

# Connexin 40 Mediates the Tubuloglomerular Feedback Contribution to Renal Blood Flow Autoregulation

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## ABSTRACT

Connexins are important in vascular development and function. Connexin 40 (Cx40), which plays a predominant role in the formation of gap junctions in the vasculature, participates in the autoregulation of renal blood flow (RBF), but the underlying mechanisms are unknown. Here, Cx40-deficient mice (Cx40-ko) had impaired steady-state autoregulation to a sudden step increase in renal perfusion pressure. Analysis of the mechanisms underlying this derangement suggested that a marked reduction in tubuloglomerular feedback (TGF) in Cx40-ko mice was responsible. In transgenic mice with Cx40 replaced by Cx45, steady-state autoregulation and TGF were weaker than those in wild-type mice but stronger than those in Cx40-ko mice. *N* $\omega$ -Nitro-L-arginine-methyl-ester (L-NAME) augmented the myogenic response similarly in all genotypes, leaving autoregulation impaired in transgenic animals. The responses of renovascular resistance and arterial pressure to norepinephrine and acetylcholine were similar in all groups before or after L-NAME inhibition. Systemic and renal vasoconstrictor responses to L-NAME were also similar in all genotypes. We conclude that Cx40 contributes to RBF autoregulation by transducing TGF-mediated signals to the afferent arteriole, a function that is independent of nitric oxide (NO). However, Cx40 is not required for the modulation of the renal myogenic response by NO, norepinephrine-induced renal vasoconstriction, and acetylcholine- or NO-induced vasodilation.

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Connexins (Cx's) are important in vascular development, cardiovascular function, and arterial pressure (AP) control.<sup>1,2</sup> Four Cx isoforms (Cx37, Cx40, Cx43, and Cx45) form gap junctions to facilitate intercellular communication in the vasculature.<sup>1,2</sup> Among these, Cx40 plays a prominent role. Cx40 is abundantly expressed in endothelial cells in most vascular beds.<sup>1,2</sup> Genetic ablation of Cx40 causes severe impairment of conducted vasodilator responses in arterioles,<sup>3,4</sup> uncoordinated vasomotion,<sup>5</sup> and hypertension.<sup>5–7</sup> Furthermore, vascular expression of Cx40 is reduced in genetically hypertensive rats.<sup>8</sup>

Within the kidney, gap junctions are prevalent in the juxtaglomerular apparatus (JGA).<sup>9</sup> The JGA is a unique structure coordinating tubular function

to the regulation of preglomerular vasomotor tone and renin release. Cx40 is the predominant connexin in the JGA, with expression in endothelial and renin-producing cells of afferent arterioles and glomerular mesangial cells.<sup>6,10–12</sup> Cx40 is thus str-

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**Table 1.** Baseline hemodynamic and excretory data<sup>a</sup>

Group	Mean AP (mmHg)	RBF (ml min <sup>-1</sup> g KW <sup>-1</sup> )	HR (min <sup>-1</sup> )	UV (μl min <sup>-1</sup> )	n
Wild-type control	85 ± 5	7.3 ± 1.2	523 ± 32	11 ± 2	6
Cx40-ko control	100 ± 5	6.7 ± 1.3	533 ± 22	13 ± 3	6
Cx40KI45 control	96 ± 6	9.1 ± 0.9	539 ± 27	18 ± 2	5
Wild-type L-NAME	122 ± 4 <sup>c</sup>	4.1 ± 0.5 <sup>b</sup>	416 ± 47	11 ± 3	4
Cx40-ko L-NAME	123 ± 7	4.1 ± 1.1 <sup>b</sup>	415 ± 9 <sup>b</sup>	19 ± 1	4
Cx40KI45 L-NAME	121 ± 8 <sup>b</sup>	5.8 ± 0.5 <sup>b</sup>	482 ± 24	18 ± 2	4

<sup>a</sup>HR, heart rate; KW, kidney weight; UV, bilateral urine flow rate. Mean ± SEM. <sup>b</sup>P < 0.05 and <sup>c</sup>P < 0.001 versus the paired control before L-NAME inhibition in the same animal (paired t test).

tegically localized for impacting GFR, tubuloglomerular feedback (TGF), and renin secretion. Indeed, deletion of Cx40 leads to increased production of renin, ectopic renin expression, and loss of pressure- and angiotensin II (Ang II)-dependent control of renin release.<sup>6,7,13</sup> A rise in plasma renin concentration is also seen after administration of a putative Cx40-inhibiting peptide.<sup>12</sup> However, Cx40 expression is increased in response to a chronic reduction of renal perfusion pressure, a common stimulus for renin synthesis.<sup>10</sup>

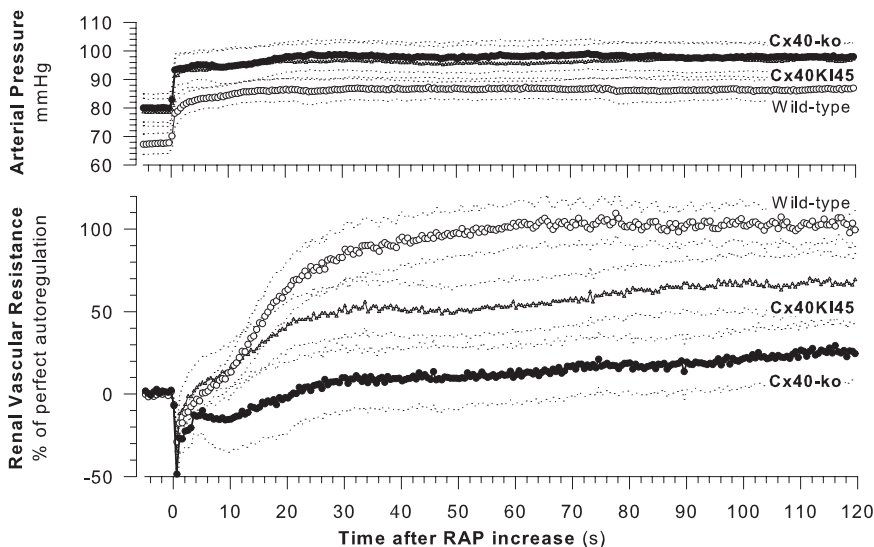
Our knowledge of the role of Cx40 in the regulation of organ blood flow and vascular resistance *in vivo* is limited. In the kidney, intrarenal infusion of peptides designed to inhibit Cx37, Cx40, or both Cx40 and Cx43 reduces basal renal blood flow (RBF) and increases AP.<sup>12,14</sup> Steady-state autoregulation of RBF and GFR is reported to be partially inhibited by peptides directed against Cx37 or Cx40.<sup>12</sup> Not known, however, is which of the three mechanisms responsible for renal autoregulation (TGF, myogenic response (MR), and an undefined third mechanism<sup>15,16</sup>) is affected. In isolated JGAs, TGF responses<sup>17</sup> and associated calcium waves<sup>18</sup> are inhibited by non-specific pharmacologic gap junction disrupters (e.g., heptanol). Such interventions also attenuate the MR to changes in vascular pressure in isolated cerebral<sup>19</sup> and mesenteric arteries,<sup>20</sup> as is the case for inhibitory peptides against Cx37 and Cx43.<sup>20</sup> However, the functional significance of genetic deletion of Cx40 for blood flow regulation and autoregulation *in*

*in vivo* in any vascular bed, including the kidney, is not known. We postulated that Cx40 is required for complete autoregulation and TGF activity.

Also poorly understood is the importance of gap junctions *in vivo* in vasoconstrictor and vasodilator responses of resistance arterioles. α-Adrenergically induced vasoconstriction is blunted by pharmacologic gap junction inhibitors in isolated arteries.<sup>20,21</sup> Gap junctions are also implicated in vasodilation,<sup>14,22</sup> although it is unclear whether or not Cx40 is involved.<sup>3,14,23</sup> Few studies have tested the participation of connexins in vasodilation *in vivo*.<sup>5,14</sup> To our knowledge, no data exist regarding connexins in vasoconstrictor responses in an animal.

Nitric oxide (NO) may interact with connexin function. NO is thought to modulate connexin conductance acutely<sup>24</sup> and expression chronically.<sup>25</sup> NO attenuates the magnitude of acute vasoconstriction elicited by receptor agonists such as Ang II and NE.<sup>26</sup> In addition, NO blunts the strength of the pressure-induced MR in RBF autoregulation, an effect specific to the kidney and dependent on JGA function and TGF.<sup>27,28</sup> Considering the possible involvement of Cx40 in JGA function, we postulated that Cx40 is critical for NO modulation of agonist-induced renal vascular responses and pressure-induced RBF autoregulation.

The present study tested the hypotheses that (a) Cx40 is essential to RBF autoregulation and is involved in MR, TGF, or



**Figure 1.** Time course of autoregulatory response of RVR to a 20 mmHg step increase of RAP in wt mice (open circles, n = 6), Cx40-ko mice (filled circles, n = 6), and Cx40KI45 (triangles, n = 5). Mean ± SEM.

**Table 2.** Characteristics of RBF autoregulation in response to a step increase in renal arterial pressure<sup>a</sup>

Group	Total Steady-State Autoregulation from Prestep to ~100 s	Autoregulatory Contributions (% of Perfect Autoregulation)			n
		Myogenic Response from ~1 to ~5 s	TGF and Fourth Mechanism from ~5 to ~25 s	Third Mechanism from ~25 to ~100 s	
Wild-type control	102 ± 11	22 ± 6	75 ± 10	27 ± 7	6
Cx40-ko control	24 ± 17 <sup>b</sup>	23 ± 7	18 ± 8 <sup>b</sup>	18 ± 7	6
Cx40KI45 control	66 ± 17	29 ± 8	40 ± 11 <sup>d</sup>	18 ± 3	5
Wild-type L-NAME	102 ± 5	78 ± 12 <sup>c</sup>	28 ± 8 <sup>c</sup>	16 ± 9	4
Cx40-ko L-NAME	58 ± 6	65 ± 8 <sup>c</sup>	4 ± 6	15 ± 6	4
Cx40KI45 L-NAME	74 ± 16	62 ± 3 <sup>c</sup>	17 ± 5	18 ± 11	4

<sup>a</sup>Mean ± SEM. <sup>b</sup>*P* < 0.01 versus the respective wt (ANOVA). <sup>d</sup>*P* < 0.05 versus the respective wt. <sup>c</sup>*P* < 0.05 versus the paired control before L-NAME in the same animal (paired *t* test).

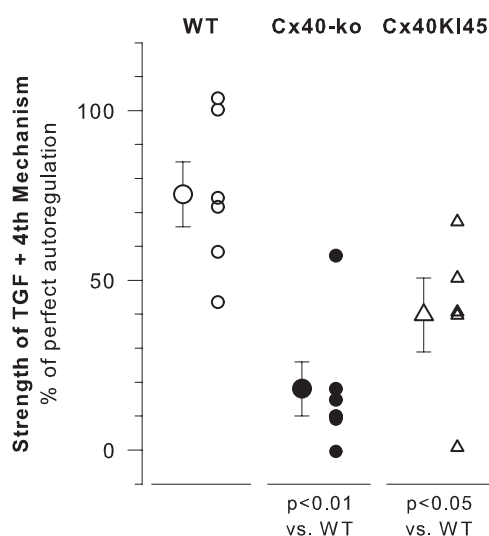
both responses to an acute change in renal perfusion pressure, (b) Cx40 contributes to agonist-induced vasodilator and constrictor responses in the renal and systemic circulation, and (c) Cx40 is involved in the signaling pathway of NO that blunts the renal MR. To this end, we conducted RBF studies on mice with genetic ablation of Cx40. Parallel studies were performed on mice with the coding region for Cx40 replaced by Cx45 regulated by the Cx40 promoter. In the latter, we postulated that Cx45 can assume Cx40 function, at least in part.

## RESULTS

### Hemodynamics

Cx40-deficient mice (Cx40-ko) mice had higher AP than those of wild-type (wt) controls (Table 1), as reported previously<sup>5–7</sup> Cx40KI45 animals tended to have an intermediate level of AP (Table 1). Baseline RBF, heart rate, and urine flow rate were similar among groups (Table 1).

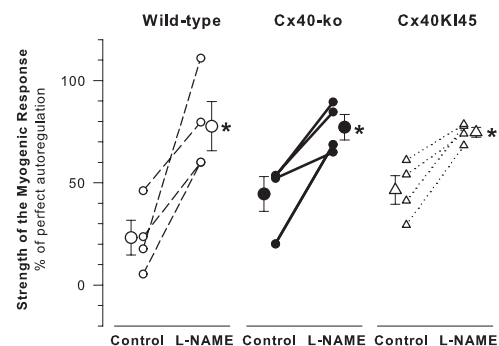
Figure 1 shows the time course of the autoregulatory re-



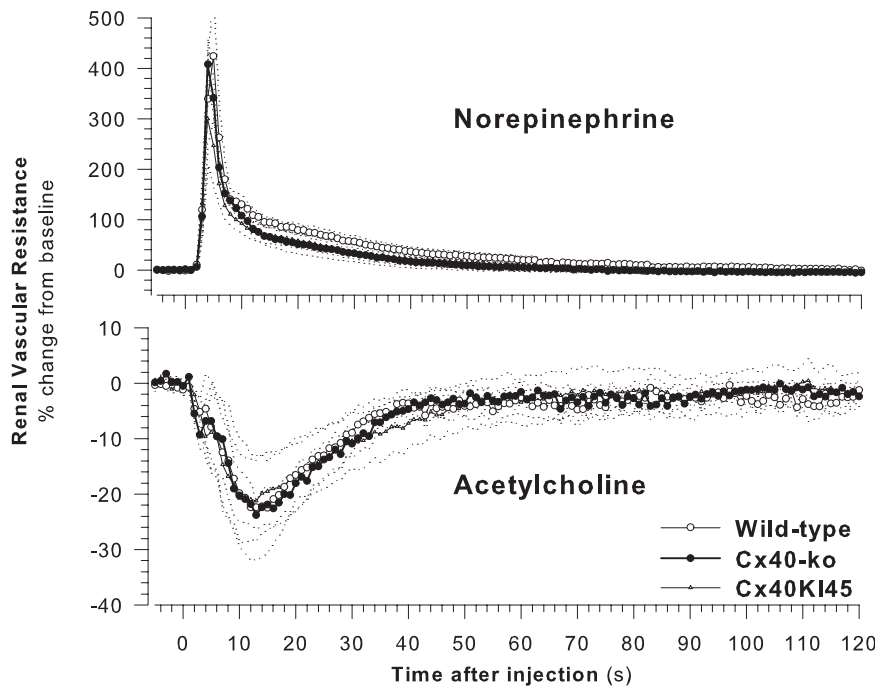
**Figure 2.** Strength of TGF and the fourth regulatory mechanism (5 to 25 s) in RBF autoregulation in wt (*n* = 6), Cx40-ko (*n* = 6), and Cx40KI45 mice (*n* = 5). Data for individual animals are shown with group mean ± SEM. *P* < 0.01 and *P* < 0.05 versus wt.

sponse of renal vascular resistance (RVR) to a sudden step increase in renal artery pressure (RAP). In wt mice, RVR initially fell and then quickly rose within 5 to 7 s. A secondary rise in RVR occurred between approximately 7 and approximately 30 s. In a third phase, RVR slowly approached the final level over the following 90 s. According to the literature and our previous analyses, the first phase of RVR rise most likely reflects the MR, the secondary rise the TGF (probably together with a “fourth” mechanism), and the tertiary phase a “third” mechanism distinct from classical TGF and the MR.<sup>16</sup> The strength and contribution of these components are assessed from RVR increases in the time windows from approximately 1 to approximately 5 s for the MR, from approximately 5 to approximately 25 s for TGF, and between approximately 25 and approximately 100 s for the third mechanism. On the basis of this analysis in wt mice, the MR provided an autoregulatory gain of 22%, TGF (and fourth mechanism) of 75%, and the third mechanism of 27% (Table 2). Overall steady-state autoregulation, apparent from the stable level reached between 90 to 120 s was very efficient (102%, Table 2). The sum of the contributions of individual mechanisms exceeds 100% because the MR is calculated from the initial minimum RVR, whereas steady-state efficiency is determined from the RVR level before the RAP increase.

Cx40-ko mice had severely impaired steady-state RBF au-



**Figure 3.** Effect of L-NAME inhibition of NO synthesis on the autoregulatory strength of the myogenic response (initial 7s) in RBF autoregulation in wt (*n* = 4), Cx40-ko (*n* = 4), and Cx40KI45 mice (*n* = 4). Data for individual animals are shown with group mean ± SEM. \*, *P* < 0.05 versus control.



**Figure 4.** Time course of renal vasoconstrictor and vasodilator responses to NE (75  $\mu$ g) and ACh (25  $\mu$ g) injected intravenously in wt ( $n = 5$ ), Cx40-ko ( $n = 4$ ), and Cx40KI45 mice ( $n = 4$ ). Mean  $\pm$  SEM.

toregulation (24 versus 102%, Figure 1 and Table 2). This was primarily due to attenuation of the second component (5 to 25 s) involving TGF (Figures 1 and 2 and Table 2). The MR and the third mechanism were essentially normal in Cx40-ko mice (Figures 1 and 3 and Table 2). Note that the nadir of RVR during the first second was lower in Cx40-ko than that in wt mice ( $-37 \pm 6$  versus  $-22 \pm 4\%$ ,  $P > 0.08$ ). The contribution of MR in Cx40-ko animals (Table 2) was therefore larger than it may appear from the level of autoregulation at 4 to 7 s in the time course (Figure 1). Replacement of the coding region for Cx40 by Cx45 (Cx40KI45) partially improved autoregulation as well as the strength of the TGF-related autoregulatory component (Figures 1 and 2). Nevertheless, both measures remained significantly less than those in wt mice (Figures 1 and 2 and Table 2). MR and the third mechanism in Cx40KI45 mice did not differ from those in wt animals (Figures 1 and 3 and Table 2).

The acute renal vasoconstrictor response to NE and the vasodilation to acetylcholine (ACh) were virtually identical in Cx40-ko and wt mice (Figure 4 and Table 3). The effects of NE and ACh on RBF were also normal in Cx40KI45 mice (Figure 4 and Table 3). Likewise, there were no differences between genotypes in the systemic AP responses to ACh (Table 3). Thus, integrated renal vascular smooth muscle function was undisturbed by either the absence of Cx40 or replacement with Cx45.

Acute nitric oxide synthase (NOS) inhibition by *N* $\omega$ -nitro-L-arginine-methyl-ester (L-NAME) increased AP (+27 to 40%,  $P > 0.1$  between genotypes) and reduced RBF ( $-39$  to  $41\%$ ,  $P > 0.7$ ) similarly in all genotypes (Table 1). L-NAME augmented the strength of the MR without affecting steady-state autoregulatory efficiency in each group, thereby shifting

the contribution of mechanisms toward the most rapid MR (Table 2). Overall autoregulation remained less efficient in Cx40-ko compared with wt and tended to persist at an intermediate level in Cx40KI45 during NOS inhibition (Table 2). The MR was increased to a similar degree in all genotypes (Figure 3 and Table 2).

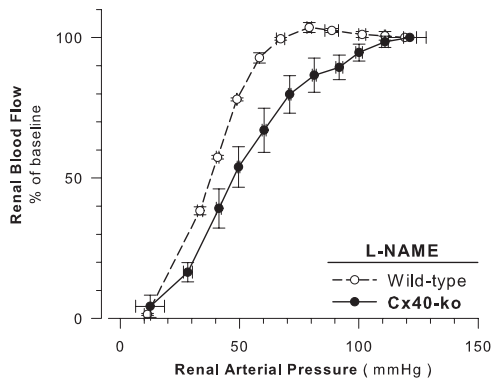
As a comparison for the overall dynamic response to a single step increase in AP, classical staircase steady-state autoregulation curves were also performed. During L-NAME inhibition, RBF autoregulation was near perfect in wt mice down to ap-

**Table 3.** Cardiovascular responses to bolus injections of NE and ACh<sup>a</sup>

Group	% Change from Baseline			n
	AP	RBF	RVR	
NE				
Wild-type control	+49 $\pm$ 3	-53 $\pm$ 8	+275 $\pm$ 60	5
Cx40-ko control	+28 $\pm$ 2 <sup>e</sup>	-61 $\pm$ 1	+255 $\pm$ 13	4
Cx40KI45 control	+48 $\pm$ 7	-46 $\pm$ 9	+207 $\pm$ 49	4
Wild-type L-NAME	+24 $\pm$ 4 <sup>d</sup>	-82 $\pm$ 6	+6741 $\pm$ 5594 <sup>f</sup>	4
Cx40-ko L-NAME	+29 $\pm$ 3	-78 $\pm$ 2 <sup>c</sup>	+581 $\pm$ 61 <sup>c</sup>	4
Cx40KI45 L-NAME	+27 $\pm$ 4	-67 $\pm$ 5	+328 $\pm$ 65	4
ACh				
Wild-type control	-43 $\pm$ 2	+23 $\pm$ 11	-25 $\pm$ 8	4
Cx40-ko control	-37 $\pm$ 1	+14 $\pm$ 4	-23 $\pm$ 3	4
Cx40KI45 control	-35 $\pm$ 5	+13 $\pm$ 3	-21 $\pm$ 7	4
Wild-type L-NAME	-38 $\pm$ 2	+20 $\pm$ 5	-38 $\pm$ 3	4
Cx40-ko L-NAME	-38 $\pm$ 2	+28 $\pm$ 5 <sup>b</sup>	-36 $\pm$ 3 <sup>b</sup>	4
Cx40KI45 L-NAME	-34 $\pm$ 8	+13 $\pm$ 4	-25 $\pm$ 5	4

<sup>a</sup>Mean  $\pm$  SEM. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$ , and <sup>d</sup> $P < 0.001$  versus the paired control before L-NAME in the same animal (paired t test). <sup>e</sup> $P < 0.05$  versus respective wild-type. <sup>f</sup>SEM is high, because in one animal RBF almost ceased in response to NE during L-NAME, resulting in a very large resistance value.





**Figure 5.** Steady-state staircase autoregulation curves of RBF to successive reductions of RAP during inhibition of NO production in wt ( $n = 2$ ) and Cx40-ko mice ( $n = 4$ ). Mean  $\pm$  SEM.

proximately 70 mmHg (Figure 5). In Cx40-ko animals, autoregulation was markedly impaired as indicated by the absence of a plateau and steadily increasing RBF with AP (Figure 5). Autoregulatory efficiencies over a range of approximately 20 mmHg below the resting level of AP were 104% (114 and 94%) in wt and  $50 \pm 19\%$  in Cx40-ko mice, values similar to autoregulation in the dynamic step responses during L-NAME inhibition (Table 2).

L-NAME tended to enhance renal vasoconstrictor responses to NE in all groups (Table 3), although it differed statistically only in Cx40-ko. ACh-induced renal vasodilation was not affected by L-NAME in wt and Cx40KI45 mice and was slightly augmented in Cx40-ko (Table 3). AP responses to NE tended to be smaller during L-NAME inhibition in all genotypes. ACh-induced reductions in AP were not affected by L-NAME ( $P > 0.1$ ). Differences between genotypes were not detected during L-NAME inhibition in the RBF or systemic AP responses to either NE or ACh (Table 3).

### Histology

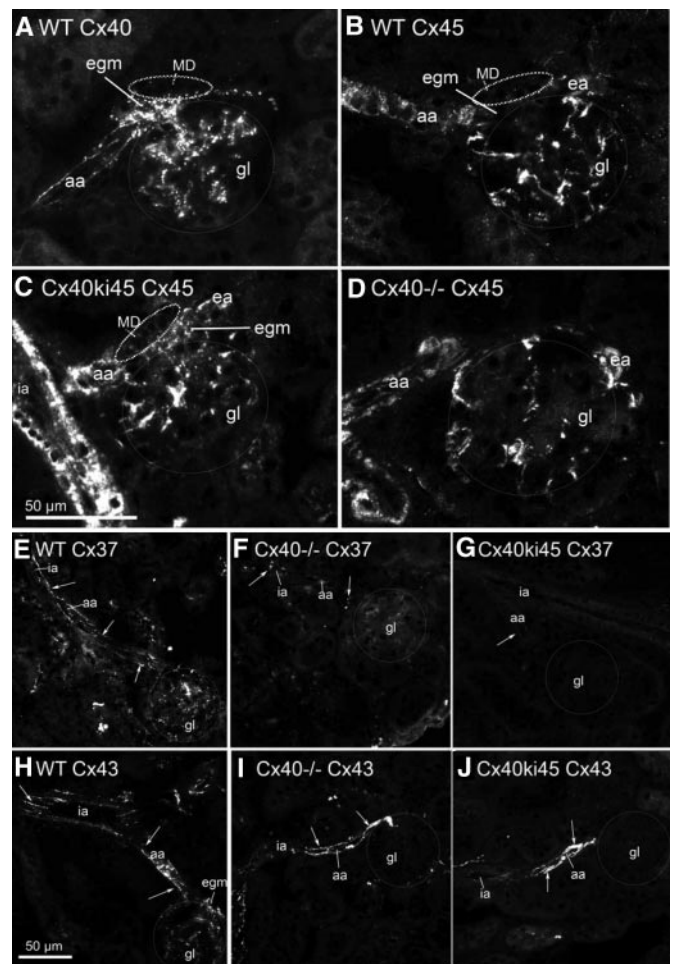
In the JGA of wt kidneys, preglomerular endothelial, renin-producing, and extraglomerular mesangial cells expressed Cx37 (Figure 6E), Cx40 (Figure 6A), and Cx43 (Figure 6H), whereas Cx45 was restricted to vascular smooth muscle cells (Figure 6B). Within glomeruli, Cx37 was found in proximity to the vascular pole (Figure 6E). Cx40 and Cx43 showed prominent expression in presumable mesangial cells (Figure 6, A and H). Cx45 staining was seen in more peripheral cells, presumably podocytes (Figure 6B).

In the JGA of Cx40-deficient kidneys, Cx40 was absent, and expression of Cx37 and Cx43 was clearly reduced in glomerular and mesangial regions (Figure 6, F and I). Cx37 was also severely diminished along the afferent arteriolar endothelium (Figure 6F). The expression pattern of Cx45 was not appreciably altered in the absence of Cx40 (Figure 6D). In Cx40KI45 kidneys, Cx45 appeared in the endothelium of preglomerular vessels, in the renin-producing cells, and in extra- and intra-glomerular mesangial cells (Figure 6C). The reduced Cx37 and

Cx43 expression in Cx40 deficient kidneys, however, was not reversed in Cx40KI45 mice (Figure 6, G and J).

### DISCUSSION

The present study demonstrates that RBF autoregulation is markedly impaired in mice lacking Cx40. This defect is primarily due to blunting of the TGF component, with normal contributions of the MR and the third mechanism. Thus, Cx40 in the JGA appears to be critical for transduction of a TGF-mediated vasoconstrictor signal to afferent arterioles participating in autoregulation. In contrast, integrated responses of renovascular resistance and systemic AP to NE, ACh, and NO remain intact. These novel *in vivo* observations indicate that there is no major defect in vasoconstrictor, endothelium-de-



**Figure 6.** Immunohistochemical localization of connexins Cx40, Cx45, Cx37, and Cx43 in renal cortical sections. Cx40 in a wt mouse (A). Cx45 in wt (B), Cx40-ko (D), and Cx40KI45 mice (C). Cx37 in wt (E), Cx40-ko (F), and Cx40KI45 mice (G) and Cx43 in wt (H), Cx40-ko (I), and Cx40KI45 mice (J). ia, interlobular artery; aa, afferent arteriole; ea, efferent arteriole; egm, extraglomerular mesangium; dotted line ellipsoid, macula densa region; solid line circle (gl), glomerulus.

pendent, or NO-mediated vasodilator properties of renal or systemic resistance arterioles lacking Cx40. Thus, the autoregulatory deficiency in Cx40 null mice is not due to a general malfunction of renal vasomotor responses. Cx45 expressed in place of Cx40 partly substitutes the function of Cx40 in TGF and renal hemodynamics. Despite the role of Cx40 in the TGF component of RBF autoregulation, the known TGF-dependent influence of NO on the renal MR does not require Cx40.

Impaired RBF autoregulation is documented both in the single step dynamic response and in the classical staircase steady-state autoregulation curve. Dynamic responses indicate that the secondary phase (between approximately 5 and approximately 25 s) is affected most markedly in mice lacking Cx40. This component has been shown to reflect the action of classical TGF in dogs,<sup>29</sup> rats,<sup>30</sup> and mice.<sup>16</sup> The present data therefore indicate that classical TGF is severely attenuated in the absence of Cx40. The 5 to 25 s time window also includes a fourth regulatory mechanism distinct from classical TGF that is resistant to furosemide and independent of A1 adenosine receptors (A1ARs).<sup>16</sup> The present results thus do not allow a definitive conclusion about the extent to which the fourth mechanism is affected by Cx40. However, because the degree of impairment of the 5 to 25 s component of Cx40-ko mice is similar to that found during complete TGF inhibition by furosemide or genetic ablation of A1AR,<sup>16</sup> it is reasonable to conclude that TGF is absent in Cx40-ko mice. Because the renal vasomotor responses to NE, ACh, and NOS inhibition do not differ between genotypes, the observed defect in autoregulation and TGF is not due to a general dysfunction of renal vasomotor responsiveness. Our findings, using a specific genetic approach, extend those of a previous pharmacologic report of impaired autoregulation of RBF and GFR during infusion of a putative inhibitory peptide against Cx40.<sup>12</sup> Importantly, our new data identify TGF as the primary target of the function of Cx40 in RBF autoregulation.

The mechanism by which Cx40 contributes to TGF-mediated autoregulation is not clear. A commonly accepted pathway of TGF signaling is that macula densa cells release ATP upon stimulation by elevated tubular NaCl concentration.<sup>31,32</sup> The released ATP may be degraded by extracellular nucleotidases in the JGA to adenosine, which then can constrict afferent arterioles via A1AR.<sup>32</sup> Alternatively or in addition, ATP may contract afferent arterioles by activating purinergic P2X receptors.<sup>18,31</sup> Given the high prevalence of gap junctions in the JGA,<sup>9</sup> the signal transduction of TGF might also follow a transcellular route from the macula densa through extraglomerular mesangial cells to the afferent arteriole.<sup>9,17,33</sup> Recent studies showed mesangial propagation of TGF-induced calcium waves from the macula densa to glomerular and arteriolar sites.<sup>18</sup> Because mesangial cells express several P2 receptors,<sup>31</sup> they may be activated by ATP released from the macula densa. Whether Cx40 primarily serves to connect mesangial cells, smooth muscle cells, or both<sup>9,17,33</sup> or acts as hemichannel to release further ATP from mesangial cells, as observed in cell culture,<sup>34</sup> warrants further investigation.

In contrast, the myogenic component of the autoregulatory response is not different in Cx40-deficient and wt kidneys. Thus, concluding that Cx40 is not required for the MR in the kidney seems reasonable. Although this may seem surprising given the involvement of Cx40 in conducted responses,<sup>3,4,23</sup> a pressure step likely reaches all parts of the vascular tree almost simultaneously. Responses to such uniform mechanical stimulation may not require conducted responses along the preglomerular vasculature. Moreover, Cx40 deficiency impairs only conduction of vasodilator but not vasoconstrictor signals.<sup>3,4,23</sup>

Replacement of Cx40 by Cx45, regulated by the Cx40 promoter, improves the defects in autoregulation and TGF but does not completely restore them to normal. The reason why Cx45 only partially replaces the function of Cx40 in the JGA is not known. Apart from the lower permeability of Cx45,<sup>35</sup> other features (secondary structure, voltage gating, signaling, and interactions with scaffolding proteins or microtubules) might be essential for the formation of heterotypic gap junctions, appropriate clustering, or regulation of their permeability.<sup>36</sup> The cellular distribution of Cx45 in Cx40KI45 mice mirrors the normal pattern of Cx40, and expression of Cx37 and Cx43 is similar in Cx40KI45 to that in Cx40-ko kidneys. Although Cx40 is essential for signal transduction from the macula densa to the afferent arteriole in TGF-mediated vasoregulation, macula densa-mediated regulation of renin release seems to be appropriate in Cx40-ko mice during changes in salt intake<sup>6,7</sup> or inhibition of macula densa sensor function by loop diuretics.<sup>13</sup> The same comparisons are obtained for A1AR-deficient mice, in which TGF is absent<sup>16,32</sup> and yet loop diuretic stimulation of renin release is preserved.<sup>37</sup> These observations suggest that two signaling pathways may emanate from the macula densa, one involving Cx40 and A1AR controlling TGF and a separate one regulating renin release.

Little is known about the contribution of Cx40 to agonist-induced vasomotor responses *in vivo*. Our results indicate that neither vasoconstrictor, nor endothelium-dependent, nor NO-mediated vasodilator responses in the intact mouse kidney are affected by the absence of Cx40. Similar results are found for AP responses, suggesting that Cx40 does not contribute to these vasodilator responses in most nonrenal vascular beds. Our results confirm previous findings on the lack of Cx40 involvement in systemic depressor responses to ACh<sup>5</sup> and extend them to vasodilator and vasoconstrictor responses in the kidney. These findings do not contrast with participation of Cx40 in conducted vasodilator responses initiated locally at a specific site, because stimulation by systemic application of these substances likely activates all sites along the vascular tree almost simultaneously. The vasomotor responsiveness to Ang II remains open. However, abnormal sensitivity to Ang II would unlikely have altered the conclusion that deficiency of Cx40 impairs RBF autoregulation, because steady-state autoregulation is usually not affected by Ang II infusion, angiotensin converting enzyme (ACE) inhibition, AT1 receptor antagonism, or chronic changes in sodium diet.<sup>15</sup> Ang II levels also do not seem to modulate the relative contributions of MR and TGF.<sup>27,38</sup>

We and others have shown that NO exerts a strong attenuating influence on the MR in the renal but not nonrenal circulations.<sup>27,28</sup> Importantly, this NO influence depends on functional TGF,<sup>27,28</sup> suggesting neuronal NOS in macula densa cells as the source. Given the probable role of Cx40 in TGF signal transduction and the possibility of NO modulation of connexin conductance,<sup>25,39</sup> we postulated that the absence of Cx40 impacts on NO effects on the renal MR. This, however, was not the case, because inhibition of NO production has similar augmenting effects on the MR in wt, Cx40-ko, and Cx40KI45 mice. Nevertheless, our current observations extend the prevalence of NO-mediated blunting of the renal MR from rats to mice, extending its relevance to additional species. To the extent that the NO responsible for this effect derives from neuronal NOS in macula densa cells, our data imply that Cx40 is not required for such NO to act on the afferent arteriole.

### Perspectives

Impaired RBF autoregulation, particularly in combination with hypertension, is known to predispose to pressure-induced damage of the kidney. Because Cx40-deficient mice have elevated AP,<sup>5–7</sup> compromised RBF autoregulation might render them prone to accelerated glomerular sclerosis. Central to an in-depth understanding of TGF function is knowledge of which particular signals are normally transduced through Cx40 in the JGA. Future investigations are warranted to understand the importance of Cx40 and autoregulation in protecting against glomerular injury and the underlying signaling pathways of the TGF-dependent influence of NO on the MR in the renal microcirculation. It is tempting to speculate that alterations in connexin-dependent pathways might be altered in diseases associated with hyperfiltration or accelerated glomerular sclerosis.

### CONCISE METHODS

Experiments were conducted on seven gene-targeted mice deficient for Cx40<sup>40</sup> (Cx40-ko, age 17 to 34 wks, body wt 25 to 29 g, left kidney wt 0.18 to 0.26 g, 5 males + 2 females) and 6 wt controls of the C57BL6 strain (wt, 9 to 34 wks, 18 to 27 g, left kidney wt 0.13 to 0.19 g, 4 males + 2 females). In addition, hemodynamics were studied in five mice from a strain in which the coding region of Cx40 had been replaced biallelically by Cx45<sup>41</sup> (Cx40KI45, 17 to 45 wks, 24 to 38 g, left kidney wt 0.19 to 0.36 g, 4 males + 1 female). Wild-type and gene-targeted mice (backcrossed for 10 generations into a C57BL6 genetic background) were bred and genotyped at the University of Lübeck. Four wt mice were purchased from Charles River, Germany. All experiments were conducted at the University of Regensburg, Germany, in accordance with German animal protection law and with the Institute of Laboratory Animal Resources (ILAR) Guide for the Care and Use of Laboratory Animals. Animals were fed a standard lab chow with free access to tap water on a 12 h:12 h light–dark cycle.

Immunohistochemistry for Cx37, Cx40, Cx43, and Cx45 was done on 5- $\mu$ m cryosections as described previously.<sup>42</sup> Renin antibody (chicken anti-mouse) was generated by Davids Biotechnologie, Re-

gensburg, Germany. Anti-Cx40 (goat anti-mouse, Santa Cruz, Santa Cruz, CA), anti-Cx37 (rabbit anti-mouse, Alpha Diagnostic International, San Antonio, TX), anti-Cx43 (rabbit anti-mouse, Sigma, St. Louis, MO), and anti-Cx45 (rabbit anti-mouse<sup>43</sup>) were purchased.

For hemodynamic studies, anesthesia (pentobarbital), surgery, experimental procedures, and data analysis were similar to previous reports.<sup>16</sup> An albumin solution (2.38 g dl<sup>-1</sup>, 12  $\mu$ l min<sup>-1</sup>) was infused intravenously. Injected into jugular vein catheters were the vasoactive agents (NE (3 mg ml<sup>-1</sup>) and Ach (1 mg ml<sup>-1</sup>) mixed in saline). Arterial pressure in a catheterized femoral artery was measured using a pressure transducer (ADInstruments, Bella Vista, NSW, Australia). A mechanical occluder was placed on the aorta. A flowprobe (0.5 PSB, Transonic) was positioned around the left renal artery to measure RBF by an ultrasound transit-time flowmeter (TS-420, Transonic, low-pass filter 40 Hz; Transonic, Ithaca, NY). All data were recorded on a computer at 100 Hz (LabChart Pro, ADInstruments).

The autoregulatory response of RBF to a rapid step increase in RAP within the autoregulatory pressure range was investigated as described previously.<sup>16,27,30</sup> The RAP increase was produced after reducing RAP 20 mmHg below the resting level for 60 to 70 s. This procedure was repeated every 5 min, thus allowing 4 min for recovery between RAP steps. Several steps were induced in each experimental period.

### Protocol A: Baseline Conditions

NE (75  $\mu$ g in 25  $\mu$ l) and Ach (25  $\mu$ g in 25  $\mu$ l), were injected intravenously over <2 s, separated by 5 min. Another 5 min later, RAP steps were induced every 5 min. Multiple responses<sup>7–9</sup> were recorded in each experimental period.

### Protocol B: NOS Inhibition

To test whether the effect of Cx40 deficiency is due to possible overproduction of NO and whether Cx40 is involved in the effects of NO on baseline RBF and the MR, autoregulation step responses were assessed 15 min after injection of the NOS inhibitor L-NAME (25 mg/kg intravenously, 4 to 6 step responses). Subsequently, NE and Ach were injected as described in protocol A. In some animals (2 male wt and 5 male Cx40-ko) classical staircase steady-state autoregulation curves were assessed by reducing RAP in a sequential step-wise fashion in 10 mmHg decrements, keeping each level stable for 100 s. Steady-state results were calculated from RBF and RAP during the final 30 s of each pressure level.

### Data Analysis

RAP, RBF, and RVR data were analyzed as described previously.<sup>16</sup> Baseline values given in Table 2 were obtained from the 10 s before each RAP reduction during a particular experimental period. First and second derivatives of RVR were calculated from the time courses of RVR by applying the Savitzky–Golay algorithm with a window size of 11 points and coefficients for third-order fitting.<sup>44</sup>

Autoregulatory efficiency is expressed as percentage of perfect autoregulation as described previously.<sup>16</sup> The transition between the initial (MR) and the secondary response (TGF) was determined from the slowest RVR change within the first 10 s after the RAP step. This was obtained from the time of zero-crossing of the second derivative



of RVR.<sup>16</sup> The zero-crossing was found at  $6.8 \pm 0.8$  s in wt,  $7.4 \pm 0.4$  s in Cx40-ko, and  $7.3 \pm 1.2$  s in Cx40KI45. The regulatory strength of each mechanism was derived from the improvement of autoregulatory efficiency within specified time intervals after the pressure step.<sup>16</sup> The MR was estimated from the autoregulatory change in RVR between  $t_0 = 0$  to 1 s and  $t_1 = 4$  to 7 s after the pressure step, TGF from  $t_1 = 4$  to 7 s to  $t_2 = 20$  to 30 s, and the third mechanism from  $t_2 = 20$  to 30 s to  $t_3 = 90$  to 120 s.

### Statistical Analysis

Statistical significance was tested by ANOVA for repeated measures in conjunction with Holm–Sidak or Tukey *post hoc* test (SigmaStat 3.5, SPSS Inc., Chicago, IL). In the case of a non-normal distribution, Tukey test for multiple comparisons was used as a *post hoc* test. For drug effects within the same animal, a paired *t* test was used. A *P* value <0.05 was considered statistically significant. Data are presented as mean  $\pm$  SEM. The numbers of animals are indicated as *n*.

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### DISCLOSURES

None.

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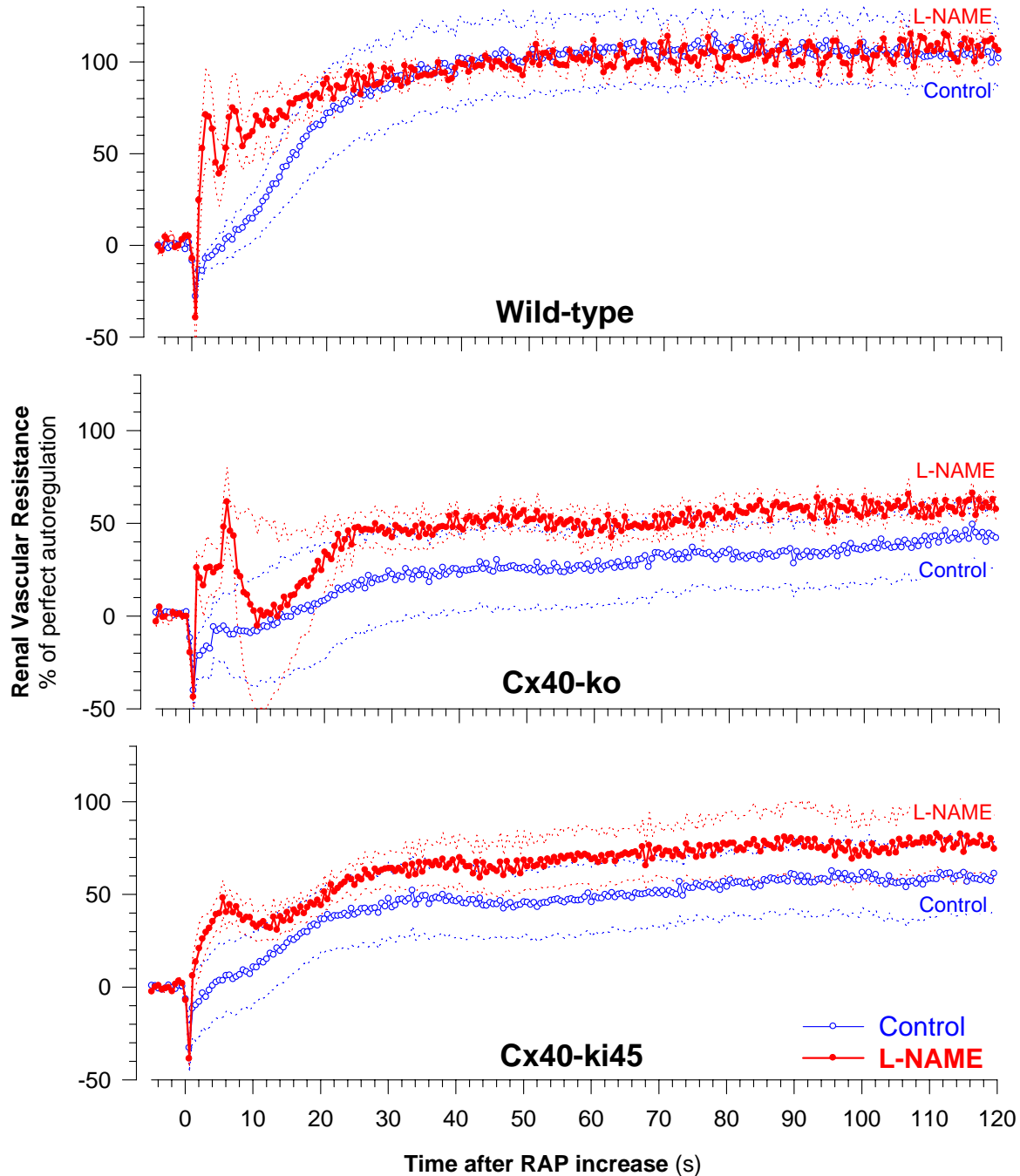
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## Online Supplement

Just, Kurtz, De Wit, Wagner, Kurtz, Arendshorst

*Connexin 40 Mediates Tubuloglomerular Feedback Contribution to Renal Blood Flow Autoregulation*

### Time course of the autoregulatory response during NOS-inhibition



**Figure S1.** Time course of renal vascular resistance (RVR) in response to a step increase in renal artery pressure initiated at time  $t=0$  s during control (Control, blue open circles) and in the same animals during NO-synthase inhibition (L-NAME, red filled circles). Data are shown for wild-type mice ( $n=4$ , upper panel), Cx40-deficient mice (Cx40-ko,  $n=4$ , middle panel) and mice with replacement of Cx40 by Cx45 (Cx40-ki45,  $n=4$ , lower panel). RVR is normalized in percent of perfect autoregulation (see main text).

Inhibition of NO-production enhances the myogenic response (rise of RVR within the first 5 s) but maintains the impairment of steady-state autoregulatory efficiency (RVR at  $t=90-120$ s) observed in Cx40-ko and Cx40-ki45 as compared to wild-type mice.

The fast rise and slow reduction of RVR between  $t=5$  and  $t=25$  s in Cx40-ko was observed in only one of the Cx40-ko mice. The reason for the atypical time course is unclear.