Role of connexin40 in the autoregulatory response of the afferent arteriole

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RENAL AUTOREGULATION PLAYS a major role in sodium and water homeostasis by maintaining a near-constant renal blood flow (RBF) and glomerular filtration rate despite acute changes in renal perfusion pressure (RPP) (49). An acute increase in RPP leads to constriction of the preglomerular vessels. Two, perhaps three, mechanisms work in concert to achieve this (25). The two most well-known mechanisms are the myogenic response and the tubuloglomerular feedback (TGF) mechanism.

Unlike the myogenic response, the TGF response is confined to the kidney (2). The response is initiated by an elevated tubular NaCl concentration at the macula densa (MD) cells in the thick ascending limb of the loop of Henle as a consequence of increased tubular flow rate. The signal is transmitted through the MD cells and the mesangial cells to the distal afferent arteriole. Adenosine has been suggested as the mediator (41, 46), possibly generated by break down of ATP released from the MD cells (40). The increase in extracellular adenosine leads to increased intracellular Ca2+ concentration ([Ca2+]i) in the extraglomerular mesangial cells (35) that spreads via cell-to-cell communication in the mesangial region to the vascular smooth muscle cells (VSMC) in the distal afferent arteriole. The constriction elicited in the distal part of the afferent arteriole initiates a vascular conducted response that can spread more than 1,000 μm upstream to the proximal afferent arteriole, neighboring afferent arteriole, and the interlobular artery, thereby constricting a substantial part of the preglomerular vessels (6, 50). An adequate TGF response therefore depends on intercellular communication within the juxtaglomerular (JG) apparatus and the preglomerular vessels.

Vascular conducted responses are characterized by a distant vasoconstriction or vasodilation caused by a local stimulation of an arteriole. They are described in several vascular beds and are believed to play a role in regulation of the microcirculation (3, 36). The conducted responses are mediated via gap junctions, which facilitate intercellular communication between neighboring cells (12, 17, 43). Gap junctions are formed by connexins. Most vascular tissues express several isoforms of connexin (Cx), namely Cx37, Cx40, Cx43, and Cx45. In the renal vasculature, Cx37, Cx40, and Cx43 are primarily expressed in the endothelial cells (1, 18, 53), whereas Cx45 is supposedly expressed in the VSMC (20, 28). Cx40 is also expressed in the renin-secreting granular cells and in the extraglomerular mesangial cells, which indicates a role for Cx40 in the TGF response as well as in the regulation of renin secretion (1, 22, 29, 48).

The Cx40 knockout (Cx40 KO) mouse is hypertensive (8), primarily because of an increased renin release from the granular cells (51). In these mice, the conduction of acetylcholine (ACh)-induced vasodilation in arterioles from the cremaster muscle is reduced significantly, whereas the conduction of KCl-induced vasoconstriction is unchanged (8). Cell-specific deletion of Cx40 in the granular cells leads to renin-induced hypertension, whereas the conduction of ACh-induced vasodilation is preserved (52). On the other hand, cell-specific deletion of Cx40 in endothelial cells causes reduced conduction of ACh-induced vasodilation (24) but not renin-induced hypertension. Taken together, these results suggest that the effect of Cx40 on vascular conduct is independent of changes in blood pressure and primarily relates to reduced coupling of the endothelial cells.

Despite the increased arterial pressure of the Cx40 KO mice, RBF is normal, but renal autoregulation is attenuated (26).
Based on an analysis of the dynamics of the autoregulatory response, Just et al. (26) concluded that it was primarily the TGF component of renal autoregulation that was affected by deletion of the Cx40 gene although an effect on the myogenic component could not be excluded. Inhibition of gap junctional communication using connexin mimetic peptides against Cx37 and -43 significantly reduced the myogenic response in mesenteric arterioles from rats (10). Thus, it is not clear whether reduced intercellular coupling primarily affects TGF or whether it also affects the myogenic response.

In the present study, we used the isolated blood-perfused juxtamedullary nephron preparation to further investigate the role of Cx40 in renal autoregulation. We hypothesized that reduced intercellular communication in the JG apparatus would reduce the TGF component of renal autoregulation possibly by reducing the vascular Ca$^{2+}$ propagation eliciting the TGF-induced vasoconstriction. We measured the afferent arteriolar diameter response to changes in RPP, close to the glomerulus (<50 μm) and further upstream (>100 μm), in Cx40 KO mice, age-matched wild-type (WT), and heterozygous (HZ) littermates. The response was measured before and after disruption of the TGF response by papillectomy. We also investigated the conduction of the intracellular Ca$^{2+}$ signal in response to electrical stimulation in isolated pregglomerular arterioles from Cx40 KO and WT mice.

**METHODS**

**Animals**

All procedures were approved by the Danish National Animal Experiments Inspectorate. All animals were kept in the animal facility and received tap water and standard chow ad libitum. Twenty-five male Sprague Dawley rats (Taconic, Lille Skensved, Denmark) were used as blood donors.

Heterozygous Cx40 KO animals (generated on a 129S4/SvJae background) for breeding were purchased from the European Mouse Mutant Archive (EMMAnet.org, Munich, Germany). Animals were genotyped from tail tip DNA using the DirectPCR kit from Viagen Biotech (Los Angeles, CA). Kidneys from a total of 34 adult age-matched female and male littermate mice were used (WT n = 7 females, n = 7 males; HZ n = 2 females, n = 3 males; Cx40 KO n = 6 females, n = 9 males). Ages ranged from 3 to 8 mo in the WT and Cx40 KO groups and from 3 to 6 mo in the HZ group.

Genotyping of the animals showed that, from the first 88 pups born, 27 were WT, 14 were homozygous knockouts, and 47 were HZ. There was a slight majority of females (52 vs. 36 males).

**Blood Collection From Rat Donors**

Blood was collected from rats anesthetized with isoflurane delivered in 35% oxygen and 65% nitrogen. A catheter was placed in the carotid artery, and the rat was exsanguinated. Blood was collected in a heparinized syringe and then centrifuged. Plasma was removed, and the buffy coat was discarded. Red blood cells (RBCs) were washed two times in 0.9% NaCl. Plasma was filtered (0.2 μm; Advantec, Tokyo, Japan) and recombined with RBCs and Tyrode buffer (in mM: 136.9 NaCl, 0.42 NaH$_2$PO$_4$, 11.9 NaHCO$_3$, 2.7 KCl, 2.2 MgCl$_2$, 5.6 d-glucose, and 1.8 CaCl$_2$; pH adjusted to 7.4) containing 5% BSA (ICPbio International, Auckland, New Zealand) to a hematocrit of 25%.

**Mouse In Vitro Blood-Perfused Juxtamedullary Nephron Technique**

Experiments were conducted based on the blood-perfused juxtedudillary nephron technique developed by Casellas and Navar (4) and adapted to mice (21, 23). Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg ip; SAD), and a catheter was placed in the abdominal aorta for arterial pressure measurements and blood sample collection. Hereafter, a cannula was inserted in the descending aorta that substituted the blood supply from the heart. The cannula system includes a 27-gauge blunt needle for introduction in the renal artery and two PE-10 lines for blood perfusion and measurement of perfusion pressure. The tips of these tubing are in close proximity to each other. The kidney was perfused immediately with Tyrode buffer containing an amino acid mixture (Sigma-Aldrich, Copenhagen, Denmark) and 5% BSA (pH 7.4). The kidneys were excised, and the cannula was advanced in the right renal artery. The left kidney was either snap-frozen in liquid nitrogen for later immunostaining or used for isolating afferent arterioles. The heart was removed for weighing, snap-frozen, and saved for later immunostaining.

A longitudinal section was carefully made along the kidney to expose the papilla without damaging it. The papilla was reflected back, thereby revealing the inner cortical surface. The venous tissue atop the cortical surface was cut open to facilitate view and access of the renal vasculature, and ligatures (10.0 sutures; Ethilon) were fastened around larger arteries to restrict perfusion to the juxtamedullary afferent arterioles of the inner cortical surface. After removal of connective tissue, the 5% albumin solution was replaced with blood from the donor rat. Perfusion pressure was maintained at 75 mmHg by adjusting the regulator controlling the flow of O$_2$–CO$_2$ mixture from the tank that pressurized the blood reservoir. The preparation was viewed and recorded using an Olympus BX50WI microscope with a PixelFly digital 12-bit charge-coupled device camera using the CamWare software (PCO, Kelheim, Germany). RPP readings were acquired with a PowerLab/8SP data acquisition system (ADInstruments, Colorado Springs, CO). During the experiment, the kidney was superfused with warmed (37°C) Tyrode solution containing 1% albumin.

**Experimental protocol.** An afferent arteriole was chosen based on intact glomerular perfusion and adequate blood flow. In the first set of experiments, measurements of afferent diameter were made >100 μm upstream from the glomerulus. In the second set of experiments, measurements were made in the afferent segment juxtaposed to the glomerulus. After initiation of blood perfusion, an equilibration period of 15 min was allowed. RPP was set to 75 mmHg and increased in steps of 20 mmHg up to 155 mmHg. Each pressure step lasted 3 min. After the first set of pressure steps, a papillectomy was performed to interrupt flow in the loop of Henle, and the pressure steps were repeated. After papillectomy, the signal for activation of TGF was interrupted, and constrictions in the afferent arteriole seen after papillectomy are presumed to be caused by the myogenic response alone (39, 47).

**Isolated Arteriole Experiments**

Preglomerular arterioles were dissected from kidneys from Cx40 KO and WT mice as previously described for rats (37). Briefly, thin slices (0.5–1 mm) of the kidney were cut from the midregion and placed in a dissection dish containing a physiological salt solution (in mM: 135 NaCl, 5.0 KCl, 1.0 CaCl$_2$, 1.0 MgCl$_2$, 10 HEPES, and 50 μM fura-2-AM with 0.01% Pluronic F-127 (Sigma-Aldrich) for 60 min at room temperature and then moved to the renal vasculature, and ligatures (10.0 sutures; Ethilon) were fastened around larger arteries to restrict perfusion to the juxtamedullary afferent arterioles of the inner cortical surface. After removal of connective tissue, the 5% albumin solution was replaced with blood from the donor rat. Perfusion pressure was maintained at 75 mmHg by adjusting the regulator controlling the flow of O$_2$–CO$_2$ mixture from the tank that pressurized the blood reservoir. The preparation was viewed and recorded using an Olympus BX50WI microscope with a PixelFly digital 12-bit charge-coupled device camera using the CamWare software (PCO, Kelheim, Germany). RPP readings were acquired with a PowerLab/8SP data acquisition system (ADInstruments, Colorado Springs, CO). During the experiment, the kidney was superfused with warmed (37°C) Tyrode solution containing 1% albumin.

**Microdissection of vessels was performed under a microscope using sharpened forceps. An interlobular artery was localized, and the tubular structures were removed. Only the most-distal part of the interlobular artery and afferent arteriole was used. If no preparation was obtained during the first 90 min, the kidney was discarded. The arteriole was loaded with 5 μM fura 2-AM with 0.01% Pluronic F-127 (Sigma-Aldrich) for 60 min at room temperature and then moved to the stage of an inverted microscope (Olympus IX 50).

**Measurements of [Ca$^{2+}$]$_i$.** A digital video camera (PCO) and Image Workbench software (INDEC Biosystems, Santa Clara, CA) or Live acquisition 2.0 (Till Photonics, Munich, Germany) were used.
The vessel was visualized on-screen and an area for measurement of 
$[Ca^{2+}]$, was chosen. The fluorescent emission was detected by the
digital video camera, and the ratio of fluorescence obtained with 340
nm excitation to that obtained with 380 nm excitation (R340/380) was
calculated. Changes in this ratio were used as an index for changes in
$[Ca^{2+}]$ (15). In preliminary experiments, autofluorescence was mea-
sured in nonloaded preparations and was found to be <10% of the
fluorescence of the fura 2-loaded vessels.

**Experimental protocol.** The viability of each preparation was as-
scussed by stimulation with norepinephrine (NE, 5 μM). Vessels not
responding promptly were discarded. Thereafter, the $[Ca^{2+}]$, response
to electrical stimulation was studied. At the end of each experiment,
the viability of the vessel was reassessed by addition of NE (5 μM).
Local electrical stimulation was performed as previously described
(38, 45). Glass pipettes, pulled to an outer tip diameter of 8 –10
μm, were filled with 2 M NaCl (resistance 0.3 –0.5 MΩ). The vessel was
stimulated by a train of continuous unipolar current pulses (2.5 Hz
frequency, 200 ms pulse duration, +90 V amplitude). The pipette tip
was positioned close to the vessel, ~500 μm proximal to the area where
$[Ca^{2+}]$, was measured. Control experiments verified that, when
the electrode was placed at the same distance (500 μm) from the area
of measurements, but removed from the vessel, the $[Ca^{2+}]$, response
to electrical stimulation was abolished. This indicates that the distant
response is due to spread along the vessel wall and not to a general-
ized electric field in the experimental chamber.

**Immunofluorescence**

Mouse kidney cryosections (12 μm) were fixed in 2% paraformal-
dehyde and preblocked with 4% BSA in PBS (4% BSA/PBS) or
blocked in PBS and blocked in 5% skim milk in PBS (for Cx37
staining). Hereafter, the sections were incubated in 4% BSA/PBS with
a primary antibody against Cx40 (AB1726, Millipore, Billerica, MA) or in 5% skim milk in PBS for Cx37 (CX37A11-A, 1:50;
Alpha Diagnostics, San Antonio, TX) at 4°C overnight. The sections
were then washed and blocked in 4% BSA/PBS and incubated for 45
min at room temperature with an Alexa 488-labeled secondary anti-
body and Alexa 555-labeled phallolidin (Invitrogen, Carlsbad, CA) to
stain F-actin. Nuclei were stained with DAPI. After being washed,
sections were mounted in Prolong Gold (Invitrogen) mounting me-
dium and imaged with either a Leica TCS SP2 (Cx40) or a Zeiss
LSM700 confocal microscope (Cx37).

**Data Analysis**

Afferent diameter was measured off-line using ImageJ (NIH,
Bethesda, MD). The edges of the arteriole were tracked, and the
diameter was measured manually every 10 s throughout the exper-
iment. Results are presented as the mean of the last minute of every
pressure step (μm ± SE).

For statistical analysis, the SigmaPlot software (Systat Software,
San Jose, CA) was used. Arterial pressure, body weight, and heart
weight were compared between groups using one-way ANOVA.
Changes within and between groups were analyzed using Student’s
paired or unpaired t-test or two-way ANOVA with repeated measure-
ments followed by the Newman-Keuls test. A $P$ value <0.05 was
considered significant.

**RESULTS**

**Animals**

Body weight, heart weight, relative heart weight (heart
weight/body weight), and mean arterial blood pressure are
presented in Table 1. Body weight tended to be lower and heart
weight higher in Cx40 KO mice compared with WT and HZ
mice. Relative heart weight was significantly higher ($P <0.05$)
in Cx40 KO mice compared with WT and HZ mice. Arterial
pressure was measured on anesthetized animals immediately
before removing the kidney. The anesthesia resulted in a lower
average arterial pressure, but, even under these conditions,
Cx40 KO mice had a significantly increased arterial pressure
(76 ± 5 mmHg; $P < 0.01$) compared with WT mice (51 ± 8
mmHg) and HZ mice (53 ± 3 mmHg).

**Immunostaining**

Staining of kidney tissue showed expression of Cx40 in the
endothelial cells of the afferent arteriole and in the JG cells in
WT and HZ mice (Fig. 1, A and C, data not shown for HZ
animals). No expression of Cx40 was seen in kidneys from
Cx40 KO mice (Fig. 1, B and D). Cx37 was expressed in the
JG cells, glomerulus (Fig. 1, G and H), and endothelial cells in
WT mice (Fig. 1E) and in Cx40 KO mice (Fig. 1F). Expression of
Cx37 was absent in the media of afferent arterioles from
both WT and Cx40 KO.

**Afferent Arteriole Diameter**

**Baseline diameter.** Afferent arteriolar diameter measure-
ments were made in WT and Cx40 KO mice both at JG sites
($n = 4$ in both groups) and further upstream ($n = 5$ and $n =
7$, respectively). Baseline diameter at 75 mmHg in WT mice
was 18.5 ± 0.7 μm at the JG site and 17.8 ± 2 μm at the
upstream site (Fig. 2A). In Cx40 KO, baseline diameter was
12.3 ± 0.9 μm at the JG site and 19.8 ± 2.2 μm at the
upstream site (Fig. 2A). The diameter at the JG site in Cx40 KO
was significantly smaller than that in the corresponding posi-
tion in WT ($P < 0.05$) and the upstream site in the Cx40 KO
arterioles ($P < 0.05$).

After papillectomy, to remove the vasoconstriction induced
by TGF, the afferent arteriolar diameter in WT mice near the
glomerulus was 19.5 ± 1.6 μm. The afferent diameter mea-
sured upstream from the glomerulus was 16.5 ± 1.9 μm after
papillectomy [Fig. 2B, not significant (NS)]. In Cx40 KO mice,
the JG diameter after papillectomy was 13.1 ± 1.0 μm. The
diameter measured upstream from the glomerulus was 19.5 ±
2.2 μm (Fig. 2B, NS). The JG diameter in Cx40 KO was
significantly smaller than that in WT after papillectomy but not
significantly different from the diameter measured upstream
in Cx40 KO mice.

**Table 1. Heart Weight, body weight, relative heart weight, and mean arterial blood pressure**

<table>
<thead>
<tr>
<th></th>
<th>Body Wt, g</th>
<th>Heart Wt, mg</th>
<th>Relative Heart Wt, mg/g</th>
<th>Mean Arterial Pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type ($n = 9$)</td>
<td>32 ± 2</td>
<td>174 ± 12</td>
<td>5.3 ± 0.3*</td>
<td>51 ± 8*</td>
</tr>
<tr>
<td>Knockout ($n = 11$)</td>
<td>31 ± 1</td>
<td>197 ± 15</td>
<td>6.5 ± 0.3</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>Heterozygous ($n = 5$)</td>
<td>32 ± 2</td>
<td>166 ± 13</td>
<td>5.4 ± 0.3*</td>
<td>53 ± 3*</td>
</tr>
</tbody>
</table>

Values are means SE; $n$, no. of animals. *$P < 0.05$ vs. Cx40 knockout mice.
Autoregulatory response. The overall response to pressure changes was similar at the JG site and the upstream site both before and after papillectomy (Table 2), and the autoregulation results were pooled (see below). Baseline afferent diameter (pooled from JG and distal sites) did not differ significantly between WT, KO, and HZ mice, averaging 18.1 ± 1.1, 17.1 ± 1.8, and 19.7 ± 0.7 μm, respectively (Fig. 2A), at a RPP of 75 mmHg. Increasing RPP from 75 to 155 mmHg in steps of 20 mmHg reduced afferent diameter significantly in WT mice (n = 9; Fig. 3). Calculating the linear regression of the individual autoregulation curves revealed an overall slope of −0.84 ± 0.32 μm/mmHg, which was significantly different from zero (P < 0.05). These results demonstrate a significant autoregulation in afferent arteriolar diameter in response to acute changes in RPP in WT animals. Returning RPP to 75 mmHg increased afferent diameter to 18.0 ± 1.7 μm, which was not significantly different from the initial diameter at 75 mmHg.

In Cx40 KO mice, afferent arteriolar diameter did not change significantly when RPP was increased from 75 to 155 mmHg (n = 11; Fig. 4). The overall slope of the autoregulation curves from the Cx40 KO mice was −0.05 ± 0.15 μm/mmHg, which was not significantly different from zero but different from the slope of the autoregulation curve in the WT mice (P < 0.05).

In HZ mice, afferent arteriolar diameter tended to decrease when RPP was increased from 75 to 155 mmHg but was not significantly different from the baseline diameter (19.7 ± 1.8 μm, results not shown, n = 5). The slope of the autoregulation curve in HZ mice was −0.47 ± 0.34 μm/mmHg (NS vs. 0) and

**Fig. 2.** Afferent arteriole diameter measured at 75 mmHg before (A) and after (B) papillectomy. Measurements were made close to the glomerulus (JG) or >100 μm upstream from the glomerulus (>100). “Total” shows the pooled results from the two groups. Black bars, WT mice (n = 9); gray bars, Cx40 KO mice (n = 11). P < 0.05 vs. WT mice (#) and vs. upstream diameter (*).
not significantly different from either the WT or KO mice curves.

After papillectomy. After the first set of pressure steps, the papilla was surgically removed to eliminate the TGF response. After papillectomy, the basal afferent arteriolar diameter at a RPP of 75 mmHg was 17.8 ± 1.3, 17.2 ± 1.7, and 18.8 ± 0.6 μm in WT, KO, and HZ mice, respectively (Fig. 2B). These values were not significantly different from each other and did not differ significantly from those measured before papillectomy (Fig. 2A).

Increasing RPP from 75 to 155 mmHg after papillectomy in WT mice did not reduce afferent arteriolar diameter (Fig. 3); however, the diameter at 95 mmHg (16.5 ± 1.3 μm) was borderline significant from the diameter at 75 mmHg (P = 0.08). The slope of the autoregulation curve after papillectomy was −0.29 ± 0.26 μm/mmHg, which was not significantly different from zero. These results indicate that TGF significantly contributes to the autoregulation of afferent arteriolar diameter during acute increases in RPP in WT mice.

In Cx40 KO mice, acute increases in RPP after papillectomy did not induce a reduction in afferent arteriolar diameter (Fig. 4). The slope of the autoregulation curve after papillectomy in the KO mice was 0.08 ± 0.16 μm/mmHg (NS vs. 0) and was not significantly different from the slope before the papillectomy.

In the HZ mice, the abolished TGF response after papillectomy also reduced the tendency toward reduced afferent arteriolar diameter when RPP increased acutely. The slope of the autoregulation curve was −0.29 ± 0.32 μm/mmHg (NS vs. 0), which was not different from either WT or Cx40 KO curves.

Nifedipine. To test whether the response in afferent arterioles from WT mice after papillectomy was a passive response, experiments (n = 4) were made using nifedipine (10 μM), an inhibitor of the voltage-dependent L-type Ca²⁺ channel. Addition of nifedipine to afferent arterioles from WT mice significantly increased the diameter measured at 75 mmHg from 18.5 ± 1.2 to 25.5 ± 2.6 μm (P < 0.01). Increasing RPP from 75 to 155 mmHg in steps of 20 mmHg lasting 3 min caused the afferent arteriole to dilate significantly (Fig. 5).

### Table 2. Afferent arteriolar diameter measured in response to changes in renal perfusion pressure before and after papillectomy in wild-type and Cx40 knockout mice

<table>
<thead>
<tr>
<th>Afferent Arteriolar Diameter, μm</th>
<th>75 mmHg</th>
<th>95 mmHg</th>
<th>115 mmHg</th>
<th>135 mmHg</th>
<th>155 mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type, JG (n = 4)</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Before</td>
<td>18.5 ± 2.0</td>
<td>17.4 ± 0.9</td>
<td>15.8 ± 1.0</td>
<td>15.5 ± 1.2</td>
<td>15.8 ± 1.4</td>
</tr>
<tr>
<td>After papillectomy</td>
<td>19.5 ± 1.6</td>
<td>17.9 ± 1.3</td>
<td>17.6 ± 1.4</td>
<td>17.8 ± 1.9</td>
<td>18.4 ± 2.1</td>
</tr>
<tr>
<td>Wild type, &gt;100 μm (n = 5)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Before</td>
<td>17.8 ± 2.0</td>
<td>15.9 ± 1.7</td>
<td>15.2 ± 1.6</td>
<td>14.3 ± 1.9</td>
<td>14.0 ± 2.2</td>
</tr>
<tr>
<td>After papillectomy</td>
<td>16.5 ± 1.9</td>
<td>15.4 ± 2.0</td>
<td>15.3 ± 2.1</td>
<td>15.0 ± 2.0</td>
<td>14.9 ± 2.0</td>
</tr>
<tr>
<td>Knockout, JG (n = 4)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Before</td>
<td>12.3 ± 0.9</td>
<td>11.5 ± 1.3</td>
<td>12.5 ± 1.3</td>
<td>12.4 ± 1.1</td>
<td>12.4 ± 1.0</td>
</tr>
<tr>
<td>After papillectomy</td>
<td>13.1 ± 1.0</td>
<td>12.5 ± 1.4</td>
<td>12.9 ± 1.5</td>
<td>13.7 ± 1.4</td>
<td>12.4 ± 1.6</td>
</tr>
<tr>
<td>Knockout, &gt;100 μm (n = 7)</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Before</td>
<td>19.8 ± 2.2</td>
<td>19.4 ± 1.9</td>
<td>19.2 ± 1.6</td>
<td>19.4 ± 1.6</td>
<td>19.1 ± 1.4</td>
</tr>
<tr>
<td>After papillectomy</td>
<td>19.5 ± 2.2</td>
<td>20.4 ± 2.2</td>
<td>20.0 ± 2.3</td>
<td>20.4 ± 2.3</td>
<td>20.1 ± 2.4</td>
</tr>
</tbody>
</table>

Values are means SE; n, no. of animals. JG, juxtaglomerular, measured next to glomerulus; >100 μm, measured upstream from glomerulus.
arteriolar diameter in WT mice after nifedipine treatment was significantly larger than in papillectomized WT mice at the corresponding pressures (P < 0.01; Fig. 5).

Isolated Arterioles

To examine the effect of reduced gap junction coupling on conducted vascular Ca^{2+} signals, renal preglomerular arterioles from WT and Cx40 KO mice were examined.

Electrical stimulation. Because of variation in baseline R_{340/380} between the different groups (WT 0.564 ± 0.058; KO 0.702 ± 0.071; P = 0.17), the responses were normalized to the ratio obtained immediately before stimulation and are presented as delta values. This variability in data is previously seen in several studies and is reflected by the large standard errors reported (13, 35).

In arterioles from WT mice (n = 5), electrical stimulation elicited a significant conducted increase in [Ca^{2+}], (as indicated by R_{340/380} 500 μm from the stimulation site (P < 0.05; Fig. 6). R_{340/380} reached a sustained plateau at 13.4 ± 4.9% above baseline. In arterioles from Cx40 KO mice (n = 4), electrical stimulation did not induce a significant conducted increase in R_{340/380} (0.8 ± 1.2%; Fig. 6). Addition of NE (5 μM) to the bath elicited an increase in Ca^{2+} concentration in arterioles from both mouse strains, verifying that all vessels were alive and responsive. Specifically, NE (5 μM) increased the R_{340/380} by 28.7 ± 9.3% in the WT mice and by 13.4 ± 2.8% in the Cx40 KO mice, values that were not significantly different.

DISCUSSION

In this study, we investigated the importance of intercellular communication via Cx40 for the autoregulatory response of the afferent arteriole. We measured changes in afferent arteriolar diameter in response to acute increases in RPP in kidneys from WT, Cx40 KO, and Cx40 HZ mice using the isolated blood perfused juxtamedullary nephron preparation. The autoregulatory response was measured before and after elimination of the TGF response by papillectomy. We also investigated the conduction of the [Ca^{2+}], response after electrical stimulation in isolated renal arterioles from these mice.

Renal vascular expression of Cx40 is primarily restricted to endothelial cells, mesangial cells, and renin-secreting granular cells (1, 19, 26). We also found expression of Cx40 in these cell types in the WT mice (Fig. 1, A and C), whereas the Cx40 KO mice showed no expression of Cx40 (Fig. 1, B and D). Cx40 KO mice have been reported to also lose their expression of Cx37 in endothelial cells from cremaster arterioles and renal arteries (7, 26, 27). In the present study, expression of Cx37 also seemed reduced in endothelial cells in the afferent arteriole from Cx40 KO mice (Fig. 1F), but we did not quantify this. Although Cx37 has been reported to be expressed in the media of the afferent arteriole in WT mice (27, 53), we were not able to confirm this (Fig. 1E).

Cx40 participates in the regulation of renin secretion (51). The electrical properties of Cx40 do not seem to be essential, since it can be substituted with Cx45, despite its significantly lower conductance (42). In Cx40 KO mice, plasma renin concentration is increased, with ectopic positioning of the renin-producing cells in the extraglomerular mesangium. Accordingly, Cx40 KO mice are hypertensive (9), a finding confirmed in the present study (Table 1).

Autoregulatory changes in juxtamedullary afferent arteriolar diameter were measured in isolated kidneys from WT and Cx40 KO mice. The initial afferent diameter was significantly different between these groups only at the JG site where Cx40 KO mice had a significantly smaller diameter compared with WT mice (Fig. 2). As RPP increased, afferent diameter was significantly reduced in WT mice (Fig. 3). In Cx40 KO mice, no significant changes in afferent diameter were seen (Fig. 4). The results demonstrate that the autoregulatory response of the afferent arteriole is attenuated in Cx40 KO mice compared with WT mice. In rats, intrarenal infusion of connexin mimetic peptides inhibiting formation of gap junctions comprised of Cx40 (40GAP27) results in an acute increase in mean arterial pressure (MAP) and a decrease in RBF (48). When MAP was restored to the initial value, a further decrease was seen in RBF, suggesting a reduced ability to autoregulate RBF in vivo in the absence of functional Cx40-containing gap junctions.
Also, an acute increase in RPP led to a concomitant increase in RBF in vivo (26) in Cx40 KO mice. Thus the results of the present study are supported by other studies indicating that Cx40 is essential for the renal autoregulatory response to acute changes in the perfusion pressure.

It has been suggested that the impaired whole kidney autoregulation in Cx40 KO mice is caused by an inefficient TGF response (26). To examine the role of TGF in the autoregulatory response, we performed a papillectomy after the first set of pressure steps. This procedure specifically eliminates the TGF response (39, 47) and is as efficient as furosemide to inhibit TGF (23). Thus, after papillectomy, any autoregulatory responses will be the result of the myogenic mechanism. Baseline afferent arteriolar diameter (at 75 mmHg) was unaltered in both Cx40 KO and WT after papillectomy (Fig. 2). Papillectomy in WT mice led to a reduced autoregulatory response (Fig. 3). This is in agreement with previous studies in both mice and rats, which have shown that the autoregulatory response of the afferent arteriole in juxtamedullary nephrons is reduced in the absence of the TGF mechanism (23, 47).

Although the vessels no longer constricted in response to the pressure increase, it is important to notice that they were able to maintain a constant diameter during the pressure increase. In contrast, in nifedipine-treated kidneys, the afferent arterioles dilated with increasing pressure (Fig. 5). Because nifedipine is an inhibitor of the L-type Ca\(^{2+}\) channels, which are necessary for both the TGF and the myogenic response (11, 32, 34), this shows that a considerable myogenic responsiveness remains after papillectomy (Fig. 5). Similar findings have been made in rat blood perfused juxtamedullary nephrons when furosemide was used to inhibit TGF (33). After furosemide treatment, the autoregulatory response of the afferent arteriole was significantly reduced, although the vessels still showed a decrease in diameter following an increase in the perfusion pressure. Following blockade of the L-type Ca\(^{2+}\) channels by nimodipine, the vessels showed passive vasodilation with increasing perfusion pressure.

In Cx40 KO mice, papillectomy had no effect on the response of the afferent arteriole to the increase in perfusion pressure. After papillectomy, the slope of the autoregulation curve (0.08 µm/mmHg) was not significantly different from the control value (−0.05 ± 0.15 µm/mmHg), suggesting that the TGF mechanism does not contribute to the autoregulatory response of the juxtamedullary nephrons in the Cx40 KO mice. Notice, however, that the afferent arterioles from the Cx40 KO did not exhibit passive dilatation like the WT arterioles following nifedipine. This indicates a preserved myogenic response that counteracts the increase in perfusion pressure, thereby making it possible to maintain a constant arteriolar diameter despite the increasing pressure.

The loss of the TGF response in the Cx40 KO mice could be due to either interference with the cell-cell propagation of the MD signal across the mesangial region or due to interference with the upstream electrotonic propagation of the TGF signal along the preglomerular vasculature. In the isolated JG apparatus, activation of the TGF mechanism elicits a depolarization (30) and an increase in mesangial cell [Ca\(^{2+}\)], that spreads toward the afferent arteriole. This combined electric and Ca\(^{2+}\) wave was blocked by 18α-glycyrrhetinic acid or heptanol, both unspecific gap junction uncouplers (35). Once the wave reaches the afferent arteriole, it spreads electrotonically upstream within the wall of the preglomerular vessels, causing what is known as a conducted vasoconstrictory response (30). Conducted vascular responses depend on gap junction-mediated coupling of the cells of the vascular wall (17, 43).

To test the integrity of vascular conducted responses in the afferent arterioles, we examined the conduction of the intracellular Ca\(^{2+}\) signal following a local electrical stimulation in preglomerular arterioles from WT and Cx40 KO mice. We have previously shown that local electrical stimulation of isolated preglomerular arterioles from rats elicits a conduction of the Ca\(^{2+}\) response along the preglomerular vessels, and this response is blocked by nonspecific inhibition of gap junctions using carbenoxolone (38, 44). In agreement with this, electrical stimulation in WT mice induced a significant increase in [Ca\(^{2+}\)], −500 µm from the stimulation site (Fig. 6). In Cx40 KO mice, conduction of the Ca\(^{2+}\) response was absent, indicating that Cx40 within the vascular wall is necessary for the conduction of renal vasoconstriction. The lack of conduction in the Cx40 KO mice was not due to a general unresponsiveness of the vessels, since the Ca\(^{2+}\) response to addition of 5 µM NE to the superfusate was not different in preglomerular arteries from Cx40 KO and WT mice. This is in accord with results obtained by Just et al. (26) showing that infusion of ACh or NE induced similar responses in renal vascular resistance in Cx40 KO and WT mice.

The reduced TGF response as well as the abolished Ca\(^{2+}\) conduction seen in the Cx40 KO mice could theoretically be caused by secondary effects due to the global knock out of Cx40. The dominant physiological change in Cx40 KO mice is an elevated renin secretion leading to increases in ANG II levels and hypertension (27, 51). However, both hypertension (5, 6) and increased ANG II levels (31) have been shown to increase the strength and the sensitivity of the TGF mechanism, and increased ANG II levels have been found to increase the strength of conducted vasoconstriction (16), which speaks against this possibility. Another possibility is that vascular remodeling in response to chronically elevated blood pressure causes the reduced afferent arteriolar response seen in Cx40 KO mice. However, in other hypertensive models, for example, the spontaneously hypertensive rat, autoregulatory afferent arteriolar constrictions are seen in response to increases in perfusion pressure (14), showing that chronic hypertension does not by itself abolish the afferent arteriolar autoregulatory response.

Although the main effect of the Cx40 KO appears to be an inhibition of the TGF mechanism, we cannot exclude that the myogenic response is also affected. Previous studies have shown that inhibition of vascular gap junctions using mimetic peptides against Cx37 and Cx43 reduced the myogenic constriction in isolated mesenteric arterioles (10). Furthermore, although not statistically significant, there was a drop in vessel diameter when going from 75 to 95 mmHg in the WT mice, which was not seen in the Cx40 KO mice. However, it is important to realize that the constancy of vessel diameter in both the WT and the Cx40 KO mice despite an increasing perfusion pressure is only possible if the vessel retains some myogenic reactivity. This becomes evident when giving nifedipine to block the L-type Ca\(^{2+}\) channels. Nifedipine is a well-known blocker of the myogenic response, and, as a result, the vessels are no longer able to resist the increasing perfusion pressure, and the result is a gradual vasodilation (Fig. 5).
In conclusion, the present study shows a role for Cx40 in renal autoregulation. The results indicate a crucial role for Cx40 in the TGF response. Also, Cx40 appears to be necessary for the Ca2+ conduction along the vascular wall. Still, further investigations are required to fully elucidate gap junction-dependent signaling pathways in the JG apparatus of juxtamedullary and cortical nephrons as well as their role in the integrated regulation of renal salt and water excretion.

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DISCLOSURES
No conflicts of interests, financial or otherwise, are to be declared.

AUTHOR CONTRIBUTIONS
Author contributions: C.M.S. conception and design of research; C.M.S., I.G., T.H.B., J.C.B., M.S., and N.-H.H.-R. interpreted results of experiments; C.M.S., T.H.B., and M.S. prepared figures; J.C.B., and M.S. analyzed data; C.M.S., J.C.B., M.S., and N.-H.H.-R. interaction for the Ca2+ conduction along the vascular wall. Still, further investigations are required to fully elucidate gap junction-dependent signaling pathways in the JG apparatus of juxtamedullary and cortical nephrons as well as their role in the integrated regulation of renal salt and water excretion.

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