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

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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.1 The damaged kidneys activate the sympathetic nervous system (SNS), the major cause of the increased peripheral vascular resistance and hypertension.2 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.3,4 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,5 similar to humans with CKD,2 which increases efferent flux of SNS and promotes progression of renal fibrosis and impairs glucose uptake in adipose tissue.5,6 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.7 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.8 However, there is two-way crosstalk between the EC and VSMC.3,8,10 Sympathetic nerves terminate on VSMCs on the outside of resistance arteries.11 Their signals can constrict VSMCs and elicit an EC-dependent anticontractile response that provides fine tuning of the changes in vascular tone.12,13 However, the specific role of this VSMC/EC/VMSC communication system (termed neurovascular transmission) in hypertension and CKD has not yet been studied.

MEJs13,14 connect VSMCs and ECs by specific gap junction channels, in which connexin 43 (Cx43) predominates. Disruption of Cx43 decreases the channel permeability of MEJs,15 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.

**Significance Statement**

Vascular smooth muscle cells (VSMCs) of resistance arteries receive sympathetic nerve signals, and subsequently elicit an endothelium-dependent anticontractile response to modulate vasoconstriction, but the specific role of this neurovascular transmission in hypertension in CKD is unknown. In this investigation, in vivo, ex vivo, and in vitro models were used to study neurovascular transmission and its contribution to elevated vascular resistance in CKD, independent of vascular structural changes. The experiments revealed that the impaired anticontractile component of neurovascular transmission relies on sustained enhancement of sympathetic discharge, which is sensed at VSMCs and impairs expression of connexin 43 in gap junctions at myoendothelial junctions. This cascade interrupts endothelium-dependent hyperpolarizing responses and increases vascular tone. The findings provide new insights into the development of hypertension in 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.16 Briefly, under anesthesia with intraperitoneal 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.16 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)5 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.17

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 occlusion18 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–sensitive potassium ion channels (apamin + charybdotoxin, EDH-dependent relaxation), or inhibitors of MEJ gap junction (18β-glycyrrhetinic 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 × 10⁶ cells for 1 day, after which the insert was inverted and the upper membrane plated with ECs at 1 × 10⁷ 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 5/6Nx 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

Supplemental Table 1. Scramble sgRNA was used as a negative control. Images were taken by confocal microscopy. 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 postoperation. (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. Activation of the SNS in 5/6Nx mice downregulates Cx43 expression that dysregulates gap junction function at the MEJ of VSMCs. To determine whether enhancement of SNS discharge regulates gap junction function in MEJs and thereby neurovascular transmission, we first 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, which resulted in reduced tension (mN)

![Graph A](image1.png)

**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.

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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 a1-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 (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...
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of TORC2 in MEJs. *MEJs of VSMCs mediated by AMPKα in this mouse model of CKD.

component that contributes to increased vascular resistance and impairs the anticontractile response that decreased Cx43 expression in 5/6Nx mice dysregulates 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.3,4 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.

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.

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NE released by SNS activation11 induces a rapid vasoconstriction followed by an offsetting anticontractile response that moderates the vasoconstriction,22 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.31 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.11 After a rapid, phasic VSMC contraction, there is an endothelium-dependent anticontractile response that depends on connexins expressed in MEJ gap

is expressed as percentage of maximal intensity in control group (%Fmax). *P<0.05 versus NE. (G) Treatment of VSMCs with NE (versus PBS) for 24 hours significantly enhanced phosphorylation of AMPKα and TORC2 in MEJs. α1-Adrenergic receptor blocker prevented NE-induced phosphorylation of AMPKα and TORC2. *P<0.05 versus PBS; #P<0.05 versus NE. (H) Knockout of AMPKα1 and -α2 by CRISPR/Cas9 in VSMCs (AMPKα 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.

JASN 31: 2312–2325, 2020 Neurovascular Transmission in CKD 2321
The new finding is that reduced expression and function 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 junctions for the impaired endothelium-dependent hyperpolarizing response to SNS signaling in the vessel wall in CKD.

An interesting new finding is that the enhanced neurovascular transmission in this CKD model depends on continuously enhanced SNS discharge. Chronic kidney damage activates the renal and cerebral sympathetic system and increases efferent flux of SNS through a reflex activation of the SNS. 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 corrected the impaired EDH response to NE. The expression of Cx43 regulated the function of the MEJ because prolonged incubation of VSMCs with NE reduced the MEJ expression of Cx43 and impaired the function of the MEJ gap junctions. 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...
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,\textsuperscript{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.\textsuperscript{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 arteries\textsuperscript{8} 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 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

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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.

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REFERENCES


AFFILIATIONS

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Supplemental data

Sympathetic overactivity in CKD disrupts buffering of neurotransmission by endothelium-derived hyperpolarizing factor and enhances vasoconstriction

Running title: Neurovascular transmission in CKD

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Supplemental methods

Measurement of GFR by inulin clearance

The GFR were determined by inulin clearance. Briefly, under isoflurane anesthesia, a catheter was placed in the bladder for urine collection, and a cannula was placed in the tail vein to infuse inulin. Normal saline solution (0.9%) containing 15% inulin, 3.75% PAH, and 1% bovine albumin was infused at a rate of 5 µl/min. After a 1-hour equilibration period, urine was collected every 30min for three times and blood was drawn at the end of the infusion. Inulin concentrations in urine and plasma were measured by using commercial kits (BioVision; Inc., Milpitas, CA and Glory Science Co., Del Rio, TX).

Vascular cell coculture

The vascular cell coculture was assembled as previously described. Briefly, VSMCs and ECs were isolated from mesenteric artery of CD-1 mice. Cells between passages 2 and 4 were used to develop a vascular cell coculture model for investigating vascular cell communication. For developing cell coculture model, a polyester transwell insert (Corning, NY, USA) was inverted and plated with VSMCs at 1×10⁶ cells for 1 day, after which the insert was inverted and the upper membrane plated with ECs at 1×10⁷ cells and stabilized for 3 day.

For isolation of VSMCs, the mesenteric artery along the small intestines was removed from the mouse under deep anesthesia with diethyl ether, then placed in Hanks balanced salt solution with 25mM HEPES and cleaned of adipose tissue and adventitia. After get rid of the endothelium, the artery was cut laterally into 1mm² explants, and incubated with 0.1%
collagenase in HBSS for 30 min at 37°C. Next, the digested solution was trituated in growth medium (Dulbecco's Modified Eagle Medium with 15% fetal bovine serum), and centrifuged, and seeded onto a 60mm FBS-coated tissue culture dish.

For isolation of ECs, the mesenteric vascular bed along the small intestines was digested with 0.02% collagenase in endothelium basal medium for 45 min at 37°C. After centrifugation at 845 g for 5 min, the cell pellets were resuspended in endothelial complete medium containing 5% fetal bovine serum and 1% endothelial cell growth supplement, and then plated onto a dish coated with 0.01% gelatin (Sigma). The adherent endothelial cells were grown in the endothelial complete medium until confluent.

**Dye transfer**

Biocytin trafficking between VSMCs and ECs was performed and quantified as previously reported. Briefly, VSMCs were loaded with biocytin (5mg/ml, 357Da) using a pinocytotic kit (Thermo Fisher Scientific, MA, USA). Biocytin transfer to ECs was assessed by cutting transverse sections of the Transwell membranes and analyzing the intensity of biocytin with fluorophore-conjugated streptavidin using an Olympus confocal microscopy. The mean pixel intensity in the XZ direction was obtained at 1μm intervals. The pixel intensity at each point along the pore length of Transwell membranes was normalized to the maximum pixel intensity in the control conditions (%Fmax).

**Histology**

Under deep anesthesia, mice were transcardially perfused, first with PBS and then with 10% neutralized formalin. The kidneys or MRAs were isolated, embedded in paraffin, sectioned at
a thickness of 5μm. Renal fibrosis was assessed on renal paraffin sections processed for Masson’s trichrome staining. The degree of tubulointerstitial fibrosis was semi-quantitated on a grading score of 0 to 4.

MRA media/lumen ratio was determined on paraffin sections using an elastic stain kit (HT-25A, Sigma). For each MRA, 3 sections 100μm apart were prepared. Measurements of Lumen or media cross-sectional area were performed using an Olympus microscope and Image-Pro Plus 6.0 (Media Cybernetics, MD, USA).

**Immunofluorescence staining**

MRAs were isolated from anesthetized, formalin-perfused mice, and 7μm paraffin sections were prepared. For flat mounts, the arteries were cut longitudinally and processed whole. Immunofluorescence staining was performed as previously described. Briefly, sections or flat-mounted arteries were permeabilized with 0.1% Triton X-100 for 15 minutes, treated with PBS containing 3% BSA, 3% goat serum, 0.1% Triton, and 0.05% Tween-20 for 1 hour, and then incubated with the primary antibody against Cx43 (1:100, ab11370, Abcam, Cambridge, UK) at 37°C for 2 hours. Sections or flat-mounted arteries were then stained with Alexa Fluor™ Plus 594-conjugated secondary antibodies (Thermo Fisher Scientific), mounted on glass slides, and visualized under an Olympus confocal microscope.

**RNA extraction and Real-time PCR**

Total RNA was extracted from the cells or MRAs using TRIzol reagent (MRC, OH, USA). 5μg total RNA was reverse transcribed with the PrimeScript RTase Kit (TaKaRa
Biotechnology, Shiga, Japan). Real-time PCR was performed by TaqMan gene expression assays (Applied Biosystems, CA, USA). The primers used were listed in Supplemental Table 2. The gene mRNA levels were calculated after normalization to GAPDH.

**Co-immunoprecipitation**

Co-immunoprecipitation was conducted as previously described.8 Briefly, cell lysates were immunoprecipitated by anti-TORC2 antibody from Santa Cruz (1:100, sc-271912, CA, USA). Immune complexes were washed five times with lysis buffer and then analyzed by western blot.

**Western blot**

Western blot was performed as previously described.9 The primary antibodies used were listed as follows: anti-α1- (1:400, ab3462), β1- (1:1000, ab3442), β2- (1:1000, ab182136), Cx43 (1:1000, ab11370), Cx37 (1:1000, ab181701), Pannxin-1 (1:1000, ab139715), Phospho-TORC2 (1:1000, ab203187) or CREB (1:1000, ab32515) antibody from Abcam, anti-AMPKα (1:1000, 2532), Phospho-AMPKα(Thr172) (1:1000, 50081), or Phospho-CREB (Ser133) (1:1000, 9198) antibody from Cell Signaling Technology (MA, USA), anti-α2-ARs (1:1000, CA1003) antibody from Cell Application (CA, USA), anti-β3-ARs (1:100, sc-1473) or TORC2 (1:100, sc-271912) antibody from Santa Cruz, and anti-Cx40 (1:1000, AB1726) antibody from Millipore (MA, USA).
References


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

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<tr>
<th>Target gene</th>
<th>Sequence</th>
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<tr>
<td><em>Gja1</em></td>
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<td><em>Prkaa1</em></td>
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<td><em>Prkaa2</em></td>
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<td><em>Creb1</em></td>
<td>GACTTATCTTTCTGATGCACC</td>
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<table>
<thead>
<tr>
<th>Gene</th>
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<th>Reverse (5’-&gt;3’)</th>
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<td>GCTTGGACCTTGTCGAGCAG</td>
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<td>TCCTTGCCAGCATCAGATTAC</td>
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<td>Gapdh</td>
<td>GCACAGTCAAGGCGAGAAT</td>
<td>GCCTTCTCCATGGGATGAA</td>
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Figure legends

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

(A and B) Male CD-1 mice were subjected to sham or 5/6Nx operation. 5/6Nx mice showed progressive tubulointerstitial fibrosis (A) and decreased GFR (B) during the 8-week observation. Scale bar, 50µm. (C-H) 5/6Nx or sham mice received intracerebroventricular (i.c.v.) infusion of clonidine (clo) or artificial cerebral spinal fluid (acsf) for 4 weeks. Intracerebroventricular (i.c.v.) infusion of clonidine improved tubulointerstitial fibrosis (C) and GFR (D), and decreased MAP (E) in 5/6Nx mice. Moreover, i.c.v. clonidine (vs acsf) reduces NE levels in MRAs of both sham and 5/6Nx mice (F and G). However, the absolute decrease in NE levels by i.c.v. clonidine was significantly greater in MRAs of 5/6Nx mice compared with sham mice (H). Error bars, mean ± SD (n=6 in each group). One-way ANOVA or t test with Bonferroni correction, *P<0.05 vs sham, #P<0.05 vs 5/6Nx+i.c.v. acsf.

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

(A) Representative images showing DiBAC4(3) fluorescence of ECs and VSMCs in a normal MRA before NE application. Scale bar, 20µm. (B and C) Expression of adrenergic receptors (ARs, B) and pannexin 1 (Panx1, C) in MRAs determined by western blot. (D) Responses of MRAs to KCl (40mM) or cromakalim (10µM). (E) Expression of Cx37 and Cx40 in MRAs determined by western blot. (F) Changes of DiBAC4(3) fluorescence in EC of MRAs after
18βGA application alone. Error bars, mean ± SD (n=6 in each group). One-way ANOVA or t

Supplemental Figure 3. Knockout of Cx43 by CRISPR/Cas9 in cultured VSMCs

impaired the function of MEJ gap junction

(A) Diagram of Transwell used for in vitro vascular cell coculture system. (B) Immunoblots

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and MEJ (D, middle) fractions, but not at EC fraction (D, right). *P<0.05 vs scramble sgRNA

(scramble). (E) Knockout of Cx43 in VSMCs reduced biocytin dye transfer from VSMCs to

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biocytin dye transfer from VSMCs to ECs. (I-K) Treatment of VSMC with NE (vs PBS) for

24 hours (I) did not alter the Cx43 expression at EC (J) or change the expression of Cx37 and

Cx40 at fractions of VSMC, MEJ and EC (K). (L) Treatment of VSMCs with NE (vs PBS)

for 24 hours significantly induced the disassociation of TORC2-CREB complex at MEJs.

Alpha1-adrenergic receptor (AR) blocker restored the NE-induced disassociation of

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(A) Diagram showing exposure of ECs to indicated doses of NE (1-10µM) for 24 hours in vascular cell coculture model. (B-E) Incubation of ECs with NE did not change the expression of Cx43 (B), Cx40 (C) or Cx37 (D), or the biocytin transfer from VSMC to EC (E). Error bars, mean ± SD (n=6 in each group). One-way ANOVA or t test with Bonferroni correction.

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(A and B) Transfections of MRAs from 5/6Nx mice with GV362-Cx43 or empty vector GV362 (vector) did not alter their expression of Cx40 and Cx37 (A), or affect their responses to KCl or cromakalim (B). (C) 5/6Nx mice transfected with plasmid did not show significant improvement in the GFR. Error bars, mean ± SD (n=6 in each group). t test.

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

(A-C) MRAs from sham mice were transfected with GV362-Cx43 or empty vector (vector). Immunoblotting showed overexpression of Cx43 in MRAs transfected with GV362-Cx43 (A).
Representative tension traces (B) and quantitative data (C) showed that overexpression of Cx43 did not affect the phasic contraction, tonic contraction, or anti-contractile component in response to NE. (D-F) Sham mice were treated with hydrodynamic-based injection of GV362-Cx43 or empty vector for 3 days. Immunoblotting showed overexpression of Cx43 in MRAs from mice injected with GSV362-Cx43 (D). Delivery of GSV362-Cx43 to sham mice did not affect the changes of SNA (Scale bar, 1 second; E), or alter the changes of MAP, mesenteric blood flow and vascular resistance (F) in response to carotid artery occlusion. Scale bar, 1 second. Error bars, mean ± SD (n=6 in each group). t test.
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