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Involvement of N-type Ca\(^{2+}\) channels in the fibrotic process of the kidney in rats

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Mishima K, Maeshima A, Miya M, Sakurai N, Ikeuchi H, Hiromura K, Nojima Y. Involvement of N-type Ca\(^{2+}\) channels in the fibrotic process of the kidney in rats. Am J Physiol Renal Physiol 304: F665--F673, 2013. First published January 16, 2013; doi:10.1152/ajprenal.00561.2012.—N-type Ca\(^{2+}\) channels are densely distributed in sympathetic nerves that innervate renal tubules. However, the role of N-type Ca\(^{2+}\) channels in renal fibrosis remains unknown. To address this issue, we examined the difference between the effects of amlodipine (an L-type Ca\(^{2+}\) channel blocker) and cilnidipine (a dual L/N-type Ca\(^{2+}\) channel blocker) on fibrotic changes using a rat unilateral ureteral obstruction (UUO) model. The expression of both L-type and N-type Ca\(^{2+}\) channels was significantly upregulated in UUO kidneys compared with that in contralateral kidneys. There were no significant differences in mean blood pressure among the rats tested. Both amlodipine and cilnidipine significantly attenuated fibrotic changes in UUO kidneys. The antifibrotic effect of cilnidipine was more potent than that of amlodipine. Amlodipine as well as cilnidipine reduced type III collagen deposition, \(\alpha\) smooth muscle actin (\(\alpha\)-SMA) expression, and interstitial cell proliferation. In addition, cilnidipine significantly reduced deposition of type I collagen and macrophage infiltration in UUO kidneys. With the use of in vivo bromodeoxyuridine labeling, label-retaining cells (LRCs) were identified as a population of tubular cells that participate in epithelial-mesenchymal transition after UUO. Some LRCs migrated into the interstitium, expressed \(\alpha\)-SMA and vimentin, and produced several extracellular matrixes in UUO kidneys. The number of interstitial LRCs was significantly decreased by cilnidipine but not amlodipine. These data suggest that N-type Ca\(^{2+}\) channels contribute to multiple steps of renal fibrosis, and its blockade may thus be a useful therapeutic approach for prevention of renal fibrosis.

N-type calcium channel; renal fibrosis; unilateral ureteral obstruction; epithelial-mesenchymal transition

THE RENAL SYMPATHETIC NERVES innervate the tubules, the vessels, and the juxtaglomerular granular cells of the kidney (5). Renal sympathetic nerve activation reduces urinary sodium and water excretion by increasing renal tubular water and sodium reabsorption throughout the nephron. It also decreases renal blood flow and glomerular filtration rate by constricting the renal vasculature and activates the renin-angiotensin system (RAS) by stimulating renin release from juxtaglomerular granular cells. Conversely, angiotensin II stimulates sympathetic nerve activity through central mechanisms and by facilitating adrenergic neurotransmission at the sympathetic nerve termini, thus suggesting significant interactions between renal sympathetic nerves and the RAS in the control of renal function (4). N-type Ca\(^{2+}\) channels are densely distributed in the sympathetic nervous system and play a predominant role in neurotransmitter release from the nerve endings of sympathetic neurons (8). In patients with chronic renal diseases, increased sympathetic nerve activity and plasma renin activity are observed (10), thus suggesting that sympathetic nerve activity via N-type Ca\(^{2+}\) channels is involved in renal injury. Renal fibrosis is the common end point of virtually all progressive kidney diseases (1). Emerging evidence indicates that a large proportion of interstitial fibroblasts, primarily responsible for extracellular matrix accumulation, originate from tubular epithelial cells via epithelial-to-mesenchymal transition (EMT) in the fibrotic kidney. In the process of EMT, tubular epithelial cells acquire a fibroblastic phenotype and migrate into the interstitium through the destroyed tubular basement membrane (9). We recently identified a population of tubular cells that participate in EMT during renal fibrosis (32). Using in vivo labeling by bromodeoxyuridine (BrDU), we detected slow-cycling cells that infrequently pass through the cell cycle to maintain a pool of cells for tissue turnover and repair. After BrDU labeling followed by a chase period, slow-cycling cells were identified as label-retaining cells (LRCs). LRCs were present in tubules of normal rat kidneys (20). After ischemic injury, LRCs divided into numerous daughter cells, which actively proliferated and differentiated into tubular epithelial cells. These data suggest that LRCs act as a source of regenerating cells during the recovery process of the kidney after injury. In vitro analysis demonstrated that LRCs show phenotypic plasticity, tubulogenic capacity, and integration capacity into the developing kidney (19). In a rat unilateral ureteral obstruction (UUO) model, LRCs were localized to both tubules and the interstitium of the fibrotic kidney. LRCs proliferated and migrated into the interstitium through the destroyed tubular basement membrane (32), thus suggesting that LRCs undergo EMT during renal fibrosis (17).

In the present study, we compared the effects of amlodipine (an L-type Ca\(^{2+}\) channel blocker) and cilnidipine (a dual L/N-type Ca\(^{2+}\) channel blocker) on fibrotic changes using a rat UUO model. We found that cilnidipine has an antifibrotic effect that was more potent than that of amlodipine. As with a previous study (32), we detected LRCs in renal tubules of normal kidneys using in vivo BrDU labeling. Some LRCs were localized in the interstitium, expressed \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) and vimentin, and produced several extracellular matrixes in UUO kidneys. Treatment with cilnidipine, but not
amldipine, significantly decreased the number of interstitial 
LRCs, suggesting the possibility that EMT in UUO kidneys 
was reduced by cilnidipine. These data imply that N-type Ca\(^{2+}\) 
channels are involved in multiple steps of renal fibrosis. Block-
ade of N-type Ca\(^{2+}\) channels may be a useful therapeutic 
approach for prevention of renal fibrosis.

MATERIALS AND METHODS

UUO. Male Wistar rats (200 g) were purchased from Oriental Bio 
Service (Kyoto, Japan). UUO was performed as described previously 
(31). Briefly, after induction of general anesthesia by intraperitoneal 
injection of pentobarbital (50 mg/kg body wt), the abdominal cavity 
was exposed via a midline incision and the left ureter was ligated at 
two points with 2–0 silk. At the indicated times after UUO, rats were 
killed and the kidneys were removed for histological examination. 
Ureteral obstruction was confirmed by observing dilation of the pelvis 
and proximal ureter, and collapse of the distal ureter. Sham-operated 
rats had their ureters exposed and manipulated but not ligated. In a 
separate experiment, cilnidipine (kindly provided by Ajinomoto, Ka-
wasaki, Japan) or amldipine (Sigma, St. Louis, MO) was adminis-
tered orally in rat chow for 14 days at an approximate dose of 3 
mg·kg\(^{-1}\)·day\(^{-1}\) based on food intake. Arterial blood pressure was 
measured at the indicated time points using a programmable apparatus 
by the tail-cuff method. Serum creatinine and blood urea nitrogen 
levels were assessed by a Hitachi 7180 autoanalyzer (Hitachi High-
Technologies, Tokyo, Japan).

The experimental protocol was approved by the Ethics Review 
Committee for Animal Experimentation of Gunma University.

Immunohistochemical analysis. Immunostaining was performed 
using VECTASTAIN ABC-kit (Vector Laboratories) as described 
previously (21). Briefly, paraffin sections (4 μm) were deparaffinized, 
hydrated according to standard methods, soaked in blocking serum, 
and incubated with primary antibody overnight at 4°C. After being 
was washed with PBS, sections were incubated with peroxidase-conju-
gated secondary antibody, followed by reaction with diaminobenzi-
dine, and were counterstained with periodic acid-Schiff.

Indirect fluorescent immunostaining was performed as follows 
(18). Briefly, sections were incubated with primary antibodies for 
overnight at 4°C. After being washed with PBS, sections were 

Fig. 1. Expression and localization of L-type and N-type Ca\(^{2+}\) 
channels in unilateral ureteral obstruction (UUO) kidneys. A: mRNA expression of L-type (Cav1.2) 
and N-type (Cav2.2) Ca\(^{2+}\) channels in normal, contralateral, and UUO kidneys was examined by real-time PCR. Values are means ± SE (n = 4). *P < 0.05, 
**P < 0.001 vs. normal. B: production of L-type and N-type Ca\(^{2+}\) channels in contralateral and UUO kidneys was examined by Western blotting. 
C: quantitative analysis of staining intensities for L-type and N-type Ca\(^{2+}\) channels is shown. Values are means ± SE (n = 5). *P < 0.05, **P < 0.01. D and 
E: localization of L-type (D) and N-type (E) Ca\(^{2+}\) channels in contralateral and UUO kidneys was examined by immunostaining. Markers: aquaporin 1 (AQP1; 
proximal tubules), Tamm-Horsfall glycoprotein (THP; thick ascending loop of Henle), AQP2 (collecting ducts), and α-smooth muscle actin (α-SMA; vessels). 
Representative images are shown. Magnification: ×1,000.
incubated with fluorescence-labeled secondary antibodies and DAPI. Fluorescent images were recorded as described previously (18). Primary antibodies were as follows: rabbit anti-rat Cav1.2 (α1C subunit of L-type Ca\(^{2+}\) channel) antibody and rabbit anti-rat Cav2.2 (α1B subunit of N-type Ca\(^{2+}\) channel) antibody (Alomone Labs, Jerusalem, Israel); mouse anti-α-AQP (AQP) 2 antibody (BD Transduction Laboratories, Franklin Lakes, NJ). Goat anti-α-SMA antibody (Sigma); goat anti-β-actin antibody (BioVision Research Products); and goat anti-β-galactosidase antibody (BD Transduction Laboratories, Franklin Lakes, NJ).

**Western blot analyses.** Tissues were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). After centrifugation, the supernatant was collected, and the protein concentration was determined with the BCA protein assay kit (Pierce). Twenty micrograms of protein from each sample were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). To reduce nonspecific antibody binding, the membrane was blocked with 5% nonfat milk, incubated with primary antibody for overnight at 4°C, and washed with Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20). After incubation with alkaline phosphatase-conjugated secondary antibodies for 2 h, the membrane was washed with Tris-buffered saline. Membranes were washed again and reacted with substrate (Sigma FAST BCIP/NBT; Sigma). Primary antibodies were as follows: rabbit anti-rat Cav1.2 (α1C subunit of L-type Ca\(^{2+}\) channel) antibody and rabbit anti-rat Cav2.2 (α1B subunit of N-type Ca\(^{2+}\) channel) antibody (Alomone Labs); mouse anti-β-actin antibody (BD Transduction Laboratories); and mouse monoclonal anti-α-SMA antibody (Sigma).

**Morphological examination.** Sections (4 μm) were cut from paraffin blocks and were stained with hematoxylin-eosin or Masson’s trichrome. With the use of Masson’s trichrome-stained sections, the collagenous, fibrotic areas were quantified in 10 random cortical fields at ×200 magnification using Image J software. All glomeruli and vessels were subtracted from a given field, yielding a target area of tubulointerstitium.

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**Fig. 2. Effects of Ca\(^{2+}\) channel blockers on fibrotic changes in UUO kidneys.** A: kidney weight (KW) of rats at 14 days after UUO. Values are means ± SE (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 vs. sham. Contra, contralateral; Cil, cilnidipine; Aml, amlodipine; BUN, blood urea nitrogen. B: renal function of rats at 14 days after UUO. Values are means ± SE (n = 5). ***P < 0.001 vs. sham. *P < 0.05 vs. UUO. C: fibrotic area in UUO kidneys of untreated, and cilnidipine-treated and amlodipine-treated rats assessed by Masson’s trichrome staining. Magnification: ×100. D: quantitative analysis of fibrotic area. Values are means ± SE (n = 5). *P < 0.05, **P < 0.001 vs. sham. E: expression of type I and type III collagen in the kidneys at 7 days after UUO was examined by immunostaining. Magnification: ×200. F and G: quantitative analysis of type I (F) and type III (G) collagen-positive area. Values are means ± SE (n = 5). *P < 0.05, ***P < 0.001 vs. none.
course. BrdU-positive cells were detected using a cell proliferation kit (Amersham, Piscataway, NJ) according to the manufacturer’s instructions and were counterstained with periodic acid-Schiff. Quantitative analysis of LRCs was performed by counting BrdU-positive nuclei in the tubular area or in the interstitial area from five randomly selected fields under a light microscope at ×200 magnification. The average of five determinants was calculated. The number of tubular or interstitial LRCs was quantified and expressed as a percentage of total tubular or interstitial cells per field, respectively. The number of PCNA-positive cells was also quantitatively analyzed in a similar manner.

Real-time PCR. Tissues were homogenized by using microhomogenizer, and total RNAs were extracted using RNAiso (Takara, Tokyo, Japan). First-strand cDNA was made from total RNA using Super Script III First-Strand (Invitrogen, Carlsbad, CA) according to the manufacturer’s instruction. Real-time PCR was performed by the ABI 7300 Real-time PCR System (Applied Biosystems, Foster City, CA). Reactions included 5 μl of a SYBR Green RealTimePCR MasterMix (TOYOBO, Osaka, Japan), 0.2 μl 3′-primer, 0.2 μl 5′-primer, and 1 μl cDNA. Samples were incubated at 50°C for 2 min and then at 95°C for 5 min, followed by 40 cycles of 15 s at 95°C, and 60 s at 58°C (Cav1.2) or at 55°C (Cav2.2). The expression of Cav1.2 or Cav2.2 and GAPDH in each sample was quantified in separate tubes with the following primers: rat Cav1.2 (sense and antisense: 5′-CAGCAGGTCCTATGTCCAGCA-3′ and 5′-CGGACAGCATGGTAAGGA-3′, respectively), rat Cav2.2 (sense and antisense: 5′-TGCCCAACATCTCCATTGGT-3′ and 5′-AGTTCTGCTGGTGTTTG-3′, respectively), and rat GAPDH (sense and antisense: 5′-TGCCCAACATCTCCATTGGT-3′ and 5′-AGTTCTGCTGGTGTTTG-3′). All data were expressed as the relative differences between normal and contralateral or UUO kidneys after normalization to GAPDH expression. Gels of the PCR products after quantification of the indicated genes by real-time PCR showed single bands (Cav1.2, 158 basepair; Cav2.2, 91 basepair; and GAPDH, 307 basepair, respectively), and rat GAPDH (sense and antisense: 5′-AGTTCTGCTGGTGTTTG-3′ and 5′-AGTTCTGCTGGTGTTTG-3′). Quantitative analysis demonstrated that production of both L-type and N-type Ca2+ channels was significantly upregulated in UUO kidneys (Fig. 1A). We also examined the expressions of the L-type Ca2+ channel and N-type Ca2+ channel in normal, contralateral, and UUO kidneys by Western blotting. Unlike the data from previous report showing that L-type Ca2+ channel was detectable in normal kidneys (33), the expressions of both L-type and N-type Ca2+ channels were undetectable in normal (data not shown) or contralateral kidneys (Fig. 1B). This discrepancy might be due to the difference in the sensitivity of the antibodies used or the difference of rat strain (Sprague-Dawley rats vs. Wistar rats). In contrast, the expressions of L-type Ca2+ channels as well as N-type Ca2+ channels were upregulated in UUO kidneys (Fig. 1B). Quantitative analysis showed that production of both L-type and N-type Ca2+ channels was significantly elevated in UUO kidneys compared with that in contralateral kidneys (Fig. 1C). We next examined the localization of L-type and N-type Ca2+ channels in kidneys after UUO. L-type Ca2+ channels were detected in AQP1-positive proximal tubular cells and THP-positive tubular cells in normal and contralateral kidneys. In contrast, in UUO kidneys, expression of L-type Ca2+ channels became detectable in AQP2-positive collecting ducts and

Statistical analysis. Differences between means were compared by Student’s t-test, with P values of <0.05 considered to be significant.

RESULTS

Expression and localization of L-type and N-type Ca2+ channels in kidney after UUO. We first studied the expressions of Cav1.2 (α1C subunit of L-type Ca2+ channel) and Cav2.2 (α1B subunit of N-type Ca2+ channel) in normal, contralateral and UUO kidneys by real-time PCR. The expressions of L-type as well as N-type Ca2+ channels were significantly upregulated in UUO kidneys (Fig. 1A). We also examined the expressions of the L-type Ca2+ channel and N-type Ca2+ channel in normal, contralateral, and UUO kidneys by Western blotting. Unlike the data from previous report showing that L-type Ca2+ channel was detectable in normal kidneys (33), the expressions of both L-type and N-type Ca2+ channels were undetectable in normal (data not shown) or contralateral kidneys (Fig. 1B). This discrepancy might be due to the difference in the sensitivity of the antibodies used or the difference of rat strain (Sprague-Dawley rats vs. Wistar rats). In contrast, the expressions of L-type Ca2+ channels as well as N-type Ca2+ channels were upregulated in UUO kidneys (Fig. 1B). Quantitative analysis showed that production of both L-type and N-type Ca2+ channels was significantly elevated in UUO kidneys compared with that in contralateral kidneys (Fig. 1C). We next examined the localization of L-type and N-type Ca2+ channels in kidneys after UUO. L-type Ca2+ channels were detected in AQP1-positive proximal tubular cells and THP-positive tubular cells in normal and contralateral kidneys. In contrast, in UUO kidneys, expression of L-type Ca2+ channels became detectable in AQP2-positive collecting ducts and

Fig. 3. Effects of Ca2+ channel blockers on α-SMA Expression in UUO Kidneys. A: Expression of α-SMA in the kidneys at 7 days after UUO was examined by immunostaining. a: Untreated UUO kidneys. b: Amlodipine-treated UUO kidneys. c: Cilnidipine-treated UUO kidneys. d: Contralateral kidneys. Magnification: ×400. B: quantitative analysis of α-SMA-positive area. Values are means ± SE (n = 5). **P < 0.01. C: production of α-SMA in UUO kidneys was examined by Western blotting. D: relative intensity of α-SMA production. Values are means ± SE (n = 5). **P < 0.01.
In normal and contralateral kidneys, N-type Ca\(^{2+}\) channels were present in THP-positive tubular cells and α-SMA-positive vessels but not in glomeruli or other portions of the nephron segment (data not shown). In contrast, expression of N-type Ca\(^{2+}\) channels was clearly detectable in AQP1-positive proximal tubular cells and AQP2-positive collecting ducts in UUO kidneys (Fig. 1B). These results suggest that both L-type and N-type Ca\(^{2+}\) channels are involved in a process of renal fibrosis.

**Effects of amlopidine and cilnidipine on histological changes in UUO kidneys.** To examine the role of N-type Ca\(^{2+}\) channels in renal fibrosis, we administered amlodipine, an L-type Ca\(^{2+}\) channel blocker, and cilnidipine, a dual L/N-type Ca\(^{2+}\) channel blocker, to UUO rats and examined the difference between the effects of amlopidine and cilnidipine on fibrotic changes after UUO. The weight of the obstructed kidney was significantly increased compared with contralateral kidney. The weight of cilnidipine-treated or amlodipine-treated obstructed kidney was significantly elevated compared with that in sham-operated rats. Both cilnidipine and amlodipine did not affect serum creatinine level. In contrast, blood urea nitrogen level in the cilnidipine-treated or amlodipine-treated UUO rats was slightly elevated compared with that in the untreated rats (Fig. 2B). UUO did not significantly alter mean blood pressure (baseline vs. 14 days: 106.4 ± 7.2 vs. 102.7 ± 7.6 mmHg). At 14 days after UUO, there was no significant difference in mean blood pressure among UUO rats in the untreated, cilnidipine-treated, and amlodipine-treated groups (data not shown).

We assessed the fibrotic area in kidneys after UUO by Masson’s trichrome staining. Interstitial fibrotic changes were observed in the untreated, cilnidipine-treated, and amlodipine-treated UUO kidneys (Fig. 2C). Quantitative analysis showed that the fibrotic area was significantly lower in the cilnidipine-treated UUO kidneys as well as in the amlodipine-treated UUO kidneys when compared with the untreated UUO kidneys (Fig. 2D). The fibrotic area in the cilnidipine-treated UUO kidneys was significantly lower than that in the amlodipine-treated UUO kidneys (Fig. 2D).

We also examined the expressions of type I and type III collagen in UUO kidneys by immunostaining. The expression of type I collagen, which was undetectable in contralateral kidneys, was upregulated in UUO kidneys (Fig. 2E). Quantitative analysis showed that type I collagen-positive area in the cilnidipine-treated and amlodipine-treated UUO kidneys was lower than that in the untreated UUO kidneys, but the difference was significant only between cilnidipine-treated and untreated UUO kidneys (Fig. 2F). Type III collagen-positive area was also undetectable in contralateral kidneys but was upregulated in UUO kidneys (Fig. 2E). Quantitatively, the expression of type III collagen in the both cilnidipine-treated and amlo-
Cilnidipine-treated UUO kidneys was significantly lower than that in the untreated UUO kidneys (Fig. 2G).

Effects of amiodipine and cilnidipine on α-SMA expression in UUO kidneys. During renal fibrosis, tubular cells transdifferentiate into fibroblastic cells and express α-SMA, a marker of myofibroblasts. We next examined the effects of amiodipine and cilnidipine on α-SMA expression in UUO kidneys. In normal (data not shown), sham-operated (Fig. 4Aa–Ac), α-SMA expression was scarcely observed. In contrast, the expression of α-SMA was upregulated in the interstitium of UUO kidneys (Fig. 3, Aa–Ac). The area of α-SMA expression was significantly reduced in cilnidipine-treated and amiodipine-treated UUO kidneys (Fig. 3B). Western blot analysis also confirmed a significant decrease in the expression of α-SMA in cilnidipine-treated as well as in amiodipine-treated UUO kidneys (Fig. 3, C and D).

Effects of amiodipine and cilnidipine on E-cadherin expression in UUO kidneys. We also examined the effects of amiodipine and cilnidipine on the expression of E-cadherin, a key epithelial cell adhesion molecule, in UUO kidneys. There were no significant differences in the expression of E-cadherin among the untreated, cilnidipine-treated, and amiodipine-treated UUO kidneys (data not shown).

Effects of amiodipine and cilnidipine on cell proliferation in UUO kidneys. We further examined the effects of amiodipine and cilnidipine on cell proliferation in UUO kidneys by PCNA staining. In normal (data not shown), sham-operated (Fig. 4Aa), and contralateral kidneys (Fig. 4Ac), PCNA-positive cells were scarcely observed. In contrast, a large number of PCNA-positive cells were present in UUO kidneys (Fig. 4Ab). Tubular and interstitial cells were positive for PCNA (Fig. 4Ac). Neither cilnidipine nor amiodipine affected the number of tubular PCNA-positive cells (Fig. 4B), but they significantly reduced the number of interstitial PCNA-positive cells (Fig. 4C).

Effects of amiodipine and cilnidipine on macrophage infiltration in UUO kidneys. Macrophage infiltration is often correlated with degree of renal fibrosis, and depletion of macrophages reduces fibrosis in several disease models (23). We finally investigated CD68-positive macrophage infiltration in UUO kidneys (Fig. 5). Macrophage infiltration was not observed in normal and contralateral kidneys but was detectable in UUO kidneys (Fig. 5A). The number of infiltrating macrophages was significantly lower in cilnidipine-treated UUO kidneys but not in amiodipine-treated UUO kidneys (Fig. 5B).

EMT in UUO kidneys. Using the BrdU labeling method, we previously demonstrated that LRCs act as renal progenitor-like tubular cells (20) and found that tubular LRCs migrated into the interstitium of the kidneys after UUO (32), suggesting that LRCs undergo EMT during renal fibrosis. We next examined the presence of LRCs in the kidney after UUO. BrdU was injected into normal rats for 7 days. After a 2-wk chase period, UUO was generated in these rats. BrdU-positive cells could be identified as LRCs, because rapidly proliferating cells divided many times during the chase period and incorporated BrdU was diluted out. LRCs were mainly localized in proximal tubular cells in normal (data not shown), sham-operated, and contralateral kidneys (Fig. 6A). No LRCs was detected in the glomeruli, capillary endothelial cells, or interstitium (data not shown). At 7 days after UUO, LRCs remained localized in the tubules, but some LRCs were found to be present in the interstitium along the destroyed basement membrane (Fig. 6A).

We also examined the localization of LRCs and laminin or type IV collagen basement membrane in the UUO kidneys (Fig. 6B). In normal and contralateral kidneys, LRCs were...
localized within the basement membrane. In contrast, LRCs were present in the interstitium adjacent to the laminin-positive or type IV collagen-positive basement membrane in the UUO kidneys, thus suggesting our previous notion (32) that tubular LRCs migrate into the interstitium of the kidney after UUO.

We investigated the phenotype of LRCs in UUO kidneys. LRCs were colocalized with α-SMA (a marker of myofibroblasts) and vimentin (a mesenchymal marker; Fig. 6C). LRCs were also found to express extracellular matrix such as type I collagen, type III collagen, and fibronectin (Fig. 6C). Collectively, these findings suggest that LRCs undergo EMT during renal fibrosis.

Effects of amlodipine and cilnidipine on EMT. We finally tested the effect of amlodipine and cilnidipine on EMT in UUO rats treated with BrdU for 1 wk, followed by a 2-wk chase period. The number of interstitial LRCs in cilnidipine-treated, but not in amlodipine-treated, UUO kidneys was significantly decreased compared with the untreated UUO kidneys (Fig. 6D), suggesting the possibility that EMT in UUO kidneys was reduced by cilnidipine. The number of tubular LRCs was reduced in both cilnidipine-treated and amlodipine-treated UUO kidneys, compared with that in the untreated UUO kidneys (Fig. 6E).

DISCUSSION

Generally, UUO-treated rats do not show hypertensive phenotype as shown in this study, making it difficult to observe blood pressure-lowering effect of antihypertensive drugs. In the present study, UUO rats were treated with cilnidipine or amlodipine at the dose of 3 mg·kg⁻¹·day⁻¹ for 14 days. Several previous reports demonstrated that cilnidipine and amlodipine had a lowering effect of blood pressure to a similar extent at the dose used in this study (3 mg·kg⁻¹·day⁻¹) in hypertensive rats (12, 25, 26). Previous pharmacological study also demonstrated that cilnidipine and amlodipine inhibited L-type Ca channel current to a similar extent using a whole cell patch-clamp technique (29). Taken together, we speculate that both Ca²⁺ channel blockers inhibited L-type Ca channel to a similar extent in this study, although the blood pressure-lowering effects of cilnidipine as well as amlodipine could not be observed at this dose.

EMT is a process by which differentiated epithelial cells undergo a phenotypic conversion that gives rise to matrix-producing fibroblasts and myofibroblasts during renal fibrosis (16). In the present study, consistent with previous reports (20, 32), we demonstrated that LRCs are present in tubules of normal rat kidneys using in vivo labeling with BrdU. LRCs migrated into the interstitium through the damaged tubular basement membrane after UUO. The number of interstitial LRCs in UUO kidneys was significantly reduced by treatment with cilnidipine but not with amlodipine (Fig. 6). In addition to L-type Ca²⁺ channels, cilnidipine inhibits N-type Ca²⁺ channels in various types of neuron (7, 28). Therefore, inhibition of LRC migration by cilnidipine may be mediated by the blockade of N-type Ca²⁺ channels. The mechanism is unclear by which cilnidipine inhibited LRC migration into the intersti-
tium. The function of Ca\(^{2+}\) channels is closely related to the actin cytoskeleton reorganization required for cell migration (24). In UUO kidneys, N-type Ca\(^{2+}\) channels are upregulated in proximal tubules (Fig. 1C), where most LRCs are localized. The changes in intracellular Ca\(^{2+}\) concentration via N-type Ca\(^{2+}\) channels may be involved in cell migration or transdifferentiation of LRCs.

Activation of the RAS is thought to be responsible for initiating tubulointerstitial fibrosis and subsequent deterioration of renal function. Angiotensin II induces infiltration of macrophages, a critical determinant of fibrogenesis, into the injured tissues (15). It has been reported that angiotensin II receptor blockers ameliorate renal function and decelerate progression of renal disease. In the kidney, sympathetic nerves are distributed to both afferent and efferent arterioles in the glomeruli (5). Renal sympathetic nerve activation decreases renal blood flow and glomerular filtration rate by constricting renal vasculature and activates the RAS by stimulating renin release from juxtaglomerular granular cells. It has been reported that cilnidipine suppresses RAS hyperactivity through its sympatholytic action (12). When compared with amlodipine, cilnidipine leads to weaker activation of the RAS in human patients (14), and the renortopic action of cilnidipine has been implicated in the reduction of RAS activity (13), suggesting that reduction of renal fibrosis, EMT, and macrophage infiltration by cilnidipine is via suppression of RAS activity in UUO kidneys. However, this is unlikely, as we could not find significant differences in expression levels of angiotensin-converting enzyme, angiotensinogen, and renin among the untreated, cilnidipine-treated, and amlodipine-treated UUO kidneys (unpublished observation). The dose of cilnidipine used in this study did not significantly alter blood pressure; thus the antifibrotic effects of cilnidipine are probably mediated by direct action on N-type Ca\(^{2+}\) channel-expressing tubular cells, rather than via its hemodynamic action or modulation of RAS activity through its sympatholytic action.

Contribution of N-type Ca\(^{2+}\) channels to tissue injury has been demonstrated in several organs. After ischemic injury, expression of N-type Ca\(^{2+}\) channels was elevated in brain regions susceptible to ischemia (3), and a selective N-type Ca\(^{2+}\) channel antagonist was found to protect against ischemic brain injury (2, 30). In DOCA-salt hypertensive rats, myocardial fibrosis was significantly improved by cilnidipine, thus suggesting the protective effects of cilnidipine on cardiovascular remodeling (11). Several reports have also demonstrated that N-type Ca\(^{2+}\) channels play a role in renal injury. Immuno-reactivity for N-type Ca\(^{2+}\) channels was observed in glomerular podocytes in spontaneously hypertensive rats, and cilnidipine suppressed the development of proteinuria by inhibiting podocyte injury (6). Cilnidipine was more effective than amlodipine at preventing the progression of proteinuria in hypertensive patients when coupled with a RAS inhibitor (27), probably because cilnidipine inhibited renal sympathetic nerve activity (13), resulting in the vasodilatation of efferent arterioles and the attenuation of glomerular hypertension (34). Cilnidipine was reported to improve renal function and arterial stiffness in patients with essential hypertension (22). Consistent with these data, we found the beneficial effect of cilnidipine on renal fibrosis in the present study. We demonstrated the upregulated expressions of L-type and N-type Ca\(^{2+}\) channels in UUO kidneys (Fig. 1). Cilnidipine (a dual L/N-type Ca\(^{2+}\) channel blocker) has antifibrotic action, which was more potent than that of amlodipine (an L-type Ca\(^{2+}\) channel blocker) (Fig. 2), suggesting the possible contribution of N-type Ca\(^{2+}\) channels to renal fibrosis. Both amlodipine and cilnidipine significantly reduced the type III collagen deposition, α-SMA expression, and interstitial cell proliferation in UUO kidneys (Figs. 2–4). Cilnidipine provided additional antifibrotic effects, including reductions in the deposition of type I collagen (Fig. 2) and macrophage infiltration (Fig. 5) and decreases in the number of interstitial LRCs (Fig. 6) in UUO kidneys. These findings suggest that N-type Ca\(^{2+}\) channels play a role in multiple steps of renal fibrosis. Its blockade may be a useful therapeutic approach for the prevention of renal fibrosis. This study did not clarify the detailed mechanism by which cilnidipine inhibited renal fibrosis. To this end, the role of N-type Ca\(^{2+}\) channels in inflammation, EMT, and extracellular matrix production during renal fibrosis should be analyzed independently. Mutant mice lacking N-type Ca\(^{2+}\) channels will also be useful. Further study will be needed to address this issue.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: K.M. performed experiments; K.M. analyzed data; K.M. and A.M. interpreted results of experiments; K.M. and A.M. prepared figures; K.M. and A.M. drafted manuscript; K.M., A.M., M.M., N.S., H.I., K.H., and Y.N. approved final version of manuscript; A.M. conception and design of research; A.M. edited and revised manuscript.

REFERENCES

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