterone (RU486), and estrogen (ICI 182,780) receptor antagonists were injected daily into mice for 3 days, and then renal NCKX3 expression was analyzed by real-time-PCR. The expression of NCKX3 was higher in female mice than in male mice, but there were no differences between the control and treatment groups in male or female mice (Fig. 4A). Similarly, treatment of male mice and OVX female mice with estrogen (E2) or progesterone (P4) daily for 3 days had no effect on renal NCKX3 expression (Fig. 4B). These results suggested that sex steroids are not involved in the differential regulation of renal NCKX3 expression in male and female mice.

The active calcium transport system is regulated by the hormonal form of vitamin D3 \([1,25(OH)_{2}D_{3}]\) and/or PTH, which have been shown to regulate the expression of renal calcium-processing genes (7, 10). PTH and 1,25(OH)2D3 were injected daily into mice for 3 days, and renal NCKX3 expression was measured by real-time PCR. The expression of NCKX3 in female mice was three times higher than in male mice, but there were no differences among the control and treatment groups of male or female mice (Fig. 4C). To determine whether dietary calcium, which is a major regulator of genes involved in active calcium transport (29), affected the expression of NCKX3, 10-wk-old male and female mice were fed a low-calcium diet for 5 wk, and then renal NCKX3 expression was examined. The levels of NCKX3 transcription in the kidneys of male mice were three times lower than in female mice at 15 wk of age, and there was no effect of dietary calcium deficiency (Fig. 4D).

Glucocorticoids and mineralocorticoids from the adrenal gland have been shown to affect the regulation of genes involved in active calcium transport and related processes (16, 25). To determine whether adrenal factors affected the expression of NCKX3 in the kidney, ADX and/or OVX mice (9.5 wk old) were treated daily with hydrocortisone or aldosterone for 3 days. As shown in Fig. 5A, treatment with either hormone significantly downregulated renal expressions of NCKX3 mRNAs in female mice but had no effect on its levels in male mice. On the other hand, the protein levels of NCKX3 were not affected by treatment with the two hormones, as seen in Fig. 5B.

**DISCUSSION**

Previously, we observed that renal active calcium transport genes were highly expressed (by ~20–100%) in female mice compared with male mice (29). We hypothesized that the elevated renal expression of calcium-processing genes in females is required to maintain normal calcium reabsorption. Women tend to suffer from a calcium imbalance after menopause, developing such conditions as postmenopausal osteoporosis, because of a deficiency in estrogen, which is a positive regulator of calcium-processing genes. In the current study, we demonstrated the differential expression of a gene involved in calcium processing in the kidneys of male and female mice.

In the current study, using microarray analysis, we demonstrated that NCKX3 is highly expressed in the female kidney. NCKX3 is a member of the family of plasma membrane \(\text{Na}^{+}/\text{Ca}^{2+}\) exchangers (NCX) (19). The genes that encode NCX comprise two families in mammalian tissues, sodium-dependent NCX (NCX1–3), and potassium-dependent NCKX (NCKX1–6) (12). Both NCX and NCKX mediate \(\text{Ca}^{2+}\) influx in response to \(\text{Na}^{+}\), \(\text{Ca}^{2+}\), and \(\text{K}^{+}\) membrane gradients and plasma membrane potential (4, 34). NCX1 is abundantly expressed in the heart, brain, kidney, and smooth muscle (35), while NCX2 and NCX3 are predominantly expressed in the brain and skeletal muscle (31, 36). NCKX1 is expressed only in retinal rod photoreceptors, whereas NCKX2 expression is restricted to brain neurons and cone photoreceptors (34). NCKX3 and NCKX4 are expressed not only in the brain but also in many other tissues, including the aorta, uterus, and intestine, which are rich in smooth muscle cells (6, 19, 30). NCKX5 has recently been described in skin and retinal pigmented epithelium, where it is thought to localize to the...
melanosome membrane rather than to the plasma membrane (22). NCKX6 has also been characterized, but the physiological function of this protein remains controversial (6, 39). The tissue distribution of \( \text{NCKX3} \) was previously examined by Northern blot analysis, and it was shown that \( \text{NCKX3} \) transcripts are abundant in various regions of the brain, as well as in several tissues that are rich in smooth muscle (aorta, intestine, and lung) (19). Relatively lower levels of transcripts were found in a variety of other tissues, whereas expression in the kidney and liver was essentially negative (19). In the current study, we demonstrated that \( \text{NCKX3} \) is expressed in the kidney and that renal expression is significantly lower than in the brain. Although it was previously reported that \( \text{NCKX3} \) transcripts were not present in the kidney (19), we detected \( \text{NCKX3} \) transcripts and protein in the kidney using RT-PCR and Western blot analysis. We also observed that renal \( \text{NCKX3} \) was differentially expressed in male and female mice. Real-time PCR showed that in female mice, the level of renal \( \text{NCKX3} \) transcripts was three times higher than in male mice. However, in the brain, which has the highest level of \( \text{NCKX3} \) expression, there were no differences between the genders. Thus \( \text{NCKX3} \) is expressed in the kidney and is particularly abundant in female mice.

\( \text{NCKX3} \) localized primarily to the basolateral layer of the distal convoluted tubules, and there was no detectable expression in the glomerulus and proximal convoluted tubules in the kidney. The distal convoluted tubules have been shown to express several calcium transport proteins, such as TRPV5/6, CaBP-9k/-28k, PMCA1b, and NCX1 (32, 37). TRPV5 and -6 are involved in calcium influx into cytoplasm (10), and these proteins have been detected in the apical domain of the distal convoluted tubules and connecting tubules (37). CaBP-9k and -28k facilitate cytosolic calcium transport (10) and are expressed in the cytoplasm of tubules (32). PMCA1b and NCX1 transfer calcium ions into the blood vessels and are distributed on the basolateral layer of the distal nephron (32). These proteins are thought to function collectively as a gatekeeper for active calcium transport in the kidney and intestine (10, 14, 29). Renal \( \text{NCKX3} \) was present in the basolateral layer of the distal convoluted tubules, suggesting that it functions similarly.
to PMCA1 and NCX1 in the regulation of calcium transport in the kidney. The renal NCKX3 gene is highly expressed in the neonatal period and decreases at 4 wk without any differential expression between genders. After that, the renal NCKX3 gene continuously decreases in males but not in females. Other calcium-processing genes are variably expressed during development. Renal TRPV5, CaBP-9k, and CaBP-28k transcription reaches a peak at 3 wk of age and declines thereafter (42). TRPV6 expression reaches a maximum in the kidney at 1 wk of age and is maintained at a relatively lower level thereafter (42). Thus, in contrast to known calcium-processing genes that tend to reach a maximum level of expression early in development, renal NCKX3 expression was highest in newborns (1 day old). In parallel with other calcium-processing genes, the level of expression of NCKX3 decreased after early development.

In mice, sexual maturation begins at 4 wk of age (20), and we hypothesized that sex steroids, such as androgen, estrogen, and progesterone, might influence the differential expression of renal NCKX3 in male and female mice. The expression of NCKX3 in both genders was unaffected by sex steroids or steroid receptor antagonists, which suggests that endogenous sex hormones are not involved in the differential regulation of NCKX3 expression in male and female mice. The calcitropic hormones 1,25(OH)2D3 and PTH have also been shown to interact with the NCKX3 gene, and the effect of glucocorticoids on calcium reabsorption in the kidney and duodenum is decreased by glucocorticoids from the adrenal gland (2, 33) through the inhibition of transcription of the duodenal calcium-processing genes TRPV6 and CaBP-9k (16, 25). The effect of glucocorticoids on calcium reabsorption in the kidney was not examined in any of these previous studies, because calcium-processing genes in the kidney did not appear to be regulated by glucocorticoids. In the current study, the treatment of ADX and/or OVX mice with hydrocortisone or aldosterone significantly reduced renal NCKX3 expression in female mice to a level that was similar to that in male mice; however, NCKX3 protein levels were not affected by these hormones. Although we could not explain the differential effect of these two hormones on its transcriptional and translational levels, we speculated the 3-day hormone injections might be not sufficient to induce NCKX3 proteins derived from its transcriptional expression. If renal NCKX3 was downregulated by adrenal gland-secreted hormones, renal NCKX3 may be regarded as a novel factor in glucocorticoid-induced calcium malabsorption in the kidney.

Active calcium transport may be facilitated by TRPV5/6, CaBP-9k/28k, PMCA1b, and NCX1, and their functions have been recently verified in gene-null mouse models. Airaksinen et al. (1) revealed that CaBP-28k knockout mice were severely impaired in tests of motor coordination, suggesting functional deficits in cerebellar pathways. These CaBP-28k KO mice showed normal development, but the study did not examine other phenotypes, such as duodenal and renal active calcium transport system. Although neural CaBP-28k was investigated in many previous studies, duodenal and renal CaBP-28k has not been thoroughly investigated (1, 8). In addition, Okunade et al. (38) reported that loss of both copies of the gene encoding PMCA1 caused embryonic lethality, suggesting that this gene is an essential housekeeping or developmental gene. As a result of the embryonically lethal phenotype in PMCA1-null mice, its role in active calcium transport has not been studied. Recently, two distinct strains of CaBP-9k knockout mice were generated by Kutazova et al. (21) and by our laboratory (29). These two
CaBP-9k-null mice appeared normal in viability, reproduction, and calcium homeostasis. Thus no CaBP-9k knockout mice presented several serious abnormalities in the active calcium transport system in the duodenum and kidney, implying that another element, like NCKX3, is a key factor in the active calcium transport system.

In summary, we demonstrated that NCKX3 is expressed in the kidney in mice and is differentially regulated in mature male and female mice. Renal NCKX3 was highly expressed in infancy and decreased just before the start of sexual maturation in both genders. Expression continued to decrease in male mice up to 10 wk of age but was maintained at relatively high levels in female mice. Renal NCKX3 localized to the basolateral layer of the distal convoluted tubules, which correlates well with previous studies (25, 32, 37), and suggests that NCKX3 plays a critical role in calcium reabsorption in the kidney in concert with or independently of other calcium-processing genes (14). Although we attested to the effect of several hormones and calcium-deficient diets on renal NCKX3 gene expression between genders, a putative regulator in the renal expression of NCKX3 remains unclear. Taken together, the results of this study indicate that the expression of renal NCKX3 in the distal convoluted tubules in female mice may be involved in active calcium transport in the kidney.

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22. Lamason RL, Mohideen MA, Mest JR, Wong AC, Horton HL, Aros M, Ji YK, Lee GS, Choi KC, Jeung EB. The classical and a non-classical pathways associated with previous studies (25, 32, 37), and suggests that NCKX3 is expressed in the kidney in mice and is differentially regulated in mature male and female mice. Renal NCKX3 was highly expressed in infancy and decreased just before the start of sexual maturation in both genders. Expression continued to decrease in male mice up to 10 wk of age but was maintained at relatively high levels in female mice. Renal NCKX3 localized to the basolateral layer of the distal convoluted tubules, which correlates well with previous studies (25, 32, 37), and suggests that NCKX3 plays a critical role in calcium reabsorption in the kidney in concert with or independently of other calcium-processing genes (14). Although we attested to the effect of several hormones and calcium-deficient diets on renal NCKX3 gene expression between genders, a putative regulator in the renal expression of NCKX3 remains unclear. Taken together, the results of this study indicate that the expression of renal NCKX3 in the distal convoluted tubules in female mice may be involved in active calcium transport in the kidney.


