Sympathetic Overactivity in CKD Disrupts Buffering of Neurotransmission by Endothelium-Derived Hyperpolarizing Factor and Enhances Vasoconstriction

Wei Cao, Liling Wu, Xiaodong Zhang, Jing Zhou, Jian Wang, Zhichen Yang, Huanjuan Su, Youhua Liu, Christopher S. Wilcox, and Fan Fan Hou

Due to the number of contributing authors, the affiliations are listed at the end of this article.

ABSTRACT

Background Hypertension commonly complicates CKD. Vascular smooth muscle cells (VSMCs) of resistance arteries receive signals from the sympathetic nervous system that induce an endothelial cell (EC)–dependent anticontractile response that moderates vasoconstriction. However, the specific role of this pathway in the enhanced vasoconstriction in CKD is unknown.

Methods A mouse model of CKD hypertension generated with 5/6-nephrectomy (5/6Nx) was used to investigate the hypothesis that an impaired anticontractile mechanism enhances sympathetic vasoconstriction. In vivo, ex vivo (isolated mesenteric resistance arteries), and in vitro (VSMC and EC coculture) models demonstrated neurovascular transmission and its contribution to vascular resistance.

Results By 4 weeks, 5/6Nx mice (versus sham) had augmented increases in mesenteric vascular resistance and mean arterial pressure with carotid artery occlusion, accompanied by decreased connexin 43 (Cx43) expression at myoendothelial junctions (MEJs), impaired gap junction function, decreased EC-dependent hyperpolarization (EDH), and enhanced contractions. Exposure of VSMCs to NE for 24 hours in a vascular cell coculture decreased MEJ Cx43 expression and MEJ gap junction function. These changes preceded vascular structural changes evident only at week 8. Inhibition of central sympathetic outflow or transfection of Cx43 normalized neurovascular transmission and vasoconstriction in 5/6Nx mice.

Conclusions 5/6Nx mice have enhanced neurovascular transmission and vasoconstriction from an impaired EDH anticontractile component before vascular structural changes. These neurovascular changes depend on an enhanced sympathetic discharge that impairs the expression of Cx43 in gap junctions at MEJs, thereby interrupting EDH responses that normally moderate vascular tone. Dysregulation of neurovascular transmission may contribute to the development of hypertension in CKD.

The hypertension that commonly complicates CKD is characterized by a relentless increase in peripheral vascular resistance and can lead to nephrosclerosis and cardiovascular events. The damaged kidneys activate the sympathetic nervous system (SNS), the major cause of the increased peripheral vascular resistance and hypertension. Endothelial cells (ECs) have been considered both the sensors of humoral factors from damaged kidneys and the regulators of vascular smooth muscle cell (VSMC) tone. We have reported that renal afferent nerves in the rat 5/6-nephrectomized (5/6Nx)
model of CKD drive a reflex activation of the SNS, similar to humans with CKD, which increases efferent flux of SNS and promotes progression of renal fibrosis and impairs glucose uptake in adipose tissue. However, it remains unclear if the activity or the responsiveness to the efferent sympathetic nerves is also enhanced in resistance arteries, and whether it is involved in CKD-associated dysfunction of resistance arteries and hypertension. This is the focus of our study.

Endothelium-derived relaxation, hyperpolarization, and contraction factors all have well established roles in communication between ECs and VSMCs. Endothelium-derived hyperpolarization is especially important in resistance arteries where ECs signal to VSMCs via myoendothelial junctions (MEJs) to induce VSMC hyperpolarization that can offset vasoconstrictor responses. However, there is two-way crosstalk between the EC and VSMC, where ECs signal to VSMCs on the outside of resistance arteries. Their signals can constrict VSMCs and elicit an EC-dependent anticontractile response that provides fine tuning of the changes in vascular tone. However, the specific role of this VSMC/EC/VSMC communication system (termed neurovascular transmission) in hypertension and CKD has not yet been studied.

MEJs connect VSMCs and ECs by specific gap junction channels, in which connexin 43 (Cx43) predominates. Disruption of Cx43 decreases the channel permeability of MEJs, but the function of Cx43 in hypertension in CKD remains to be investigated.

Here, we test the hypothesis that a defect in a specific MEJ connexin mediates an impaired EC-dependent hyperpolarization (EDH) response that enhances vasoconstriction during sympathetic activation in a model of CKD. Our studies are focused on the general problem of dysregulated neurovascular transmission in the development of hypertension. We find that this dysregulation precedes vascular remodeling and show the importance of a disrupted anticontractile component of neurovascular transmission due to loss of EDH in the increased arterial contractility and its dependence on ongoing SNS discharge. This disruption of EDH is accompanied by reduced permeability of Cx43 gap junctions and is likely a component of the increased peripheral vascular resistance in hypertension from CKD.

**METHODS**

**Animal Model**

Male CD-1 mice (6 weeks old) weighing 20–24 g were obtained from the Institutional Animal Experiment Center. Animal experiments were approved by the Institutional Animal Ethics Committee, and the National Institutes of Health criteria for the use and treatment of laboratory animals were followed.

The CKD model of 5/6Nx was induced in male CD-1 mice. Briefly, under anesthesia with intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg body wt), both poles of the left kidney (approximately two thirds of the left kidney mass) were ablated via a left flank incision. One week later, the entire right kidney was removed via a right flank incision. Sham-operated mice (sham) were subject to a similar procedure without the removal of kidneys. All animals were followed up for 8 weeks. The CKD model was validated by measurement of decreased GFR and progressive renal fibrosis during the 8-week observation (Supplemental Figure 1, A and B).

To study the effects of inhibiting central sympathetic outflow, groups of 5/6Nx mice received continuous intracerebroventricular (i.c.v.) infusions of clonidine (5.76 μg/kg per day; Sigma, St. Louis, MO) or artificial cerebral spinal fluid (Sigma) from the first day after operation for 4 weeks, using Micro Infusion Pumps (iPrecio SMP/IMS-310R model, Durect Corporation, Cupertino, CA). Micro Infusion Pumps were implanted in mice under anesthesia 7 days before the 5/6Nx operation, and were programmed wirelessly using proprietary iPrecio pump software (Durect Corporation). The accuracy of the i.c.v. infusion was confirmed by the tracer Evans blue.

**Measurement of Mean Arterial Pressure**

A telemetry system (Data Sciences International, New Brighton, MN) was used to measure mean arterial pressure (MAP) in conscious mice. The sensor tip of the telemetry probe (TA11PA-C10 probes; Data Sciences International) was placed into the left carotid artery under anesthesia with i.p. sodium pentobarbital 7 days before the start of experiments.

For the measurement of MAP in anesthetized mice during carotid artery occlusion, a pressure catheter (FTH 1211B-0018; Transonic Systems, Ithaca, NY) was inserted into the left femoral artery under anesthesia with urethane (1.0–1.3 g/kg body wt, i.p.). MAP was recorded continuously during bilateral carotid artery occlusion using the AcqKnowledge software (Biopac Systems, Goleta, CA).
Gene Delivery in Animals

Construction of Cx43 Expression Vector
Full-length mouse Cx43 cDNA was synthesized and subcloned into a mammalian expression vector GV362 (GeneChem, Shanghai, China) using routine molecular cloning techniques. The empty expression GV362 vector was used as a negative control.

Transfection of GV362-Cx43 Plasmid into Isolated Resistance Arteries
Mice were euthanized by carbon dioxide asphyxiation. Mesenteric resistance arteries (MRAs; about 2 mm in length with a luminal diameter <200 μm) were isolated and transfected with GV362-mouse Cx43 or empty vector GV362 (all from GeneChem) by electroporation as previously described.19 Briefly, isolated MRAs were transfected by electroporation (program A033) with 5 μg of plasmid DNA diluted in 100 μl VSMC Neucleofection Reagent (Lonza, Basel, Switzerland) using a Nucleofector 2b device (Lonza).

Transfection of Mice In Vivo with GV362-Cx43 Plasmid
GV362-mouse Cx43 or empty vector GV362 (GeneChem) was injected into mice by a hydrodynamic gene transfer technique as previously described.20 Briefly, 20 μg of plasmid DNA was diluted in 1.8 ml of saline and injected rapidly over 10 seconds into the tail vein of each mouse.

Evaluation of SNS Activity

Recording of SNS Activity in Mesenteric Artery
SNS activity in the mesenteric artery was measured as described previously21 with minor modifications. Briefly, under anesthesia with urethane (1.0–1.3 g/kg body wt, i.p.), the sympathetic nerve fiber of superior mesenteric artery was isolated, and a pair of platinum electrodes (Biopac Systems) was attached to the nerve fiber to record SNS activity. The nerve signal was amplified 5000 times with an ERS 100C amplifier and filtered with a band-pass filter between 1 and 3000 Hz.21 This amplified and filtered nerve signal was stored and analyzed on a personal computer and recording software (Acqknowledge; Biopac Systems). SNS activity was expressed as mean number of bursts per second.

Measurement of NE by ELISA
The concentrations of NE in tissue homogenates were assessed using an ELISA kit (Demeditec Diagnostics, Kiel, Germany) according to the manufacturer’s instructions.

Measurement of Vascular Responses to SNS Activation by Bilateral Carotid Artery Occlusion
Mice were anesthetized by urethane (1.0–1.3 g/kg body wt, i.p.) for insertion of a pressure catheter (FTH 1211B-0018) into the left femoral artery and the placement of a 0.7-mm Transonic Flow Probe (MA-0.7PSB; Transonic Systems) around the superior mesenteric artery. MAP and mesenteric blood flow were recorded continuously and mesenteric vascular resistance was calculated online after bilateral carotid artery occlusion18 using the Acqknowledge software (Biopac Systems).

Responses of MRAs to NE
Mice were euthanized by carbon dioxide asphyxiation and MRAs were separated from the superior mesenteric bed and then mounted in a wire myograph (Model M610; Darush Myo Technology A/S, Aarhus, Denmark).7,10 MRAs were allowed to equilibrate for 30 minutes in physiologic salt solution, followed by a stimulation with a physiologic salt solution containing 60 mM potassium chloride (KCl) and 10 μM NE (NAK).7,10 Next, MRAs were washed in Krebs solution and subjected to cumulative concentrations of NE (1–10 μM) in the absence or presence of EC, inhibitors of nitric oxide (NO) synthase (L-NAME, NO-dependent relaxation), inhibitors of intermediate/small conductance calcium ion–sensitive potassium ion channels (apamin+charybdotoxin, EDH-dependent relaxation), or inhibitors of MEJ gap junction (18β-glycerolretic acid; 18βGA).22 The MRA tension induced by different doses of NE was expressed as the percentage of the tension induced by NAK.

Measurement of Membrane Potential
Membrane potential was evaluated using a fluorescent dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC4 [3]).23 DiBAC4(3) enters the cytosol during decreases in membrane potential, where its fluorescence intensity elevates via binding to cytosolic proteins.23 When there are increases in membrane potential, DiBAC4(3) dissociates from cytosolic proteins and exits the cell, leading to a reduction in fluorescence.23 For measurement of EC or VSMC membrane potential in MRAs,24 MRAs were isolated, cut open longitudinally with fine scissors, and pinned EC-side up on the Sylgard-coated base of a custom microscope chamber. The MRAs were then loaded with 2 μM DiBAC4(3) at 37°C for 30 minutes. DiBAC4(3) signals were observed using an Olympus (Tokyo, Japan) confocal microscope. Changes of DiBAC4(3) fluorescence after NE application were normalized to the fluorescence intensity at the beginning of that experiment.

Vascular Cell Coculture
The vascular cell coculture was assembled as previously described.14 Briefly, VSMCs and ECs were isolated from the mesenteric artery of CD-1 mice.25,26 Cells between passages 2 and 4 were used to develop a vascular cell coculture model for investigating vascular cell communication. For developing the cell coculture model, a polyester transwell insert (Corning, Corning, NY) was inverted and plated with VSMCs at 1×10^6 cells for 1 day, after which the insert was inverted and the upper membrane plated with ECs at 1×10^5 cells and stabilized for 3 days.

Gene Transfection of Cultured Cells
CRISPR/Cas9 AMP-activated protein kinase α (AMPKα), cAMP response element-binding protein (CREB), and Cx43 knockouts were
Figure 1. Hypertension in 5/6Nx mice is accompanied by enhanced SNS activity and increased mesenteric vascular resistance in response to carotid artery occlusion. Male CD-1 mice were subjected to sham operation or 5/6Nx. (A) MAP in mice at various time points post operation. (B) SNS activity (SNA) in mesenteric artery from mice: representative raw records (scale bar, 1 second; left) and quantitative data (right). (C) Level of NE in homogenates of MRAs from 5/6Nx and sham mice. (D) Representative cross-sectional
generated using CRISPR/Cas9. Small guide RNAs (sgRNAs) were designed to knock out mouse AMPKα1 and AMPKα2 as previously described.27 sgRNAs targeting mouse CREB or Cx43 were designed using the optimized CRISPR design tool (http://crispr.mit.edu).27 sgRNAs targeting AMPKα1 were cloned into U6-sgRNA-EF1a-Cas9-FLAG-P2A-hygro, whereas other sgRNAs were cloned into U6-sgRNA-EF1a-Cas9-FLAG-P2A-puro by Genechem. The lentivirus sgRNAs were then applied to VSMCs according to the manufacturer's instructions (Genechem). sgRNA sequences are listed in Supplemental Table 1. Scramble sgRNA was used as a negative control.

Statistical Analyses
Continuous variables were expressed as mean±SD. The normality of data were confirmed using Shapiro–Wilk goodness of fit test. Differences between two groups were tested by an unpaired t test. Differences among groups were tested by one-way ANOVA or unpaired t test with Bonferroni correction for multiple testing. A P value of <0.05 was considered statistically significant. Power analyses for independent t tests were used to assess the number of animals required (nQuery version 8.2.1, Statistical Solutions Ltd., Cork, Ireland).

Detailed methods, including information about materials and reagents, isolation of VSMCs and ECs, immunofluorescence staining, Western blot, and real-time PCR are described in the Supplemental Methods (Supplemental Table 2, Supplemental References).

RESULTS

Hypertension in CKD Mice Is Accompanied by Enhanced SNS Activity and Impaired EDH Responses, Leading to Dysregulation of Neurovascular Transmission

The CKD (5/6Nx) mice had a progressive increase in MAP from 2 to 8 weeks (Figure 1A), accompanied by a persistent rise in SNS activity (Figure 1B) and NE levels in MRAs (Figure 1C) from 2 to 8 weeks. However, the MRA median/lumen ratio was increased significantly only at week 8 (Figure 1, D and E). This highlights the importance of functional vascular factors in this early phase of hypertension in this model.

Neurovascular transmission was assessed in MRAs from 5/6Nx or sham mice at week 4 in response to activation of SNS by bilateral carotid artery occlusion, a strategy that reduces baroreceptor afferent activity.18 Although the SNS activity and heart rate increased similarly in 5/6Nx and sham mice during carotid occlusion (Figure 1, F and G), the increases in MAP and mesenteric vascular resistance were greater in the 5/6Nx mice (Figure 1H).

To dissociate neurovascular reactivity from hypertension, the reactivity of isolated MRAs to NE was determined using a wire myograph (Figures 2 and 3). The tension of MRAs from normal mice during NE application rose rapidly (“phasic contraction”), followed by a reduction in tension (“anticontractile component”) to a steady state (“tonic contraction”) (Figure 2A). The anticontractile component is taken as the difference between the phasic and tonic constriction. Figure 2B depicts representative tension traces of MRAs from sham or 5/6Nx mice in response to cumulative NE (1–10 μM). Although the phasic contractions were similar in all groups (Figure 2C, left), the tonic contraction in MRAs from 5/6 Nx mice was enhanced (Figure 2C, middle), suggesting a curtailed anticontractile component of neurotransmission (Figure 2C, right). This was largely dependent on continuous SNS enhancement because MRAs from 5/6Nx mice infused i.c.v. with clonidine (versus artificial cerebral spinal fluid), which resulted in lower SNS activity (Figure 2D), had an unchanged phasic contraction (Figure 2C, left) but a reduced tonic contraction (Figure 2C, middle), resulting in near normalization of the impaired anticontractile component (Figure 2C, right). In addition, inhibition of central sympathetic outflow by i.c.v. clonidine reduced renal dysfunction and fibrosis, and decreased MAP in 5/6Nx mice (Supplemental Figure 1, C–E).

This impaired anticontractile response during NE in MRAs from 5/6Nx mice was abolished after EC denudation (Figure 3A). Furthermore, this relaxation phase was maintained after NO blockade with L-NAME (Figure 3B), but was lost after blockade of EDH with apamin and charybdotoxin in L-NAME–pretreated arteries (Figure 3C). The impaired anticontractile component in response to NE in 5/6Nx mice was accompanied by reduced hyperpolarization of ECs and enhanced depolarization of VSMCs, as indicated by changes in DiBAC4(3) fluorescence (Figure 3, D and E). These data suggest that CKD impairs the anticontractile component of the response of MRAs to NE and that this is dependent on an EDH response originating in the endothelium.

I.c.v. infusion of clonidine decreased NE concentration in the MRAs from both sham and 5/6Nx mice (Supplemental Figure 1, F and G). Compared with the sham mice, the absolute decrease in NE levels from CKD mice treated with clonidine was significantly greater (Supplemental Figure 1H). The expression of adrenergic receptors and pannexin 1 in MRAs images of MRAs from mice at various time points postoperation. Scale bar, 50 μm. (E) Quantitative data of media area (left), lumen area (middle), and media/lumen ratio (right) in MRAs from mice at various time points postoperation. (F) Changes of SNA in mesenteric artery after carotid artery occlusion in mice at week 4 postoperation: representative raw records (scale bar, 1 second; left) and quantitative data (right). (G) Changes of heart rate after carotid artery occlusion in mice at week 4 post operation. (H) Changes of MAP (left), mesenteric blood flow (middle), and vascular resistance (right) after carotid artery occlusion in mice at week 4 postoperation. Error bars, mean±SD (n=6 in each group). One-way ANOVA or t test with Bonferroni correction. *P<0.05 versus sham.
was comparable among 5/6Nx and sham groups of mice (Supplemental Figure 2, B and C). MRAs from 5/6Nx and sham mice had similar contractions with KCl or relaxations with cromakalim (Supplemental Figure 2D), suggesting 5/6Nx treatment did not affect the polarization of VSMCs. Therefore, we conclude that enhanced vasoconstriction in this model of CKD depends on continuously enhanced SNS activity that impairs an EDH anticontractile response and thereby leads to enhanced neurovascular transmission.

Activation of the SNS in 5/6Nx Mice Downregulates Cx43 Expression That Dysregulates Gap Junction Channel Function at the MEJ of VSMCs

Communication between VSMCs and ECs is dependent on connexins in MEJs.28 To determine whether enhancement of SNS discharge regulates gap junction function in MEJs and thereby neurovascular transmission, we firstly examined the expression of connexins in MRAs of 5/6Nx mice. The expression of Cx43 was downregulated in MRAs from 5/6Nx mice (Figure 4, A–D), but the expression of Cx37 and Cx40 were unchanged (Supplemental Figure 2E). Cx43 was predominantly localized to VSMCs (Figure 4A) around MEJs (indicated by holes in the internal elastic lamina; Figure 4, B and C). Prolonged inhibition of central sympathetic outflow by i.c.v. clonidine restored Cx43 expression in MRAs of 5/6Nx mice (Figure 4, A–D).

Interestingly, the anticontractile component of MRAs from both sham and 5/6Nx mice during NE application was abolished by disruption of gap junctions with 18βGA (Figure 4E), an inhibitor of gap junction,22 which resulted in reduced

Figure 2. Enhanced SNS activation in 5/6Nx mice impairs anticontractile response to NE, leading to dysregulation of neurovascular transmission. 5/6Nx mice received i.c.v. infusion of clonidine (clo) or artificial cerebral spinal fluid (acsf) for 4 weeks. (A) A representative tension trace showed that application of 1 µM NE to normal MRA induced a rapid rise in vascular tension (phasic contraction), followed by a reduction (anticontractile component) in tension to a steady state (tonic contraction). The anticontractile component is taken as the difference between the phasic and tonic constriction. (B) Representative tension traces showed the response of MRAs to cumulatively applied NE (1–10 µM). (C) Quantitative data showed comparable phasic contraction among groups (left), but enhanced tonic contraction (middle), and thus impaired anticontractile component (right) in MRAs from 5/6Nx mice during NE application. I.c.v. infusion of clonidine (versus acsf) improved the anticontractile response of MRAs to NE. (D) I.c.v. infusion of clonidine (versus acsf) in 5/6Nx mice decreased SNS activity (SNA) in mesenteric artery: representative raw records (scale bar, 1 second; left) and quantitative data (right). Error bars, mean±SD (n=6 in each group). One-way ANOVA or t test with Bonferroni correction. *P<0.05 versus sham, #P<0.05 versus 5/6Nx+i.c.v. acsf.
hyperpolarization of ECs and enhanced depolarization of VSMCs (Figure 4F). By contrast, the baseline EC or VSMC fluorescence was not altered by 18βGA (Supplemental Figure 2F), indicating that MEJ gap junction specifically mediates an EDH anticontractile response to NE and that this is impaired in MRAs from 5/6Nx mice.

A VSMC/EC coculture was used to investigate Cx43 function further (Figure 5) and to isolate it from systemic

**Figure 3.** Impaired anticontractile response to NE in 5/6Nx mice is due to decreased EDH responses. MRAs were isolated from sham or 5/6Nx mice, and were stimulated with NE. (A) Responses of MRAs to NE after EC denudation: phasic contraction (left), tonic contraction (middle), and anticontractile component (right). (B and C) Responses of MRAs to NE in the presence of L-NAME (10 μM) or L-NAME (10 μM)+charybdotoxin (CTX, 1 μM)+apamin (1 μM): phasic contraction (left), tonic contraction (middle), and anticontractile component (right). (D and E) Changes of DiBAC4(3) fluorescence (reflecting membrane potential) in ECs and VSMCs after 10 minutes of NE application in the (D) absence or (E) presence of L-NAME+CTX+apamin. Error bars, mean±SD (n=6 in each group). One-way ANOVA or t test with Bonferroni correction. *P<0.05 versus sham, #P<0.05 versus 5/6Nx i.c.v. artificial cerebral spinal fluid (acsf). Clo, clonidine.
influences. Cx43 was the major connexin expressed at MEJs of VSMCs (Supplemental Figure 3B). Knockout of Cx43 in VSMCs by CRISPR/Cas9 markedly decreased Cx43 expression at MEJs and inhibited biocytin dye transfer from VSMCs to ECs, as shown by a failure to normalize the dye intensity across the MEJ between cells (Supplemental Figure 3, C–E). However, deletion of Cx43 from ECs did not affect the signal transfer from VSMCs to ECs (Supplemental Figure 3, F–H). Importantly, incubation of VSMCs with NE (3 μM NE for 24 hours) downregulated Cx43 expression at both VSMCs and MEJs (Figure 5, B and C) and decreased biocytin trafficking through the MEJ pore (Figure 5D). Incubation of ECs with NE did not change the expression of Cx43 or the channel permeability at the MEJ (Supplemental Figure 4).

Downregulation of Cx43 expression and decreased biocytin trafficking in response to NE were prevented by α1-adrenergic receptor blockade with prazosin (Figure 5, E and F), whereas incubation of VSMCs with NE did not change the expression of Cx37 or Cx40 (Supplemental Figure 3K).

Incubation of VSMCs with NE for 24 hours enhanced the phosphorylation of AMPKα and target of rapamycin complex 2 (TORC2) at the MEJs (Figure 5G) and dissociated the TORC2-CREB complex that forms a transcriptional factor regulating Cx43 gene expression (29,30) (Supplemental Figure 3L). These changes in the NE signaling pathway via AMPKα/TORC2/CREB were prevented by prazosin (Figure 5G, Supplemental Figure 3L). Knockout of AMPKα1 and -α2 in VSMCs restored the ability of NE to reduce Cx43 expression (Figure 5H) and its ability to disassociate CREB and TORC2 (Supplemental Figure 3M), whereas knockout of CREB in VSMCs decreased Cx43 expression at the MEJ.

**Figure 4.** Activation of the SNS in 5/6Nx mice downregulates Cx43 expression in MRAs. (A) Immunostaining for Cx43 (red) in cross sections of MRAs. Internal elastic lamina (IEL), green. Scale bar, 20 μm. (B) Immunostaining for Cx43 in flat-mounted MRAs. Cx43 (red) located in MEJs (small holes within the IEL). IEL, green. Scale bar, 20 μm. (C) Number (Num) of holes in IEL (HIEL) that contains Cx43 (left), number of HIEL (middle), and percentage of HIEL that contains Cx43 (right). (D) Cx43 expression in MRAs determined by Western blot (upper) and real-time PCR (lower). (E) Responses of MRAs to NE in the presence of 18βGA (30 μM). (F) Changes of DiBAC4(3) fluorescence in ECs and VSMCs after NE application in the presence of 18βGA alone. Error bars, mean ± SD (n = 6 in each group). One-way ANOVA or t test with Bonferroni correction. *P < 0.05 versus sham, #P < 0.05 versus 5/6Nx i.c.v. artificial cerebral spinal fluid (acsf). Clo, clonidine; DAPI, 4′,6-diamidino-2-phenylindole.
Figure 5. Incubation of VSMCs with NE downregulates Cx43 expression at MEJs and reduces biocytin trafficking between VSMCs and ECs. (A) Diagram of transwell used for vascular cell coculture system. (B and C) Exposure of VSMCs to NE downregulates Cx43 expression at VSMCs and MEJs in a (B) dose- and (C) time-dependent manner. *P<0.05 versus PBS. (D) Treatment of VSMCs with NE (versus PBS) for 24 hours decreased biocytin transfer from VSMCs to ECs. *P<0.05 versus PBS. (E and F) α1-Adrenergic receptor blocker (prazosin) restored NE-induced reduction of Cx43 expression in (E) MEJs and (F) biocytin transfer from VSMCs to ECs. Biocytin transfer...
(Supplemental Figure 3N). Thus, the impairment of MEJ gap junction function by NE or SNS activity entails activation of \( \alpha_1 \) adrenergic receptors that interrupt the function of Cx43 in MEJs of VSMCs mediated by AMPK\( \alpha \)/TORC2/CREB signaling.

**Restoration of Arterial Cx43 Expression Improves Neurovascular Transmission in MRAs from 5/6Nx Mice**

Both *ex vivo* (Figure 6) and *in vivo* (Figure 7) experiments were designed to test the hypothesis that rescue of the decreased Cx43 expression in the MRAs from 5/6Nx mice could restore a normal neurovascular transmission.

Isolated MRAs from 5/6Nx mice that were transfected with a GV362-Cx43 plasmid (versus empty vector) had upregulated immunolabeling of Cx43 expression in MEJs of VSMCs (Figure 6, A–C) accompanied by an improved anticontractile component of the response to NE (Figure 6, D and E), improved EC hyperpolarization, and reduced VSMC depolarization (Figure 6F). Transfections with plasmid did not alter the expression of Cx40 or Cx37 (Supplemental Figure 5A) in MRAs, or the responses to KCl or cromakalim (Supplemental Figure 5B).

*In vivo* transfection of 5/6Nx mice with a GV362-Cx43 plasmid (versus empty vector) (Figure 7A) enhanced Cx43 expression in the MRAs (Figure 7B) and moderated the rise in vascular resistance and MAP (Figure 7E) in 5/6Nx mice after carotid artery occlusion despite similar elevations of SNS activity and heart rate (Figure 7, C and D). This short-term restoration of arterial Cx43 in 5/6Nx mice did not improve the GFR (Supplemental Figure 5C).

Overexpression of Cx43 in sham mice did not alter the neurovascular transmission in their MRAs in response to NE or carotid artery occlusion (Supplemental Figure 6). Thus, both *ex vivo* and *in vivo* studies concur in demonstrating that decreased Cx43 expression in 5/6Nx mice dysregulates neurovascular transmission and impairs the anticontractile component that contributes to increased vascular resistance in this mouse model of CKD.

**DISCUSSION**

The major new finding from our study is of a novel role for disrupted buffering of neurovascular transmission in the development of enhanced vasoconstriction in CKD (illustrated in Figure 8). We show enhanced neurovascular transmission and thus enhanced vascular tone in CKD, independent of vascular structural changes that depend on a prolonged enhancement of SNS discharge. This is sensed by VSMCs and impairs the function of Cx43-containing gap junction at MEJs, thereby inhibiting an EC-dependent hyperpolarizing response that offsets the vasoconstriction. Inhibition of central sympathetic outflow or restoration of arterial Cx43 expression in CKD improves the neurovascular transmission and reduces vascular tone. These data demonstrate for the first time that the disruption of Cx43-mediated communication between VSMCs and ECs might be a critical mechanism underlying CKD-associated hypertension.

Previous studies of the EC in hypertension have considered it as both the sensor of humoral factors and the regulator of VSMC constriction. However, in this study, we reveal the importance of an additional pathway initiated by prolonged sympathetic activation of VSMCs. This was modeled by the increased SNS discharge accompanying carotid occlusion that caused an augmented increase in vascular resistance and MAP in 5/6Nx mice. This finding was extended by demonstrating an augmented contraction to NE by 4 weeks of CKD that preceded detectable remodeling that was seen only at week 8. Therefore, an enhanced neurovascular transmission because of a reduced offsetting by EDH could enhance the early phase of hypertension in CKD.

NE released by SNS activation induces a rapid vasoconstriction followed by an offsetting anticontractile response that moderates the vasoconstriction, as confirmed in this study. The enhanced vasoconstriction to NE in mice with CKD was dependent on the endothelium because EC denudation increased vasoconstriction and abolished the differences in the offsetting anticontractile component of response to NE in 5/6Nx versus sham mice. The two well-established endothelial pathways that moderate vascular tone are EC-dependent relaxation mediated by NO and EDH generating VSMC hyperpolarization. The impaired EC response in the 5/6Nx resistance arteries was not affected by NO blockade but was accompanied by reduced hyperpolarization in VSMCs and was blocked by apamin and charybdotoxin which prevent hyperpolarization of VSMCs. The defect in VSMC function in mice with CKD was related to defective signal transmission from the EC to the VSMC because the VSMC responses to direct hyperpolarization or depolarization were intact.

The vascular response from activation of the SNS originates from perivascular sympathetic nerves located in the outside of the wall of resistance arteries. After a rapid, phasic VSMC contraction, there is an endothelium-dependent anticontractile response that depends on connexins expressed in MEJ gap

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*is expressed as percentage of maximal intensity in control group (%F\textsubscript{max}). *\( P<0.05 \) versus NE. (G) Treatment of VSMCs with NE (versus PBS) for 24 hours significantly enhanced phosphorylation of AMPK\( \alpha \) and TORC2 in MEJs. \( \alpha_1 \)-Adrenergic receptor blocker prevented NE-induced phosphorylation of AMPK\( \alpha \) and TORC2. *\( P<0.05 \) versus PBS; \( ^{\dagger} P<0.05 \) versus NE. (H) Knockout of AMPK\( \alpha_1 \) and \( \alpha_2 \) by CRISPR/Cas9 in VSMCs (AMPK\( \alpha \) KO) restored NE-induced reduction of Cx43 expression and inhibits NE-induced phosphorylation of TORC2 in MEJs. *\( P<0.05 \) versus scramble sgRNA. Error bars, mean±SD (n=6 in each group). One-way ANOVA or t test with Bonferroni correction.
flects the renal and cerebral sympathetic system and increases ously enhanced SNS discharge. Chronic kidney damage acti-
cular transmission in this CKD model depends on continu-
response to SNS signaling in the vessel wall in CKD.

In this study, SNS activity was increased from 2 to 8 weeks after 5/ 6Nx and i.c.v. clonidine that prevented the increase in out-
fow of SNS activity, restored arterial Cx43 expression, and correc-
ted the impaired EDH response to NE. The expression of Cx43 regulat ed the function of the MEJ because prolonged

The study has some limitations. We cannot exclude some spillover effects of clonidine in activating the α2-adrenergic receptor on peripheral blood vessels. However, intravenous administration of clonidine in pithed animals, which presumably activates only peripheral α2-adrenergic receptors, increases, rather than decreases, BP.32,33 Therefore, a dominant effect of peripheral activation of α2-adrenergic receptors does not seem to play a role in our model. Secondly, i.c.v. clonidine

Figure 6. Restoration of Cx43 expression in MRAs from 5/6Nx mice improves the anticontractile response to NE. (A and B) MRAs from 5/6Nx mice were transfected with either GV362-Cx43 or empty vector GV362 (vector). Immunostaining of transfected MRAs revealed that Cx43 expression (red) was modiﬁed in (A) VSMCs and (B) MEJs. Internal elastic lamina (IEL), green. Scale bar, 20 μm. (C) ImmunobLOTS showed restoration of Cx43 expression in MRAs transfected with GV362-Cx43. (D) Representative tension traces and quantitative data showed that restoration of Cx43 expression did not affect (E) the phasic contraction (left), but reduced tonic con-
traction (middle), and improved anticontractile component (right) of MRAs in response to NE. (F) Restoration of Cx43 expression in MRAs enhanced EC hyperpolarization (upper) and reduced VSMCs depolarization (lower) after NE application. Error bars, mean±SD (n=6 in each group). t test, *P<0.05 versus empty vector. DAPI, 4′,6-diamidino-2-phenylindole; HIEL, holes in IEL.

An interesting new finding is that the enhanced neurovas-
cular transmission in this CKD model depends on continu-
ously enhanced SNS discharge. Chronic kidney damage activat es the renal and cerebral sympathetic system and increases efferent flux of SNS through a reﬂex activation of the SNS.5 In this study, SNS activity was increased from 2 to 8 weeks after 5/ 6Nx and i.c.v. clonidine that prevented the increase in outflow of SNS activity, restored arterial Cx43 expression, and correc-
ted the impaired EDH response to NE. The expression of Cx43 regulated the function of the MEJ because prolonged

juncti0ns.13 The new finding is that reduced expression and func-
tion of Cx43 at MEJs is responsible for the diminished offsetting response mediated by EDH in the vessel wall of mice with CKD. Supporting this notion, knockout of Cx43 in VSMCs in a vascular cell coculture impaired the function of the MEJ gaps junctions, whereas restoration of arterial Cx43 expression in CKD mice rescued the impaired EDH response to NE, and corrected the enhanced vascular resistance after carotid artery occlusion. These findings imply a critical role of reduced Cx43 in MEJ gap junc-
tions for the impaired endothelium-dependent hyperpolarizing response to SNS signaling in the vessel wall in CKD.

incubation of VSMCs with NE reduced the MEJ expression

of Cx43 and impaired the function of the MEJ gap junctions. This MEJ response to NE was mediated via the α1-adrenergic receptor because its blockade with prazosin attenuated all of the effects of NE on MEJs. We assigned the α1-adrenergic receptor intracellular signaling underlying the downregulation of Cx43 in MEJs to phosphorylation of AMPKα and TORC2, leading to a reduced combination of TORC2 with CREB, which is known to regulate Cx43 gene transcription.29,30 Interruption of this signaling pathway corrected the reduction in Cx43 in MEJs with NE. Thus, the persistent enhanced SNS discharge in CKD impairs the offsetting by EDH of neurovascular transmission and enhances vasostriction.
also decreased MAP. However, our previous studies support that inhibiting central sympathetic outflow by clonidine in CKD improves renal fibrosis and insulin resistance in adipose tissue, but normalization of MAP with hydralazine does not,5,6 suggesting the protective effect of i.c.v. clonidine may not depend on BP. Consistently, a previous study reports that vascular dysfunction persists after normalization of MAP with hydralazine in 5/6Nx rats.34

Thirdly, we did not investigate the effect of the vascular renin-angiotensin system which is activated in 5/6Nx rats and involved in impairment of EDH in MRAs. Finally, only male mice were used in this study. Thus, sex-specific differences in SNS regulation and anticontractile mechanisms cannot be addressed, and caution should be used when extrapolating to females.

In sum, our study demonstrates a disrupted regulation of neurovascular transmission in CKD that contributes to the enhanced vascular resistance. The normal link between VSMC activation by NE and the offsetting endothelial buffering via EDH is disrupted in CKD, thereby enhancing vasoconstriction. The sustained activation of the SNS discharge in CKD downregulates Cx43 in gap junctions of MEJs and thereby impairs the transmission of EDH signals that normally offset the vasoconstriction. Our findings provide a translation of the normal role of heterocellular vascular communication in resistance arteries8 to CKD and provide new insights into the development of hypertension in CKD.

Figure 7. Delivery of GV362-Cx43 plasmid to 5/6Nx mice moderates the rise in mesenteric vascular resistance in response to carotid artery occlusion. (A) Outline of experimental protocol: 5/6Nx mice were treated with hydrodynamic-based injection of GV362-Cx43 plasmid or empty vector GV362 (vector) for 3 days (day 26–28 postoperation). (B) Immunoblotting showed restoration of Cx43 expression in MRA homogenates from 5/6Nx mice injected with GV362-Cx43. (C–E) Transfection of 5/6Nx mice with GV362-Cx43 plasmid did not affect (C) the changes of SNS activity (SNA) in mesenteric artery and (D) heart rate, (E) but moderated the rise in MAP (left), mesenteric blood flow (middle), and vascular resistance (right) after carotid artery occlusion. Error bars, mean±SD (n=6 in each group). t test, *P<0.05 versus empty vector.
ACKNOWLEDGMENTS

Dr. Wei Cao performed the experiments, analyzed the data, and drafted part of the manuscript; Dr. Fan Fan Hou designed and financed the study and wrote and edited the manuscript; Dr. Youhua Liu and Dr. Jian Wang gave suggestions to the study design; Dr. Huanjuan Su, Dr. Xiaodong Zhang, Dr. Jing Zhou, and Dr. Zhichen Liu and Dr. Jian Wang gave suggestions to the study design; and Dr. Liling Wu performed biochemical experiments; Dr. Christopher S. Wilcox gave suggestions to the study design and revised the manuscript; and Dr. Liling Wu performed the experiments and analyzed the data.

DISCLOSURE

F.F. Hou reports personal fees from AbbVie and personal fees from AstraZeneca, outside the submitted work. All remaining authors have nothing to disclose.

FUNDING

This study was supported by the National Natural Science Foundation of China Foundation for Innovation Research Groups grant 81521003 (to H.Y.Liu); and the National Natural Science Foundation of China grants 81922014 and 81870473 (to W. Cao).

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2020030234/-/DCSupplemental.

Supplemental Table 1. List of sgRNAs used to knock out the indicated targets.

Supplemental Table 2. List of primers used for real-time PCR.

Supplemental Figure 1. 5/6Nx mice showed progressive renal injury, while i.c.v. infusion of clonidine in 5/6Nx mice reduced NE levels in MRAs and improved renal injury.

Supplemental Figure 2. 5/6Nx or i.c.v. infusion of clonidine did not affect the expression of pannexin-1 and adrenergic receptors in MRAs.

Supplemental Figure 3. Knockout of Cx43 by CRISPR/Cas9 in cultured VSMCs impaired the function of MEJ gap junction.

Supplemental Figure 4. NE treatment in cultured ECs did not alter the expression of connexins or the biocytin trafficking between VSMCs and ECs.

Supplemental Figure 5. Transfections of ECs from 5/6Nx mice with GV362-Cx43 did not alter the expression of Cx40 and Cx37.

Supplemental Figure 6. Overexpression of Cx43 in MRAs from sham mice did not affect the neurovascular transmission in response to NE or carotid artery occlusion.

REFERENCES


Figure 8. Schematic diagram summarizing that continuously increased SNS discharge in CKD, through activation of α1-adrenergic receptors (α1-AR) in VSMCs, impairs the function of Cx43-containing gap junction at MEJs, thereby inhibiting an EDH response that offsets the vasoconstriction and promotes hypertension.


**AFFILIATIONS**

1Division of Nephrology, Nanfang Hospital, Southern Medical University, State Key Laboratory of Organ Failure Research, National Clinical Research Center of Kidney Disease, Guangzhou Regenerative Medicine and Health Guangdong Laboratory, Guangzhou, People’s Republic of China

2State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Disease, The First Affiliated Hospital, Guangzhou Medical University, Guangzhou, Guangdong, People’s Republic of China

3Division of Nephrology and Hypertension, Georgetown University Medical Center, Washington, DC