Replacement of connexin 40 by connexin 45 causes ectopic localization of renin-producing cells in the kidney but maintains in vivo control of renin gene expression

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The media layer of renal afferent arterioles contains two cell types, vascular smooth muscle and renin-producing cells, which are located at the terminal end in the juxtaglomerular position (4). It is thought that smooth muscle cells and renin-producing cells switch into each other by metaplastic transformation (2, 4). Major structural and functional differences exist between these two cell types. The vascular smooth muscle cells, which lay circularly in the vessel wall, contain a number of smooth muscle actin filaments, while the renin-producing juxtaglomerular cells contain less smooth muscle actin, but instead are composed of prominent secretory granules (4). Both cell types are coupled to each other via gap junctions (14, 20, 21). In smooth muscle cells, the gap junctions are formed by connexin 45 (Cx45) (6, 12), whereas they are primarily formed by connexin 40 (Cx40) in juxtaglomerular cells (5, 7, 11, 12, 24). Deletion of Cx40 causes the disappearance of renin-producing cells from the media layer of afferent arterioles (12, 13). Compensatory smooth muscle cells extend up to the branching of the arteriole into the capillary loop (13). Renin-expressing cells in Cx40-deficient kidneys are located outside the vessel wall in the periarteriolar and periglomerular interstitium (13), and they appear irregularly shaped. The number of renin-expressing cells is increased, and renin secretion has escaped its physiological negative feedback control that is normally established by blood pressure and by angiotensin II (11, 22). As a consequence, Cx40-deficient mice are hyperreninemic and hypertensive (11, 22). Replacement of the coding sequence of the Cx40 gene by that of Cx45 produces Cx45 expression under the control of the Cx40 promoter, i.e., replaces Cx40 by Cx45 protein. In renin-producing cells, this maneuver normalizes the renin phenotype of Cx40-deficient mice to a major extent (17).

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Materials and Methods

Animals. All animal experiments were approved by an ethics committee and were conducted according to the National Institutes of Health guidelines for the care and use of animals in research. Kidneys were sampled from 16 Cx40+/− (9) and 16 Cx40kiCx45 male mice (1), in which the coding region of the Cx40 was replaced by the coding region of Cx45.

Sixteen wild-type mice served as controls. The genetic background of Cx40+/− and Cx40kiCx45 mice and wild-type animals was considered identical since Cx40-deficient and Cx40kiCx45 mice were backcrossed for seven generations on a C57BL/6 background.
Eight mice of each genotype were assigned to a treatment control group, and the other eight mice of each genotype were pretreated with a low-salt diet (0.02% wt/wt) for 1 wk. In addition, these mice on low salt received the angiotensin-converting enzyme (ACE) inhibitor enalapril (10 mg/kg) via their drinking water for the last 3 days of the dietary treatment.

At the end of the experiments, the animals were anesthetized with a lethal dose of pentobarbital sodium. In four animals of each experimental group, kidneys were perfusion-fixed with 4% paraformaldehyde, removed, cut in half, and processed for paraffin embedding. The kidneys of the remaining four animals of each group were removed without fixation, cut in half, and snap-frozen in liquid nitrogen for further RNA extraction.

Immunohistochemistry for renin, α-smooth muscle actin, and Ki-67. The expression of renin, α-smooth muscle actin, and the nuclear antigen Ki-67 was localized by immunohistochemistry. In brief, kidneys were perfusion-fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Immunolabeling was performed on 5-μm paraffin sections. After blocking with 10% horse serum, 1% BSA in PBS, sections were incubated with either anti-renin (generated by Davids Immunotechnologie, Regensburg, Germany), anti-smooth muscle actin (Beckman Coulter, Immunotech, Marseille, France), or anti-Ki-67 (DakoCytomation, Glostrup, Denmark) antibodies overnight at 4°C, followed by incubation with a fluorescent secondary antibody (Dianova, Hamburg, Germany).

Quantification of renin immunoreactive areas was performed using the freely available software program Image J (Rasband JW, National Institutes of Health). For analysis, the total renin-immunoreactive area per kidney section was related to the number of glomeruli visible on the sections. From each kidney used for morphometric analysis, 10 well-visible afferent arterioles were selected for measurement of the lengths of segments, in which renin-expressing cells were found. Since four kidneys of each experimental group were analyzed, a total of 40 afferent arterioles were measured per experimental group. The results of the measurements are given as means ± SE.

3D reconstruction. Serial sections of kidney specimens were fixed and stained for renin and α-smooth muscle actin as described above. A 3D reconstruction of renin immunoreactivity and of α-smooth muscle immunoreactivity was performed using the Amira 3.3 visualization program (Mercury Systems) as described elsewhere (16).

Determination of mRNA by real-time PCR. Total RNA was isolated from the frozen kidneys, and cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Superscript, Invitrogen) as described elsewhere (10). For quantification of renin mRNA expression, real-time RT-PCR was performed using a Light Cycler Instrument (Roche Diagnostics) and the QuantiTect SYBR Green PCR kit (Quiagen), with β-actin as a control. To verify the accuracy of the amplicon, a melting-curve analysis was implemented after amplification, and PCR products were analyzed on an ethidium bromide-stained agarose gel.
RESULTS

In wild-type mice, renin-expressing cells were located at the typical juxtaglomerular position of afferent arteriole glomeruli (Fig. 1A). Renin-immunoreactive cells were located in the media layer of the arterioles at the entrance to the glomeruli (Fig. 2A). Immunoreactivity for α-smooth muscle actin was weaker in renin-containing cells than in smooth muscle cells of the afferent arterioles. Activation of the renin system by treating the mice with a combination of a low-salt diet with an ACE inhibitor increased renin mRNA levels and renin-immunoreactive areas in the kidneys of wild-type mice five- and fourfold, respectively (Figs. 3, 4, A and B). Plasma renin concentration increased from 92 ± 12 to 950 ± 180 ng ANG I·h⁻¹·ml⁻¹ (P < 0.05). Renin-expressing cells displayed a pattern of upstream recruitment in the media layer of all afferent arterioles (Figs. 1B and 2B). Under normal conditions, renin-expressing cells in the afferent arterioles extended 15 ± 2 μm upstream from the vascular pole. This figure was increased to 34 ± 4 μm after treatment with a low-salt diet in combination with the ACE inhibitor (P < 0.05).

In Cx40⁻/⁻ mice under normal conditions, kidney renin mRNA levels were increased 2.8-fold relative to wild-type mice (Fig. 3, top). In parallel, renin-immunoreactive areas in kidney sections were increased fourfold relative to wild-type controls (Figs. 3, bottom, and 4C). Renin-immunoreactive cells were located outside the media layer of afferent arterioles in a spotlike fashion in the periglomerular interstitium (Fig. 5A). Smooth muscle cells extended to the branching of afferent arterioles into glomerular capillaries (Fig. 5A). Spots of renin-immunoreactive fibroblast-like cells were heterogeneously distributed in the renal cortex, in a manner that was not found at all the glomeruli (Fig. 1C). Treatment of Cx40⁻/⁻ mice with a combination of low salt with ACEI marginally increased kidney mRNA levels (Fig. 3, top), while it did not increase renin-immunoreactive areas (Figs. 3, bottom, and 4D) nor plasma renin concentration (1,150 ± 190 ng ANG I·h⁻¹·ml⁻¹ under normal conditions vs. 1,241 ± 253 ng ANG I·h⁻¹·ml⁻¹ after stimulation). The renin-expressing cells remained ectopically localized, and no retrograde recruitment of renin-expressing cells in the media layer of afferent arterioles was visible (Fig. 5B). The heterogeneous distribution of the renin-expressing cell spots remained unchanged (Fig. 1D). In view of the localization of renin-expressing cells outside of the afferent arterioles, no data on the lengths of renin-positive segments of afferent arterioles could be elaborated.

In Cx40ki45 mice under normal conditions, kidney renin mRNA abundance (Fig. 3, top), renin-immunoreactive areas...
(Figs. 3, bottom, and 4E), and plasma renin concentrations (110 ± 22 ng ANG 1·h⁻¹·ml⁻¹) were not different from those in wild-type controls. Renin-immunoreactive cells were localized at the juxtaglomerular pole. In some arterioles, renin cells were located in the media layer (Fig. 6A), while in others renin cells resided outside the media layer but still had contact with the media layer (Fig. 6B). In contrast to Cx40−/− mice and similarly to wild-type mice, juxtaglomerular areas of most glomeruli in Cx40ki45 mice showed renin expression under normal conditions (Fig. 1E). Due to the inconsistent localization of renin-expressing cells in the walls of afferent arterioles, the lengths of renin-positive segments of the afferent arterioles were very variable (12 ± 4 μm). Upon treatment of Cx40ki45 mice with low salt/ACE inhibitor, renin mRNA abundance and renin-immunoreactive areas increased five- and fourfold, respectively (Figs. 3 and 4F). Plasma renin concentration increased ~10-fold to 1,010 ± 230 ng ANG 1·h⁻¹·ml⁻¹ (P < 0.05). Renin immunoreactivity became visible in the vast majority of juxtaglomerular areas (Fig. 1F). However, no clear retrograde recruitment of renin-expressing cells in the afferent arterioles could be observed (Fig. 6C). The lengths of renin-positive segments of the afferent arterioles was not increased (10 ± 4 μm). Instead, renin expression appeared in vessel-associated or interstitially localized, fibroblast-like cells (Fig. 6, D and E). Electron microscopic analysis revealed that in between the extraglomerular cells granular cells with giant confluent granules were frequently encountered. Additionally, many granular cells were seen around the dilated afferent arterioles (Fig. 7). Renin-expressing cell plaques similar to those seen in Cx40 −/− kidneys appeared in Cx40ki45 kidneys upon stimulation (Fig. 6E).
In view of the obvious recruitment of renin-producing cells in wild-type and in Cx40ki45 mice upon challenge by salt deficiency, we looked for potential signs of cell proliferation in the renin-expressing cells. For this purpose, we examined nuclear Ki-67 immunoreactivity as an indicator of cell division. Although we found a few Ki-67-positive cells in the proximal tubule, we found no Ki-67 immunoreactivity associated with renin-immunoreactive cells (data not shown).

**DISCUSSION**

This study confirms findings from previous studies demonstrating that chronic stimulation of the renin system by renal ischemia, salt deficiency, and by inhibition of the renin-angiotensin system induces an increase in the number of renin-expressing cells (2–4). This might result from a renin reexpression in the media layer of afferent arterioles, rather than from proliferation of renin-expressing cells. Our study furthermore confirms the appearance of renin-expressing cells in the periglomerular interstitium lacking Cx40 protein (13). We have recently shown that the expression of Cx40 is a characteristic of renin-expressing cells in the kidney at any developmental stage (12). Our data show that the absence of Cx40 not only leads to renin reexpression in cells beyond the media layer of the afferent arteriole, but it also prevents an increase in the number of renin cells in response to a strong renin-stimulatory challenge. The data also illustrate that the interstitial periglomerular accumulation of renin cells in the absence of Cx40 is not homogeneously distributed over the kidney cortex. This heterogeneous pattern is maintained also during challenge of the renin system, indicating that the lack of Cx40 probably somehow suppresses the development of potential renin-producing cells in certain renocortical areas. This finding might suggest that the development of juxtaglomerular renin-producin-
renin-producing cells by Cx45, which is typically found in vascular smooth muscle cells (6, 12), maintains the ability of renin system activity to be physiologically controlled by both renal perfusion pressure and ANG II (17). Also, the localization of renin-expressing cells in Cx40ki45 kidneys without further challenge appeared not to be unusual, at least at first glance. Our more detailed analysis now reveals that under normal conditions some derangements of renin-expressing cells can already be seen in Cx40ki45 mice, in the sense that the renin-expressing cells are not exclusively located in the media layer of the afferent arterioles. Upon challenge of the renin system, the number of renin-expressing cells strongly increases both in Cx40ki45 and in wild-type mice to similar extents, although in quite different locations. In contrast to wild-type mice but similarly to Cx40+/− mice, newly recruited renin-expressing cells are found outside the afferent arteriole in the interstitium in Cx40ki45 mice. Extravascular, mostly irregularly shaped renin-expressing cells appeared, resembling the expression pattern of Cx40+/− kidneys. A contrast to Cx40+/− kidneys, however, was that renin expression in challenged Cx40ki45 kidneys was more homogenously distributed over the kidney cortex. We obtained no evidence for divisions of renin-expressing cells in either wild-type mice or in Cx40−/− or Cx40ki45 mice. This finding confirms, in part, previous results (2, 13) and suggests that the expansion of renin cell masses in wild-type kidneys is in fact the result of a transformation of preexisting cells, which switch from a nonsecretory to a renin-secretory phenotype. A similar explanation should hold for the appearance of ectopic renin-producing cells in challenged Cx40ki45 kidneys. This would indicate that the ectopic potential, renin producers had already developed and were hibernating. Such a conclusion would fit with the concept that renin cells are precursors for multiple cell types that switch to the renin phenotype when homeostasis is threatened (18). As a consequence, we infer that Cx45 cannot fully replace Cx40 in its function of a proper homing of renin-producing cells in the media layer of afferent arterioles. Interestingly, however, Cx45 appears to be able to mediate a suppressive effect on renin gene expression in these ectopic cells, at least under ambient laboratory conditions of the mice. This finding suggests that the dislocation of renin-expressing cells in Cx40−/− kidneys per se might not be the predominant reason why renin secretion runs out of physiological control in Cx40−/− mice. This negative feedback control is apparently lost if Cx40 is not replaced by Cx45. Gap junctions composed of Cx40 or Cx45 differ in their electrical conductance, their permeability for uncharged molecules, and their modes of regulation (15, 19). Which of these properties accounts for the compensatory or noncompensatory features of Cx45 for the function of Cx40 for renin expression remains to be clarified. It should be noted that neither in the cardiac conduction system (1), the vascular endothelium (23) or for the autoregulation of renal blood flow (8) can Cx45 replace the function of Cx40.

Since we do not yet know the molecular signals that trigger or disinhibit renin expression in response to low salt/ACE inhibition, we presently cannot distinguish whether Cx45 does replace Cx40 in its function to regulate renin expression at the cellular level, or whether Cx45 can replace Cx40 in its function to generate a yet unknown extracellular signal that secondarily affects renin gene activity. Distinguishing between these tempting possibilities remains a task for future research.

GRANTS

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REFERENCES


