Renal Nerves Drive Interstitial Fibrogenesis in Obstructive Nephropathy

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ABSTRACT

The signals that drive fibrogenesis after an initiating insult to the kidney are incompletely understood. Here, we report that renal nerve stimulation after ureteral obstruction is the primary profibrotic signal and that renal denervation prevents both fibrogenesis and the inflammatory cascade. Local infusion of neural factors, norepinephrine, and calcitonin gene-related peptide (CGRP) in denervated kidneys mimicked the fibrotic response observed in innervated obstructed kidneys. Norepinephrine and CGRP act through the α2-adrenergic receptor and CGRP receptor, respectively, because blocking these receptors prevented fibrosis, the inflammatory response, and tubular cell death. In tubular epithelial cells, both norepinephrine and CGRP induced apoptosis and the release of profibrotic factors capable of stimulating the differentiation of fibroblasts to myofibroblasts. In conclusion, these data suggest that nerve-derived signaling molecules may drive renal fibrosis and that their suppression may be a therapeutic approach to fibrosis prevention.


Fibrosis is responsible for chronic tissue injury in various organs. The histopathology of tubulointerstitial fibrosis in CKD is characterized by loss of tubular cells, influx of leukocytes, and deposition of extracellular matrix consequent to myofibroblast accumulation.1,2 During renal fibrosis, the tubular cell loss occurs mainly via apoptotic cell death.3 The injured tubular cells release cytokines that generate inflammatory and fibrotic responses.3 Although fibrogenesis is the final common pathway that leads to ESRD, there is little information available regarding the primary signals that drive fibrogenesis. Several profibrogenic factors, including TGF-β and connective tissue growth factor (CTGF), are well established inducers of fibrosis via stimulation of myofibroblast differentiation and extracellular matrix production in multiple organs.4–6 These factors are upregulated in both tubular and tubulointerstitial cells after kidney injury, resulting in kidney fibrogenesis;7,8 however, the primary signaling stimuli that induce the generation of profibrogenic factors in the injured tissue have not been identified.

Sympathetic efferent and primary sensory nerves are key players in the regulation of peripheral inflammation and the immune response.9 During tissue injury, neuropeptides are released by tissue peripheral nerve terminals and have local functions, such as neurogenic inflammation.10 Although renal sympathetic and sensory nerves are important effectors of renal dysfunction in CKD,11,12 their role in the development of renal injury and tubulointerstitial fibrosis remains poorly defined. The kidney is innervated by efferent sympathetic nerves as well as peptidergic sensory afferent nerves, in which several neuroactive substances have been identified.13 Given the pronounced effect of the renal nerves on CKD, we sought to determine whether efferent and afferent nerve-derived neuropeptides/neurotransmitters and their signaling pathways are
Figure 1. Renal denervation (DNx) prevents tubulointerstitial fibrogenesis and inflammation during UUO. Renal denervation in left kidneys of male 129S1/SvImJ mice aged 8–10 weeks was carried out; 2 days after the onset, the left ureters were obstructed for 3 or 10 days. (A) Immunohistochemistry of TH and CGRP in renal denervation or intact kidneys (n=4 in each group). (B) TH expression in renal denervation or intact kidneys using Western blot analysis (n=4 in each group). (C and D) Norepinephrine (NE) and CGRP protein level in renal denervation or intact kidneys using ELISA. (E–G) Collagen deposition using Sirius red staining (E and F) and hydroxyproline measurement (G) after UUO or sham operation in renal denervation or intact kidneys (n=4 in each sham-group, n=6 in each UUO-group). (F) The Sirius red-positive area was measured in five randomly chosen high-power (×200) fields per kidney using NIH ImageJ software. (H and I) Immunohistochemistry of α-SMA in UUO or sham kidneys after renal denervation or intact kidneys (n=4 in each sham group, n=6 in each UUO group). The visible blue color indicates nuclei stained by DAPI. (I) The α-SMA–positive area was measured in five randomly chosen high-power (×200) fields per kidney using NIH ImageJ software. (J) α-SMA, fibronectin, TGF-β, and p-Smad3 expression using Western blot analysis in UUO or sham kidneys after renal denervation or intact kidneys (n=4 in each sham group, n=6 in each UUO group). Anti-β-actin antibody served as a loading control. (K–M) Immunohistochemistry of PMN and F4/80 in UUO or sham kidneys after renal denervation or intact kidneys (n=4 in each sham group, n=6 in each UUO group).
RESULTS

Renal Denervation Prevents Fibrogenesis and Inflammation

We confirmed that renal denervation eliminated tyrosine hydroxylase (TH)–positive sympathetic nerve fibers and calcitonin gene-related peptide (CGRP)–positive sensory nerve fibers in adventitia of intrarenal arteries (Figure 1A). Furthermore, renal denervation markedly decreased TH expression, norepinephrine, and CGRP levels in mouse whole kidneys (Figure 1, B–D). To determine the effect of renal denervation on renal fibrosis, we performed unilateral ureteral obstruction (UUO) after renal denervation. Intact UUO kidneys showed a time-dependent increase of collagen deposition as measured by Sirius red–positive area and hydroxyproline level, whereas renal denervation markedly reduced collagen deposition during UUO (Figure 1, E–G). Immunohistochemistry demonstrated that tubulointerstitial expression of α-smooth muscle actin (α-SMA) was diminished by renal denervation compared with that in intact UUO kidneys (Figure 1, H and I). The denervated kidneys also reduced expressions of profibrotic proteins, including α-SMA, fibronectin, TGF-β, and phosphorylated Smad3 (p-Smad3) during UUO (Figure 1J and Supplemental Figure 1A). Infiltration of polymorphonuclear neutrophil (PMN)–positive neutrophils and F4/80-positive macrophages was increased after UUO in intact kidneys, whereas renal denervation inhibited the infiltration of neutrophils and macrophages (Figure 1, K–M). In addition, denervated kidneys showed decreased expression of proinflammatory factors, including intercellular adhesion molecule-1 (ICAM-1), TNF-α, IL-1β, IL-6, monocyte chemotactic protein-1 (MCP-1), KC, interferon γ–inducible protein of 10 kDa (IP-10), and toll-like receptor 4 (TLR4) during UUO, compared with that in intact kidneys (Figure 1N and Supplemental Figure 1B). However, renal denervation after UUO was not renoprotective against fibrosis and inflammation (Supplemental Figure 1, C and D). These data suggest that renal nerve activation is required to instigate fibrogenesis, but it may not play a significant role during the progression of the injury.

Norepinephrine and CGRP Contribute to Fibrosis and Inflammation

To identify neurotransmitters that contribute to renal fibrosis, we treated denervated kidneys with exogenous norepinephrine, neuropeptide Y (NY), CGRP, or substance P. Ten days after UUO, the denervated kidneys treated with either norepinephrine or CGRP showed a dose-dependent induction of collagen deposition; however, the collagen deposition was unaltered by NY and substance P (Figure 2A and Supplemental Figure 2A). The α-SMA–positive area, α-SMA expression, and p-Smad3 level were also increased by norepinephrine or CGRP treatment of denervated UUO kidneys (Figure 2, B and C, and Supplemental Figure 3B). Treatment of renal denervation kidney with norepinephrine or CGRP, but not NY or substance P, led to neutrophil and macrophage infiltration after UUO (Figure 2D). The expression of ICAM-1 and TNF-α was also increased by norepinephrine or CGRP in denervated UUO kidneys (Figure 2E and Supplemental Figure 2C). In contralateral uninjected kidneys, however, neurotransmitters had no effect on fibrosis (Supplemental Figure 2D).

Norepinephrine and CGRP Induce Caspase-Dependent Tubular Cell Death

In innervated kidneys, the norepinephrine level was not changed by UUO, but the CGRP level was significantly increased 1–24 hours after UUO (Figure 3A). Renal denervation persistently suppressed renal levels of norepinephrine and CGRP during UUO (Figure 3A). Twenty-four hours after UUO, immunohistochemical localization revealed CGRP present at the apical and basolateral membranes of epithelial cells in dilated or tubules positive on terminal deoxynucleotidyl transferase–mediated digoxigenin–deoxyuridine nick-end labeling (TUNEL), as well as on nerve fibers in the adventitia of intrarenal arteries (Figure 3B). To assess whether neuropeptides/neurotransmitters are involved in the tubular cell death, we assessed histologic damage and apoptosis. Renal denervation lessened the histologic damage score based on periodic acid-Schiff (PAS) staining and the number of apoptotic cells assessed by TUNEL staining during UUO, compared with intact kidneys (Supplemental Figure 3, A and B). Additionally, renal denervation abolished the UUO-induced increase in expression of full-length poly(ADP-ribose) polymerase 1 (PARP1), an indicator of necrosis,14 as well as the expression of cleaved PARP1 and cleaved caspase-3, markers of apoptosis (Supplemental Figure 3C). In denervated kidneys, the treatment with norepinephrine or CGRP, but not NY or substance P, evoked significant histologic damage and TUNEL-positive apoptotic cell death at 10 days after UUO (Figure 3C). In UUO kidneys without renal denervation, treatment with norepinephrine or CGRP aggravated tubular dilation and TUNEL-positive apoptosis (Supplemental Figure 3D). Consistent with the data of histologic damage and
Figure 2. Norepinephrine and CGRP contribute to kidney fibrosis and inflammation during UUO. Renal denervation (DNx) in left kidneys of male 129S1/SvImJ mice aged 8–10 weeks was carried out; 2 days after the onset, norepinephrine (NE), NY, CGRP, or substance P (SP) (0, 1.2, 6, or 30 ng/kg per day) was continuously infused into kidneys via a mini-osmotic pump, and the left ureters were obstructed for 10 days. (A) Norepinephrine- and CGRP-induced collagen deposition using Sirius red staining in denervated UUO kidneys. The Sirius red–positive area was measured in five randomly chosen high-power (×200) fields per kidney using NIH ImageJ software. (B) Immunohistochemistry of α-SMA in denervated UUO kidneys after treatment. The visible blue color indicates nuclei stained by DAPI. The α-SMA–positive area was measured in five randomly chosen high-power (×200) fields per kidney using NIH ImageJ software. (C) α-SMA and p-Smad3 expression in norepinephrine- or CGRP-treated UUO kidneys using Western blot analysis. Anti–β-actin antibody served as a loading control. (D) Immunohistochemistry of PMN and F4/80 in norepinephrine- or CGRP-treated UUO kidneys. The number of PMN-positive neutrophils and the area of F4/80-positive macrophages were evaluated in five randomly chosen high-power (×200) fields per kidney. (E) ICAM-1 and TNF-α expression using Western blot analysis. Anti–β-actin antibody served as a loading control. n=6 in each group. Scale bar, 50 μm. *P<0.05, **P<0.01, ***P<0.001 versus 0 ng/kg per day. Error bars represent SDs.
TUNEL assay, exogenous norepinephrine or CGRP increased the expression of PARP1, cleaved PARP1, and cleaved caspase-3 in denervated UUO kidneys (Figure 3D). Infusion of norepinephrine or CGRP, however, had no effect on systolic BP, indicating that the UUO injury in renal denervation kidneys treated with norepinephrine or CGRP is not a consequence of hemodynamic alterations (Supplemental Figure 3E).

The in vivo data on the role of norepinephrine and CGRP in tubular cell death are complemented by our studies in mouse renal proximal tubular (MCT) cells. Treatment with norepinephrine or CGRP time-dependently induced both TUNEL-positive apoptotic cell death and propidium iodide (PI)–positive necrotic cell death; however, the increase in the number of TUNEL-positive cells occurred earlier than did the increase in the number of PI-positive cells (Figure 3E). Cleaved PARP1 and cleaved caspase-3 levels were also increased by norepinephrine or CGRP treatment, but PARP1 expression was not significantly altered (Figure 3F).

To determine whether CGRP-mediated tubulointerstitial fibrosis is dependent on its receptor, we used a specific CGRP receptor antagonist. CGRP receptor antagonist treatment markedly inhibited collagen deposition in mouse kidneys after 10 days of UUO, as shown by reduced Sirius red–positive area and hydroxyproline content (Figure 7, A and B). The α-SMA–positive area was significantly reduced in CGRP receptor antagonist–treated kidneys after UUO, compared with that in vehicle-treated UUO kidneys (Figure 7C). The α-SMA and p-Smad3 expression was also lessened by treatment with CGRP receptor antagonist (Figure 7D). UUO kidneys treated with the CGRP antagonist also showed reduction in proinflammatory responses, as demonstrated by decreased PMN-positive neutrophil influx into kidney, ICAM-1, and TNF-α expression (Figure 7, E and F). Additionally, the CGRP receptor antagonist treatment significantly diminished induced histologic damage assessed from PAS-stained sections and TUNEL-positive apoptotic cell death in UUO kidneys (Figure 7G). The effects of UUO on PARP1, cleaved PARP1, and cleaved caspase-3 protein levels were reduced by CGRP receptor antagonist treatment (Figure 7H). In tubular cell culture, the treatment with the CGRP receptor antagonist reduced the percentages of TUNEL-positive apoptotic and PI-positive necrotic cell death (Supplemental Figure 5C). Furthermore, the CGRP receptor antagonist treatment significantly diminished induced histologic damage assessed from PAS-stained sections and TUNEL-positive apoptotic cell death in UUO kidneys (Figure 7G). The effects of UUO on PARP1, cleaved PARP1, and cleaved caspase-3 protein levels were reduced by CGRP receptor antagonist treatment (Figure 7H). 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Figure 3. Norepinephrine (NE) and CGRP contribute to tubular cell death in UUO kidneys. (A and B) Renal denervation (DNx) or intact in left kidneys of male 129S1/SvImJ mice aged 8–10 weeks was carried out; 2 days after the onset, the left ureters were obstructed for 0, 1, 3, 6, 12, 24, 72, or 240 hours. (A) The kidney norepinephrine and CGRP protein levels after UUO using ELISA (n=6 in each group). **P<0.01 versus 0 hours, ##P<0.01 versus intact kidneys. (B) Immunohistochemistry of CGRP and TUNEL assay of apoptotic cells in UUO kidneys after renal denervation or intact kidneys (n=6 in each group). The visible blue color indicates nuclei stained by DAPI. (C and D) Renal denervation in left kidneys of male 129S1/SvImJ mice aged 8–10 weeks was carried out; 2 days after the onset,
DISCUSSION

Interstitial fibrosis is the hallmark of CKD regardless of its cause.\textsuperscript{1} During the past two decades, causative roles for inflammation, fibroblast activation, tubular and microvascular injury, and apoptosis have been established in the development of tubulointerstitial fibrogenesis.\textsuperscript{16} Several molecules are implicated in the progression of renal fibrosis, including various cytokines, chemokines, angiogenic factors, and growth factors and the renin-angiotensin system.\textsuperscript{1,17} These molecular responses and signaling events indicate the importance of a cytokine milieu and an inflammatory cascade, including infiltration of leukocytes during fibrogenesis. Despite these great strides, there remains a gap in the our understanding of the processes that induce these myriad signaling events after an initial insult to the kidney.\textsuperscript{18}

Therefore, identification of the primary signal or the core signaling pathway that instigate renal fibrogenesis after an initial stimulus is essential for better understanding of the syndrome and in developing effective therapeutic strategies for preventing, reversing, or limiting progression of fibrogenesis.\textsuperscript{19}

In cultured renal tubular cells, presence of CGRP or norepinephrine induced the generation and release of TGF-β1 and CTGF. Furthermore, CGRP and norepinephrine induced the production of extracellular matrix, including fibronectin, but did not induce epithelial-mesenchymal transition because no expression of α-SMA or p-Smad3 was observed. One of the main mediators of fibronectin production is through TGF-β signaling;\textsuperscript{20} however, p-Smad3 was not induced by CGRP or norepinephrine in cultured renal proximal tubular cells, suggesting that autocrine signaling through TGF-β was not activated in renal tubular cells. Fibronectin can probably be produced by alternate pathways, such as activation of c-Jun N-terminal kinase signaling pathway.\textsuperscript{21} On the other hand, culturing of fibroblasts in the presence of the supernatants from CGRP- or norepinephrine-treated tubular cells induced myofibroblast differentiation via p-Smad3, suggesting that TGF-β induced by norepinephrine or CGRP in tubular epithelial cells triggers fibrosis by driving phosphorylation of Smad3 in adjacent fibroblasts.

Our data revealed that renal CGRP levels were increased soon after UUO, with maximal levels observed during the first day. CGRP protein was evident at apical or basolateral membranes of epithelial cells in dilated or TUNEL-positive tubules, as well as in sensory nerve fibers. Inhibition of CGRP receptor prevented injury, whereas long-term administration of CGRP in denervated UUO kidneys induced tubular cell apoptosis, inflammatory cascade, as well as fibrogenesis. In vitro, treatment of tubular cells with CGRP also induced apoptosis, suggesting that the mechanism by which CGRP induces apoptosis may be due to its direct effect in tubular cells and not due to indirect effects, such as fibrosis or inflammation. CGRP enhances apoptosis in thymocytes\textsuperscript{22} and inhibits apoptosis in retina cells,\textsuperscript{23} suggesting that CGRP-mediated apoptosis depends on cell types.

Sensory activity has been shown to be associated with inflammation.\textsuperscript{24} The autonomic nervous system has been implicated in acute and chronic inflammation of various organs.\textsuperscript{25,26} Because CGRP has potent vasodilator activity, it has long been considered to be involved in aggravation of inflammation by increasing the blood flow, the number of circulating cells, and chemotactic factors. This possibility is supported by in vitro data demonstrating that exposure of endothelial cells to CGRP increases the expression of adhesion molecules for binding to neutrophils.\textsuperscript{27} Consistent with this finding, our data demonstrate that CGRP induces ICAM-1 expression after UUO. Although the kidney encounters various immune-mediated diseases, such as GN, the role of neuroimmune interactions has not been well studied. Our data showed that inhibition of CGRP receptor prevented the expression of inflammatory molecules as well as infiltration of leukocytes into the renal parenchyma, suggesting that neuroimmune interactions can occur in UUO kidneys.

Afferent nerve activation due to an initial insult could activate renal efferent sympathoexcitation. Our data implicating norepinephrine, NY, CGRP, substance P (SP) (30 ng/kg per day), or vehicle (0.9% saline) was continuously infused into kidneys via a miniosmotic pump, and the left ureters were obstructed for 10 d (n=6 in each group). (C) Kidneys were fixed for PAS staining to measure the histologic damage in tubules. TUNEL assay to detect apoptotic cells was done using In Situ Cell Death Detection kit. The visible blue color indicates nuclei stained by DAPI. The number of TUNEL-positive apoptotic cells in tubules was counted in five randomly chosen high-power (×200) fields per kidney. *P<0.05, **P<0.01 versus vehicle. (D) PARP1, cleaved PARP1, and cleaved caspase-3 expression using Western blot analysis. Anti–β-actin antibody served as a loading control. *P<0.05, **P<0.01 versus vehicle. (E-G) MCT cells were maintained in DMEM/high-glucose medium containing 10% FBS at 37°C with 5% CO₂. The cells were grown until 70% confluence on culture plates and then changed to serum-free medium. After serum starvation for 6 hours, norepinephrine or CGRP was incubated to the culture at a final concentration of 1 nM in PBS (vehicle) (n=4 in each group). A pan-caspase inhibitor Q-VD-OPh was also added at the same time at a final concentration of 20 μM in DMSO (n=4 in each group). (E) TUNEL and PI staining in MCT cells treated with norepinephrine, CGRP, or vehicle. The visible blue color indicates nuclei stained by DAPI. The number of TUNEL-positive apoptotic or PI-positive necrotic cells was counted in five randomly chosen high-power (×200) fields per plate. *P<0.05, **P<0.01, ***P<0.001 versus vehicle. (F) PARP1, cleaved PARP1, and cleaved caspase-3 expression in MCT cells treated with norepinephrine, CGRP, or vehicle using Western blot analysis. Anti–β-actin antibody served as a loading control. **P<0.01, ***P<0.001 versus vehicle. (G) The number of TUNEL-positive apoptotic or PI-positive necrotic cells cotreated with Q-VD-OPh plus norepinephrine, CGRP, or vehicle was counted in five randomly chosen high-power (×200) fields per plate. **P<0.01, ***P<0.001 versus DMSO. Scale bar, 50 μm. Error bars represent SDs.
norepinephrine signaling through upregulated α2-AR subtypes to induce fibrogenesis in UUO kidneys are intriguing because α2-AR subtypes were initially identified as feedback inhibitors of neurotransmitter release in adrenergic and other neurons. In the mouse heart-failure model, deletion of sympathe inhibitory α2-ARs results in increased norepinephrine levels, which elicit typical cardiovascular consequences, including aggressive remodeling of the left ventricle, cardiac hypertrophy, and fibrosis. Furthermore, α2A- and α2C-ARs seem to play essential roles in the prevention of heart failure progression. In contrast, our data indicate that activation of the α2-AR subtypes, especially α2A- and α2C-ARs, are detrimental in the progression of renal injury, inflammation, and fibrogenesis. Inhibition of α2-ARs with their antagonists had no effect on norepinephrine levels but decreased chemokine expression in the UUO kidney, suggesting a possible mechanism by which norepinephrine may promote leukocyte recruitment and inflammation. Further, activation of the α2-AR subtypes on tubular cells in the UUO kidney may trigger signaling events other than sympathoinhibition, such as activation of TGF-β and CTGF signaling pathways to instigate fibrogenesis. The reason for the inhibitory effects of multiple α2-AR subtypes on UUO injury measures is not clear. It has been shown that the α2C-AR can alter α2A-AR signaling by heterodimerization, with these complexes acting as a functional signaling unit in cells in which both subtypes are expressed. Further studies are needed to determine whether such heterodimerization, and the resulting signaling events, may explain the similar renoprotective effects of inhibiting α2A- or α2C-AR subtypes during UUO.

Elevated plasma norepinephrine is observed in patients with CKD and ESRD. In the present study, UUO did not significantly increase renal norepinephrine levels; however, inhibition of ARs prevented UUO-induced injury, whereas long-term administration of norepinephrine prompted injury in denervated UUO kidneys. Norepinephrine has previously been shown to induce apoptosis in neonatal cardiomyocytes through a reactive oxygen species/TNF-α/caspase signaling pathway. In our study, exogenous norepinephrine induced TNF-α and caspase activation via the α2-AR-mediated process. On the other hand, exogenous norepinephrine has been reported to induce proximal tubule necrotic cell death in dogs. Our in vivo data indicate that norepinephrine infusion increased PARP1 expression in the denervated kidney, supporting a possible explanation for the necrotic cell death observed after UUO. The signaling pathway by which norepinephrine induces apoptosis and necrosis was not delineated in this study and requires further investigation.

In conclusion, this study provides incontrovertible evidence that renal nerve activation is a primary mechanism instigating fibrogenesis in the UUO kidney. Our data implicate both renal afferent nerve–derived CGRP and efferent nerve–derived
norepinephrine in triggering tubular damage, the inflammatory cascade, and fibrogenesis. Inhibiting the actions CGRP or norepinephrine, alone or in combination, might represent a novel effective therapeutic strategy to prevent or limit progression of renal fibrogenesis at its onset in CKD.

CONCISE METHODS

Mice and Surgical Preparation
Male 129S1/SvImJ mice aged 8–10 weeks were purchased from Jackson Laboratories. All mouse experiments were performed in accordance with the animal protocols approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. UUO was conducted as previously reported. Briefly, the mice were anesthetized with an intraperitoneal injection of a cocktail containing ketamine (200 mg/kg body weight) and xylazine (16 mg/kg body weight). After the left kidney was exposed through the left flank incision, the left ureter was ligated completely near the kidney pelvis using a 5-0 silk tie. Sham-operated mice underwent the same surgical procedure without the ureter ligation. Renal denervation was carried out 2 days before UUO or sham operation, as described previously. Briefly, the left kidney artery and vein were exposed through the abdominal incision and isolated from the surrounding connective tissue under anesthesia with ketamine and xylazine. For stripping the nerves, the kidney vessels were painted for 2 minutes with 95% ethanol and for 2 minutes with PBS. Intact mice underwent the same surgical procedure without stripping of the kidney nerves. For local treatment of neurotransmitters, norepinephrine (R&D Systems, 12 mg/kg body weight per day) against CGRP or norepinephrine, alone or in combination, might represent a novel effective therapeutic strategy to prevent or limit progression of renal fibrogenesis at its onset in CKD.

Collagen Deposition
Collagen deposition was assessed by both Sirius red staining and hydroxyproline assay as previously described. The area of positive Sirius red staining was measured in five randomly chosen high-power (×200) fields per kidney using National Institutes of Health (NIH) ImageJ software (http://rsb.info.nih.gov/ij/).

Kidney Norepinephrine and CGRP Levels
For measurement of kidney norepinephrine contents, the kidneys were homogenized using 1 mM EDTA and 4 mM sodium metabisulphite in 0.1 N HCl. After centrifugation of the homogenates, norepinephrine concentration in the supernatants was determined by ELISA (ALPCO Diagnostics, Windham, NH), as suggested by the manufacturer. For measurement of kidney CGRP contents, the kidneys were homogenized in 2 N acetic acid. The homogenates were then incubated at 90°C for 10 minutes and centrifuged. After extraction of the supernatants using solid phase extraction cartridges (Waters, Milford, MA), CGRP concentration in the samples was determined by ELISA (Cayman, Ann Arbor, MI) as suggested by the manufacturer.

Cell Culture and Treatment
MCT cells were maintained in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) at 37°C with 5% CO₂. The cells were grown until 70% confluence on culture plates and then changed to serum-free medium. After serum starvation for 6 hours, norepinephrine or CGRP was incubated for 48 hours to the culture at a final concentration of 1 nM in PBS (vehicle). This concentration and time were chosen after TUNEL and PI staining in dose- and time-dependent experiments (0.1, 1, 10, or 100 nM norepinephrine or CGRP for 0, 1, 3, 6, 12, 24, or 48 hours). Pan-caspase inhibitor Q-VD-OPh (Sigma, final concentration of 20 μM in DMSO), α2A-AR antagonist BRL44408 (R&D System, final concentration of 10 μM in DMSO), α2B-AR antagonist imiloxan (R&D System, final concentration of 1 μM in DMSO), α2C-AR spiroxatrine (R&D System, final concentration of 100 nM in DMSO), or CGRP receptor antagonist CGP3738 (R&D System, final concentration of 100 nM in PBS) was also added at the same time of norepinephrine or CGRP treatment. This concentration was chosen after TUNEL and PI staining in dose-dependent experiments (0.1 or 10 nM and 1, 10, or 50 μM). NRK-49F cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM/F-12 medium supplemented with 10% FBS (Invitrogen) at 37°C with 5% CO₂. After 6 hours of treatment with norepinephrine or CGRP in MCT cells at a final concentration of 1 nM in PBS (vehicle), the culture plate was washed using PBS, changed to serum-free media, and continued for 48 hours. The conditioned medium was then collected and added to NRK-49F cells starved for 24 hours in the conditioned medium, norepinephrine and CGRP levels were not detected by ELISA (ALPCO Diagnostics and Cayman, respectively). The dose-dependent treatment (0.1, 1, 10, or 100 nM) with norepinephrine or CGRP alone did not change α-SMA expression in NRF-49F cells starved for 24 hours (data not shown). The released level of TGF-β1 and CTGF in the supernatant of MCT cells treated with norepinephrine, CGRP, or vehicle for 48 hours was examined by ELISA (BioLegend, San Diego, CA) and Western blot analysis, respectively.
Figure 5. α2-AR antagonists reduce tubulointerstitial fibrosis and inflammation after UUO. Male 129S1/SvImJ mice aged 8–10 weeks was continuously treated with doxazosin (α1-AR antagonist, 12 mg/kg per day), metoprolol (β1-AR antagonist, 12 mg/kg per day), ICI118551 (β2-AR antagonist, 2.4 mg/kg per day), BRL44408 (α2A-AR antagonist, 12 mg/kg per day), L748337 (α2B-AR antagonist, 2.4 mg/kg per day), imiloxan (α2B-AR antagonist, 12 mg/kg per day), spiroxatrine (α2C-AR antagonist, 12 mg/kg per day), or vehicle (10% DMSO in PBS) via an intraperitoneal implantation of a mini-osmotic pump 24 hours before UUO (n=5). (A–C) Kidney collagen deposition was evaluated by Sirius red staining (A and B) and hydroxyproline measurement (C) in UUO kidneys treated with AR antagonists. (B) The Sirius red–positive area was measured in five randomly chosen high-power (×200) fields per kidney using NIH ImageJ software. (D) α-SMA expression using immunohistochemistry in UUO kidneys treated with AR antagonists. The visible blue color indicates nuclei stained by DAPI. (E) The α-SMA–positive area was measured in five randomly chosen high-power (×200) fields per kidney using NIH ImageJ software. (F) α-SMA and p-Smad3 expression in UUO kidneys treated
with AR antagonists using Western blot analysis. Anti-β-actin antibody served as a loading control. (G) Immunohistochemistry of PMN in UUO kidneys treated with AR antagonists using Western blot analysis. Anti-β-actin antibody served as a loading control. Scale bar, 50 μm. Error bars represent SDs.
Immunohistochemistry, Histology, TUNEL Assay

Immunohistochemical staining of the kidneys was performed on paraffin sections or cryosections as previously described.36–38 Briefly, 4% paraformaldehyde-fixed kidney sections were rehydrated and labeled with antibodies against TH (Abcam, Cambridge, MA), CGRP (Santa Cruz Biotechnology, Santa Cruz, CA), α-SMA (Sigma), F4/80 (Abcam), α3A-AR (Cell Applications, San Diego, CA), α2B-AR (Santa Cruz Biotechnology), α2C-AR (Santa Cruz Biotechnology), or PMN (Accurate, Westbury, NY). The sections were then incubated to fluorescein or peroxidase-conjugated secondary antibodies (Vector...
Laboratories, Burlingame, CA). The α-SMA–positive area was measured in five randomly chosen high-power (×200 magnification) fields per kidney using NIH Image J software. The number of PMN-positive cells was counted in five randomly chosen high-power (×200 magnification) fields per kidney. PAS-stained sections were used for histologic damage score as described previously. Histologic damage of tubular injury was scored by percentage of tubules that displayed tubular necrosis, cast formation, and tubular dilation as follows: 0 = normal, 1 = 10%–25%, 2 = 26%–50%, 3 = 51%–75%, and 4 = >75%. Ten randomly chosen high-power (×200 magnification) fields per kidney were used for the counting. TUNEL assay in the kidneys and cells was carried out using In Situ Cell Death Detection kit, Fluorescein (Roche, Mannheim, Germany), following the manufacturer’s protocol, as previously described. Norepinephrine- or CGRP-treated MCT cells were stained with 1 µg/ml PI for 10 minutes, and the percentage of PI-positive cells was counted by fluorescence microscopy.

**Western Blot**

We performed electrophoresis of protein extracts using tris-tricine or Herrnstein–41 membranes were incubated with antibodies against TH (Abcam), α-SMA (Sigma), fibronectin (Cedarlane), TGF-β (Santa Cruz Biotechnology), p-Smad3 (Cell Signaling), ICAM-1 (Santa Cruz Biotechnology), TNF-α (Abcam), IL-1β (Abcam), IL-6 (Abcam), MCP-1 (Abcam), KC (Abcam), IP-10 (Abcam), α2A-AR (Cell Applications, San Diego, CA), α2b-AR (Santa Cruz Biotechnology), α2c-AR (Santa Cruz Biotechnology), or TLR4 (Imgenex, San Diego, CA). Peroxidase-conjugated secondary antibodies (Vector Laboratories) were applied, and a chemiluminescence reagent (PerkinElmer, Boston, MA) was used to detect proteins. Anti-β-actin antibody (Sigma) was used for loading controls on stripped membranes. The bands were quantified using Lab Works analysis software (Ultra-Violet Products, Cambridge, United Kingdom).

**BP Measurement**

Systolic BP of mice was measured by a noninvasive tail cuff method (CODA; Kent Scientific, Torrington, CT). Mice were placed on a heated platform (30°C) in an isolated chamber, and systolic BP levels were obtained. Mice were trained for 5 days before renal denervation followed by daily recording for experimental days as previously described.

**Statistical Analyses**

ANOVA was used to compare data among groups. Differences between two groups were assessed by two-tailed unpaired or paired Student t test using SigmaPlot for Windows Version 12.0 (Systat Software, San Jose, CA). P values < 0.05 were considered to represent statistically significant differences.

REFERENCES


This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2012070678/-/DCSupplemental.
Supplemental Figure 1
**Supplemental Figure 2**

A. Hydroxyproline (μg/mg kidney)

<table>
<thead>
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<th>CGRP</th>
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<td>0 ng/kg/d</td>
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DNx + UUO 10 d

B. Expression (fold vs. 0 ng/kg/d)

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C. Expression (fold vs. 0 ng/kg/d)

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D. Contralateral uninjected kidneys after 10 d of UUO

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Expressio

Vehicle NE NY CGRP SP

Contralateral uninjected kidneys after 10 d of UUO

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Supplemental Figure 3
A

TUNEL; DAPI stain

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B

Released TGF-β1 (ng/ml)

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Supplemental Figure 5

C

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D

Released TGF-β1 (ng/ml)

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SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: DNx preconditioning prevents expressions in pro-fibrotic proteins and pro-inflammatory proteins.  (A and B) DNx in left kidneys of male 129S1/SvImJ mice aged 8 to 10 weeks was carried out; then 2 d after the onset, the left ureters were obstructed for 3 or 10 d (n=4 in each sham-group, n=6 mice in each UUO-group).  (A) Kidney levels of α-SMA, fibronectin, TGF-β and p-Smad3 protein were measured by Western blot analysis after UUO or sham-operation in DNx or intact kidneys.  Anti-β-actin antibody served as a loading control.  The bands were quantified using Lab Works analysis software.  (B) Kidney levels of ICAM-1, TNF-α, IL-1β, IL-6, MCP-1, KC, IP-10 and TLR4 protein expression were measured by Western blot analysis after UUO or sham-operation in DNx or intact kidneys.  Anti-β-actin antibody served as a loading control.  The bands were quantified using Lab Works analysis software.  (C and D) UUO in left ureters of male 129S1/SvImJ mice aged 8 to 10 weeks were obstructed for 15 d, and DNx was carried out 1, 3 or 5 d after UUO (n=4 in each group).  (C) Collagen deposition using hydroxyproline measurement after UUO in DNx or intact kidneys (n=4 in each sham-group, n=6 in each UUO-group).  (D) The Sirius red-positive area was measured in 5 randomly chosen high-power (x 200) fields per kidney using NIH ImageJ software.  *P < 0.05, **P < 0.01, ***P < 0.001 versus intact.  Error bars represent SD.

Supplemental Figure 2: NE and CGRP contribute to fibrosis and inflammation in UUO kidneys.  (A-C) DNx in left kidneys of male 129S1/SvImJ mice aged 8 to 10 weeks was carried out; 2 d after the onset, NE, NY, CGRP or SP (0, 1.2, 6 or 30 ng/kg/d) was continuously infused into kidneys via a mini-osmotic pump; and the left ureters were obstructed for 10 d (n=6 in each group).  (A) NE- and CGRP-induced collagen deposition using hydroxyproline measurement in denervated UUO kidneys.  (B and C) α-SMA, p-Smad3, ICAM-1 and TNF-α expression in either NE-, NY-, CGRP- or SP-treated UUO kidneys using Western blot analysis.  The bands were quantified using Lab Works analysis software.  (D) Fibronectin, α-SMA and p-Smad3 expression in either NE-, NY-, CGRP- or SP-uninjected contralateral kidneys after 10 d of UUO using Western blot analysis (n=6 in each group).  The bands were quantified using Lab Works analysis software.  *P<0.05, **P<0.01 versus 0 ng/kg/d.  Error bars represent SD.

Supplemental Figure 3: NE and CGRP contribute to tubular cell death in UUO kidneys.  (A-C) DNx in left kidneys of male 129S1/SvImJ mice aged 8 to 10 weeks was carried out; then 2 d after the onset, the left ureters were obstructed for 1, 3 or 10 d (n=4 in each sham-group, n=6 in each UUO-group).  (A) The kidneys were fixed for PAS staining to measure the histological damage in tubules.  The histological damage in tubules on PAS-stained kidney sections was scored by counting the percentage of tubules.  Five randomly chosen high-power (x 200) fields per kidney were used for the counting.  (B) The kidneys were fixed for TUNEL assay to detect apoptotic cells using In Situ Cell Death Detection kit.  The visible blue color
indicates nuclei stained by DAPI. The number of TUNEL-positive apoptotic cells in tubules was counted in 5 randomly chosen high-power (x 200) fields per kidney. (C) Kidney expression levels of PARP1, cleaved PARP1 and cleaved caspase-3 protein were measured by Western blot analysis. Anti-β-actin antibody served as a loading control. *P<0.01, **P<0.05, ***P<0.001 versus intact. (D) NE or CGRP (30 ng/kg/d) was continuously infused into kidneys via a mini-osmotic pump, and the left ureters were obstructed for 10 d (n=4 in each group). The kidneys were fixed for PAS and TUNEL staining to measure the tubular dilation and detect apoptotic cells in tubules, respectively. The visible blue color indicates nuclei stained by DAPI. The lumen area of tubular dilation on PAS-stained kidney sections was measured in 5 randomly chosen high-power (x 200) fields per kidney using NIH ImageJ software. The number of TUNEL-positive apoptotic cells in tubules was counted in 5 randomly chosen high-power (x 200) fields per kidney. *P<0.05 versus vehicle. (E) Systolic blood pressure of mice was measured by a noninvasive tail cuff method. Mice were placed on a heated platform (30°C) in an isolated chamber, and systolic blood pressure levels were obtained. Mice were trained for 5 d before DNx followed by daily recording for experimental days (n=5 in each group). Scale bar, 50 μm. Error bars represent SD.

Supplemental Figure 4: α2-AR antagonists reduce tubulointerstitial fibrosis and inflammation after UUO. Male 129S1/SvImJ mice aged 8 to 10 weeks was continuously treated with doxazosin (α1-AR antagonist, 12 mg/kg/d), atipamezole (α2-AR antagonist, 2.4 mg/kg/d), metoprolol (β1-AR antagonist, 12 mg/kg/d), ICI118551 (β2-AR antagonist, 2.4 mg/kg/d), L748337 (β3-AR antagonist, 2.4 mg/kg/d), BRL44408 (α2h-AR antagonist, 12 mg/kg/d), imiloxan (α2b-AR antagonist, 12 mg/kg/d), spiroxatrine (α2c-AR antagonist, 12 mg/kg/d) or vehicle (10% DMSO in PBS) via an intraperitoneal implantation of a mini-osmotic pump 24 h before UUO (n=5). (A) α-SMA, p-Smad3, ICAM-1, TNF-α, MCP-1 and KC expression in UUO kidneys treated with α2-AR antagonists using Western blot analysis. The bands were quantified using Lab Works analysis software. (B) PARP1, cleaved PARP1 and cleaved caspase-3 expression in UUO kidneys treated with α2-AR antagonists using Western blot analysis. Anti-β-actin antibody served as a loading control. The bands were quantified using Lab Works analysis software. (C) NE level in UUO kidneys using ELISA assay. *P<0.05, **P<0.01, ***P<0.001 versus vehicle; #P<0.05 versus imiloxan. Error bars represent SD.

Supplemental Figure 5: NE and CGRP induce renal tubular apoptosis via α2-ARs and CGRP receptor, respectively. (A and B) Mouse renal proximal tubular cell line (MCT) was maintained in DMEM-high-glucose medium containing 10% FBS at 37°C with 5% CO2. The cells were grown until 70% confluence on culture plates and then changed to serum-free medium. After serum starvation for 6 h, 1 nM of NE and either 10 μM of α2A-AR antagonist BRL44408, 1 μM of α2B-AR antagonist imiloxan, 100 nM of α2C-AR antagonist spiroxatrine, or vehicle (10% DMSO in PBS) was co-treated to the culture (n=4). (A) The MCT cells co-treated with NE and either α2A-AR antagonist, α2B-AR antagonist, α2C-AR antagonist, or vehicle
for 48 h were fixed for TUNEL and PI staining to detect apoptotic and necrotic cells, respectively. The visible blue color indicates nuclei stained by DAPI. The number of TUNEL-positive apoptotic or PI-positive necrotic MCT cells was counted in 5 randomly chosen high-power (x 200) fields per plate. (B) The released level of TGF-β1 and CTGF in the supernatant of MCT cells co-treated with NE- and respective antagonists of α2-AR subtypes for 48 h was examined by ELISA assay and Western blot analysis. The protein bands were quantified using Lab Works analysis software. *P < 0.05, **P < 0.001 versus vehicle. (C and D) MCT cells were grown until 70% confluence on culture plates and then changed to serum-free medium. After serum starvation for 6 h, 1 nM of CGRP plus/minus either 10 nM of CGRP antagonist (CGRP8-37) was incubated for 48 h (n=4). (C) The MCT cells co-treated with NE and CGRP8-37 for 48 h were fixed for TUNEL and PI staining to detect apoptotic and necrotic cells, respectively. The visible blue color indicates nuclei stained by DAPI. The number of TUNEL-positive apoptotic or PI-positive necrotic MCT cells was counted in 5 randomly chosen high-power (x 200) fields per plate. (B) The released level of TGF-β1 and CTGF in the supernatant of MCT cells co-treated with CGRP and CGRP8-37 for 48 h was examined by ELISA assay and Western blot analysis. The protein bands were quantified using Lab Works analysis software. *P < 0.05, **P < 0.001 versus no treatment. Error bars represent SD.