Connexin43-dependent mechanism modulates renin secretion and hypertension

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To investigate the function of *Cx43* during hypertension, we studied the mouse line Cx43KI32 (KI32), in which the coding region of *Cx32* replaces that of *Cx43*. Within the kidneys of homozygous KI32 mice, Cx32 was expressed in cortical and medullary tubules, as well as in some extra- and intraglomerular vessels, i.e., at sites where Cx32 and Cx43 are found in WT mice. Under such conditions, renin expression was much reduced compared with that observed in the kidneys of WT and heterozygous KI32 littermates. After exposure to a high-salt diet, all mice retained a normal blood pressure. However, whereas the levels of renin were significantly reduced in the kidneys of WT and heterozygous KI32 mice, reaching levels comparable to those observed in homozygous littermates, they were not further affected in the latter animals. Four weeks after the clipping of a renal artery (the 2-kidney, 1-clip [2K1C] model), 2K1C WT and heterozygous mice showed an increase in blood pressure and in the circulating levels of renin, whereas 2K1C homozygous littermates remained normotensive and showed unchanged plasma renin activity. Hypertensive, but not normotensive, mice also developed cardiac hypertrophy. The data indicate that replacement of *Cx43* by *Cx32* is associated with decreased expression and secretion of renin, thus preventing the renin-dependent hypertension that is normally induced in the 2K1C model.

Introduction

Channels located at gap junctions represent one way in which vertebrate cells communicate (1–3), by sharing ions, second messengers, small metabolites, and other signaling molecules (4, 5). This type of intercellular communication permits coordinated cellular activity, including secretion (6–13), by allowing cells to review the functional state of their neighbors, a critical feature for the homeostasis of multicellular systems (14). Intercellular gap junction channels result from the association of 2 half channels, named connexons, which are separately contributed by each of 2 adjacent cells. Each connexon is an assembly of 6 membrane proteins, named connexins, which are encoded by a gene family consisting of at least 20 members (15).

The kidney provides a challenging model to relate connexin diversity to the function of different cell types (1), particularly in the context of the control of blood pressure. This control is mostly achieved by the juxtaglomerular apparatus, which accommodates, in small regions of the kidney, several cell types, including smooth muscle, endothelial, mesangial, macula densa, and renin-producing cells of the afferent arterioles. Cells of each type are connected by gap junctions (16–22), and other gap junctions further link the ECs, the smooth muscle cells, and the renin-producing cells of the afferent arteriole (19, 23, 24). Connexin43 (Cx43) and Cx40 appear to be the prominent connexins forming these junctions in vivo (22, 23).

We have previously reported that renin-dependent hypertension, which is experimentally induced by clipping of 1 renal artery in the 2-kidney, 1-clip (2K1C) model, is associated with a vessel-specific increase in the expression of Cx43 (25–27). Consistent with this view, the endothelium-specific ablation of Cx43 in at least some vessels resulted in either no change (28) or a drop of blood pressure (29). The reason why Cx43 mediates the response of the vascular wall to excessive hemodynamic conditions remains to be understood. In view of the increasing evidence that distinct connexin species impart different biophysical and regulation characteristics to cell-to-cell channels (30), we hypothesized that Cx43 channels are particularly well suited for the exchanges of cytoplasmic ions and metabolites that take place during hypertensive conditions between ECs, between smooth muscle cells, and, possibly, between these 2 cell types. Therefore, replacing the Cx43 channels with the different channels made by another connexin isoform would be expected to alter cell-to-cell signaling. In turn, this alteration would be anticipated to result in an inappropriate response of the vascular wall.

Here, we have tested this hypothesis in vivo by studying the knock-in mice of the Cx43KI32 line, hereafter referred to as KI32, in which the coding region of *Cx43* was replaced by that of *Cx32*, under control of the native *Cx43* promoter (31). We first show that the levels of renin were decreased by about half at both the transcript and the protein level in the kidneys of homozygous KI32 mice. After feeding of a high-salt diet, we found that, in contrast to WT and heterozygous KI32 mice, homozygous KI32 littermates did not show the expected downregulation in the expression of the renin gene. After the experimental induction of a renin-dependent renovascular hypertension in the 2K1C model (32), we further show that, whereas WT and heterozygous KI32 mice, which expressed Cx43, became hypertensive and featured cardiac hypertrophy as a result of increased plasma renin activity, homozygous littermates, which expressed Cx32 instead of the native Cx43, failed to develop these changes, being unable to normally increase renin expression and release.

Nonstandard abbreviations used: Cx, connexin; 2K1C, 2-kidney, 1-clip (model).

Conflict of interest: The authors have declared that no conflict of interest exists.

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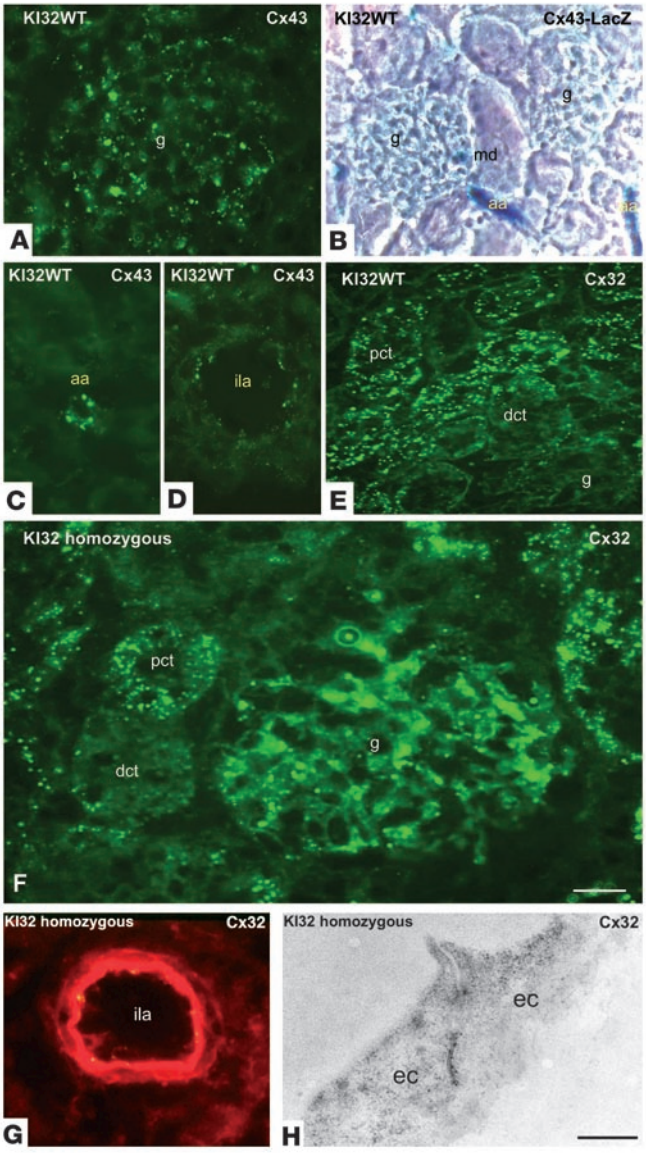

Figure 1
Cx43 and Cx32 were differentially distributed in the kidneys of KI32 mice. (**A**–**D**) Antibodies (**A**, **C**, and **D**) and a *lacZ* reporter gene construct (**B**) revealed the presence of Cx43 between the ECs of both interlobular renal arterioles (**D**) and afferent arterioles (**B** and **C**). Cx43 was also expressed by the ECs of the glomeruli (**A** and **B**). (**E**) In contrast, antibodies against Cx32 showed no staining of glomeruli and arterioles, but abundant levels of Cx32 in cortical proximal tubules. (**F**) In kidneys of homozygous KI32 mice, Cx32 was immunolocalized in both proximal and distal convoluted tubules, as well as in ECs of glomeruli. (**G**) The protein was also immunolabeled at gap junctions of ECs in interlobular arteries. In these vessels, the protein A–coated gold particles that were reacted with the Cx32 antibodies selectively decorated minute areas of apposition between EC membranes characteristic of gap junctions. g, glomerulus; aa, afferent arteriole; md, macula densa; ila, interlobular arteriole; pct, proximal convoluted tubule; dct, distal convoluted tubule; ec, endothelial cells. Scale bar: 30 μm (**A**, **C**, **D**, and **G**); 50 μm (**B** and **E**); 20 μm (**F**); 215 nm (**H**).

Results

Cx43 and Cx32 are differentially expressed in the kidneys of KI32 mice. Immunofluorescence labeling demonstrated that, in the kidneys of WT mice, Cx43 was expressed by the ECs of interlobular (Figure 1D) and intralobular vessels, including the afferent arteriole (Figure 1C). The latter localization was confirmed using a highly sensitive reporter gene approach of endothelium-specific replacement of *Cx43* by *lacZ*. The transcriptional expression of the reporter gene was also localized in the afferent arteriole (Figure 1B). This approach, as well as immunostaining, further indicated the presence of Cx43 in the ECs of renal glomeruli (Figure 1, B and A, respectively).

Antibodies to Cx32 showed abundant levels of this protein in proximal convoluted tubules, with no detectable signal in either vessels or glomeruli (Figure 1E). In homozygous KI32 mice, no detectable Cx43 was observed (not shown), and Cx32 was found expressed in ECs of glomeruli (Figure 1F) and interlobular vessels (Figure 1, G and H), i.e., at sites where Cx43 is normally expressed, as well as in cortical tubules, i.e., at sites of native Cx32 expression (Figure 1F).

Renin expression is altered after replacement of Cx43 by Cx32. Northern blot analysis revealed that the levels of the renin transcript were decreased by about half in the kidneys of homozygous KI32 mice, in which all *Cx43* has been replaced by *Cx32*, relative to those of *GAPDH*, which were unchanged (Figure 2A). Consistent with this finding, in situ hybridization and immunofluorescence labeling both indicated a reduction of renin expression and content in the afferent arterioles of homozygous KI32 mice (Figure 2B).

Cx43 is involved in the control of renin mRNA expression. WT, heterozygous, and homozygous KI32 mice were fed a high-salt diet (6%) during 3 weeks; at the end of this treatment, all mice showed a mean blood pressure similar to that of control mice fed a normal-salt diet (Figure 3A). As a result of the high-salt diet, the kidneys of WT and heterozygous KI32 mice displayed levels of renin mRNA that were 40% lower than those of controls maintained on a normal-salt diet, as evaluated by both Northern blots (Figure 3B) and quantitative RT-PCR (Figure 3C). In contrast, the levels of renin mRNA were unaffected in the kidneys of homozygous KI32 mice (Figure 3, B and C).

Full replacement of Cx43 by Cx32 protects mice against hypertension and cardiac hypertrophy. The characteristics of the mice that underwent the renal artery clipping, as evaluated on the experimental day, are shown in Table 1. Homozygous KI32 mice were about 15% smaller than WT mice. There was no difference in heart rate between 2K1C and sham-operated mice. In all 2K1C animals, the left, clipped kidney weighed less than that of the sham-operated mice, whereas the contralateral kidney was significantly larger than that of these controls (Table 1).

Mean arterial blood pressure of WT and heterozygous 2K1C mice was significantly higher than that of the cognate, normotensive sham-operated controls (Figure 4). In contrast, no change in blood pressure was observed in homozygous 2K1C animals, which displayed an average blood pressure (120 ± 8 mmHg) similar to that of sham-operated controls (115 ± 5 mmHg) (Figure 4).

The hearts of hypertensive WT and heterozygous 2K1C mice were hypertrophied compared with those of normotensive controls, as indicated by a 20–30% increase in cardiac weight index (Figure 5B). In contrast, no such change was observed in 2K1C homozygous KI32 mice (Figure 5B). In the latter animals, Cx32 was expressed at intercalated disks, fully replacing the native Cx43, which was observed in cardiomyocytes of WT mice (Figure 5A). The 2 connexins colocalized at intercalated disks of heterozygous KI32 littermates (Figure 5A).

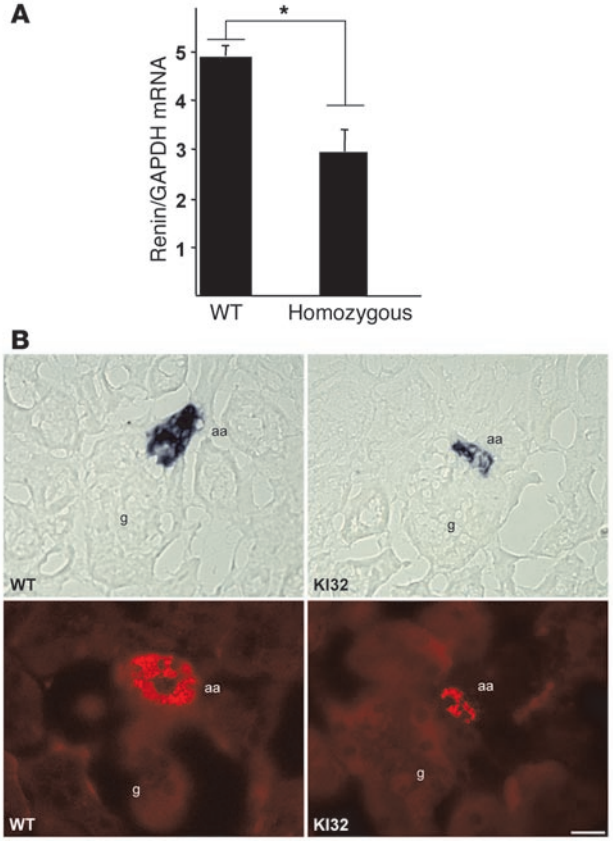

Figure 2
Renin expression was reduced after replacement of *Cx43* by *Cx32*. (**A**) Northern blots revealed that the levels of the renin transcript were decreased by about half in the kidneys of homozygous KI32 mice, relative to those of GAPDH. *$P < 0.05$ versus WT mice. (**B**) Consistent with this finding, in situ hybridization (top panels) and immunofluorescence labeling (bottom panels) of the afferent arteriole of homozygous KI32 mice indicated reduced levels of the hormone transcript and of the cognate protein, respectively. Scale bar: 30 μm.

Replacement of Cx43 by Cx32 alters the control of renin secretion. Four weeks after clipping, hypertensive WT and, to a somewhat lesser degree, heterozygous 2K1C mice showed increased plasma renin activity, compared with the cognate sham-operated, normotensive controls (Figure 6). In contrast, no difference in plasma renin activity was observed between 2K1C and sham-operated homozygous mice, which, after surgery, showed basal levels of the hormone (Figure 6).

At the same time point, the levels of renin mRNA were similar in the left and right kidneys of sham-operated mice, but markedly increased in the left, clipped kidneys of WT 2K1C mice, which also showed decreased renin expression within the contralateral kidney (Figure 7A). In contrast, such a differential regulation was not observed in the homozygous 2K1C mice, which showed control levels of renin mRNA in both kidneys (Figure 7A). Quantitative assessment of Northern blots showed that the clipped kidneys of WT, hypertensive mice contained levels of renin mRNA that were 10-fold higher than those in the contralateral kidneys, whereas such levels were similar in the homozygous, normotensive littermates (Figure 7A). Comparable observations were made by quantitative RT-PCR (Figure 7B). This approach further demonstrated an intermediate profile of the heterozygous 2K1C mice. Hence, these animals featured a 5- to 6-fold increase in renin expression within the clipped kidney (Figure 7B).

Parallel measurements of mRNA levels of *Cx32* and *Cx43* by quantitative RT-PCR failed to demonstrate a significant change in the expression of the transcripts of these 2 connexins in both WT and heterozygous KI32 mice, whether the animals had been sham-operated or had had a renal artery clipped (Figure 8).

Discussion

We have previously reported that the renin-dependent hypertension that is experimentally induced by clipping of 1 renal artery in the 2K1C model is associated with a vessel-specific increase in the expression of Cx43 (25–27). These findings, the specific distribution of Cx43 between smooth muscle cells and selected ECs of vessels, and the regulation of Cx43 levels in both vascular endothelial and smooth muscle cells exposed to shear stress and other mechanical challenges (33–37) provide evidence that the cell-to-cell communication mediated by Cx43 channels contributes to

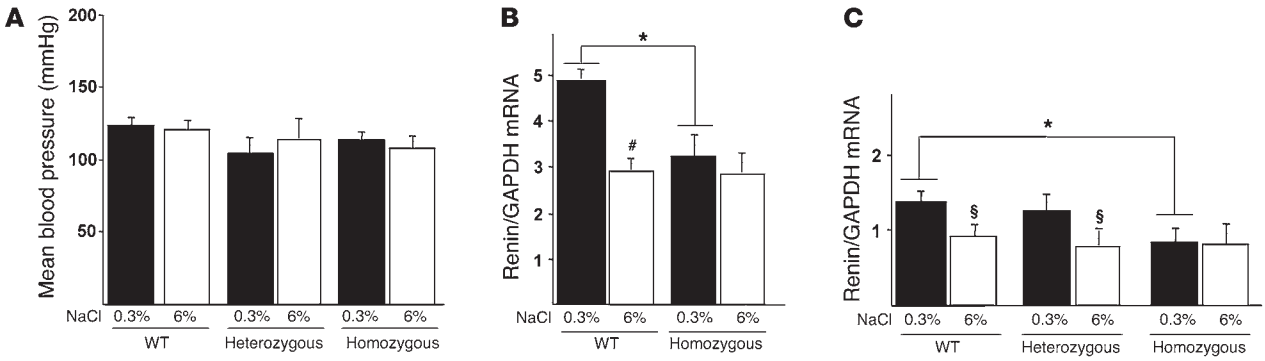

Figure 3
Transcription of the renin gene was differentially regulated after replacement of *Cx43* by *Cx32*. (**A**) Mean blood pressure measurements remained normal in KI32 mice fed a high-salt (6%) diet. (**B**) Under these conditions, Northern blots revealed that the expression of the renin gene was decreased by about 40% in the kidneys of WT mice but was not altered in the kidneys of homozygous KI32 animals. #$P < 0.01$ versus value observed under a control NaCl diet (0.3%); *$P < 0.05$ versus WT mice. (**C**) A similar difference was found by quantitative RT-PCR. This approach further revealed that the renin expression of heterozygous KI32 mice was decreased by the high-salt diet, as seen in WT. §$P < 0.05$ versus controls fed a normal-salt diet; *$P < 0.05$ versus WT mice. Data are mean ± SEM values of 5 mice per group.

Table 1
Characteristics of the 2K1C and sham-operated KI32 mice

Animal	Groups	*n*	Body weight (g)	Heart rate (bpm)	LKi (mg/g)	RKi (mg/g)
WT	Sham	6	24.8 ± 0.7	617 ± 41	6.0 ± 0.2	6.2 ± 0.2
	2K1C	7	27.7 ± 0.5	616 ± 43	5.4 ± 0.7[A]	8.7 ± 0.8[A]
KI32 heterozygous	Sham	12	26.4 ± 1.0	576 ± 25	7.7 ± 0.4	7.2 ± 0.4
	2K1C	9	27.2 ± 1.1	644 ± 18	4.7 ± 0.5[A]	8.0 ± 0.5[A]
KI32 homozygous	Sham	4	22.3 ± 2.3[B]	555 ± 55	6.0 ± 0.4	6.3 ± 0.4
	2K1C	5	23.0 ± 0.6[B]	597 ± 15	4.9 ± 0.4[A]	6.8 ± 0.4[C]

LKi, left kidney index (ratio between the weight of the left, clipped kidney [mg] and body weight [g]); RKi, right kidney index. [A]$P < 0.01$ versus sham-operated animals; [B]$P < 0.05$ versus WT or heterozygous KI32 mice; [C]$P < 0.05$ versus sham-operated animals.

the response of the vascular wall to excessive mechanical loads (24, 38). Consistent with this view, the endothelium-specific ablation of Cx43 in at least some vessels resulted in either no change (28) or a drop of blood pressure (29).

The reason why Cx43 mediates the response of the vascular wall to excessive hemodynamic conditions remains to be understood (38). In view of the increasing evidence that distinct connexin species impart different biophysical and regulation characteristics to cell-to-cell channels (30, 39), we hypothesized that Cx43 channels are particularly well suited to integrate the response of the cells making a vascular wall to hypertensive conditions. An implication of this hypothesis is that replacing Cx43 with another connexin species should alter the normal response of the vascular wall to such conditions. We have tested this implication in vivo, by subjecting knock-in KI32 mice (31) to conditions of a chronic, renin-dependent hypertension.

Here, we document that, in their WT form, KI32 mice are normotensive and show, within the kidneys, the differential, cell-specific distribution of Cx43 and Cx32 that has been reported in the kidneys of other normal rodents (21, 22, 40). Thus, Cx32 was prominent among cells of proximal convoluted tubules, whereas Cx43 was mostly found in collecting medullary ducts (22); ECs of inter- and intralobular vessels, including the afferent arterioles (23, 41); and glomerular capillaries. In contrast, homozygous KI32 mice did not express Cx43, which was replaced by Cx32 in all organs. Thus, this protein was found at locations where Cx32 and Cx43 are natively expressed, including in kidneys and heart, 2 major organs targeted during hypertension.

In heterozygous KI32 mice, these changes were associated with a major loss in the levels of kidney renin, due to a transcriptional downregulation of the cognate gene. To assess whether this change was due to a perturbed function of the renin-angiotensin system, we studied the effect of dietary salt loading, which is known to suppress the renin-angiotensin system and to decrease circulating levels of renin without changes in blood pressure. We found that, in contrast to WT and heterozygous KI32 mice, which displayed these features, homozygous KI32 littermates did not change renin transcription or blood pressure. The data indicate that replacement of Cx43 by Cx32 did not affect the counterregulatory mechanism involved in the control of blood pressure in the presence of an increased sodium load but altered both the basal and the downregulated transcriptional activity of the renin gene, resulting in a marked loss of the hormone.

To assess whether this alteration also affected the upregulation of renin transcription, we subjected the animals to the clipping of 1 renal artery, a procedure (2K1C) that induces a renin-dependent hypertension in control rodents (32). Four weeks after this clipping, blood pressure and plasma renin levels were increased in both WT and heterozygous littermates, indicating that KI32 mice expressing 1 or 2 alleles of the *Cx43* gene responded, as observed in controls, by increasing renin production within the clipped, hypoperfused kidney. As a result, these mice also developed cardiac hypertrophy, as previously reported in the 2K1C model (27, 32). Strikingly, however, homozygous KI32 mice, which no longer expressed Cx43 and were subjected in parallel to rigorously identical experimental conditions, remained normotensive and failed to develop the increased plasma renin levels and cardiac alterations observed in WT and heterozygous littermates. Further analysis showed that these animals also did not increase the transcription of the renin gene in the clipped kidney and featured plasma renin levels that, before and after surgery, were significantly lower than those of both WT and heterozygous controls.

Because the direct monitoring of mean blood pressure in mice requires the surgical insertion of an in-dwelling arterial catheter and the restriction of the animal during the measurement, it may be argued that the blood pressure values we report were affected by the short anesthesia and surgery that took place the day preceding the measurement, and/or by the restraint of the nonanesthetized animals during the 15-minute duration of this measurement. We cannot exclude that these factors may have marginally affected our measurements, but this effect, if any, is most likely to be similar in all mice, since all littermates were studied in parallel by the very same procedure. Furthermore, several lines of evidence concur to indicate that the method we used, which is the gold standard for direct measurements of blood pressure in mice (refs. 42, 43; also http://www.mmpc.org/uc/cardiotests.html), could not artifactually induce the difference we observed between the homozygous

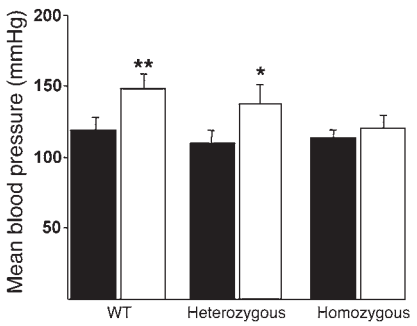

Figure 4
Renal artery clipping increased blood pressure in WT and heterozygous KI32 mice, but not in homozygous littermates. Four weeks after clipping of a renal artery, mean intra-arterial blood pressure of WT, 2K1C mice (white bar) was higher than that of sham-operated controls that remained normotensive (black bar). Similar observations were made in heterozygous KI32 mice. In contrast, no change in blood pressure was observed in homozygous KI32 littermates. Data are mean ± SEM values of the number of mice indicated in Table 1. White bars, 2K1C mice; black bars, sham-operated mice. *$P < 0.05$; **$P < 0.01$.

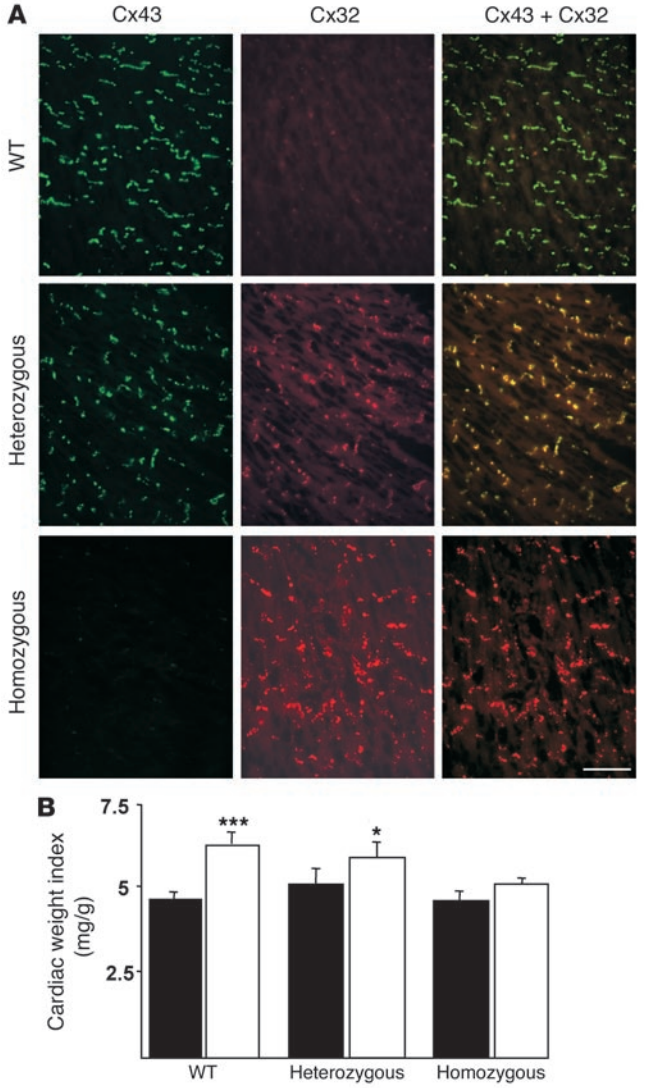

Figure 5
Heart weight increased in control and heterozygous KI32 mice, but not in homozygous littermates. (**A**) Immunofluorescence labeling for Cx43 and Cx32 showed the exclusive presence of the former and latter connexins in sections of ventricular myocytes of WT and homozygous KI32 mice, respectively. In the myocardium of heterozygous littermates, the 2 connexins colocalized at intercalated discs. Bar: 120 μm. (**B**) Four weeks after clipping of a renal artery, the cardiac weight index of WT, 2K1C mice that were hypertensive (white bar) was higher than that of sham-operated controls that remained normotensive (black bar), reflecting cardiac hypertrophy. Similar observations were made in heterozygous 2K1C mice. In contrast, no change in cardiac index was observed in homozygous 2K1C littermates. Data are mean ± SEM values of the number of mice indicated in Table 1. White bars, 2K1C mice; black bars, sham-operated mice. $^{*}P < 0.05$; $^{***}P < 0.001$.

KI32 animals and their WT and heterozygous littermates, after the 2K1C procedure. First, in the hands of trained operators (32, 44, 45), the method provided for reproducible and rather stable measurements of normal blood pressure in all control and high-salt-fed littermates, whatever their KI32 genotype. Second, the 25% increase in blood pressure that was induced by the 2K1C procedure in WT and heterozygous KI32 mice correlated with a similar increase in the cardiac weight index, a totally independent parameter that reflects the chronic effect of hypertension. Third, homozygous KI32 mice that were found to be normotensive after the 2K1C procedure, as indicated by the pressure measurements, also featured a normal heart index. Thus, the data show that replacement of Cx43 by Cx32 altered the in vivo function of renin-secreting cells, resulting in a full protection of the homozygous KI32 animals against the chronic hypertension that is normally induced by renin in the 2K1C model.

The mechanism whereby the replacement of Cx43 by Cx32 resulted in an inhibition of renin expression and secretion in KI32 mice remains to be elucidated. Conceivably, this inhibition could be due either to the loss of Cx43, which connects both the endothelial and the smooth muscle cells of most vessels, including the afferent arteriole of the kidney (23), or to the gain of Cx32 between these cells. In the former case, our data documenting an impairment of the renin response of the homozygous KI32 mice would be consistent with a positive or at least a permissive effect of Cx43 on the production and release of the hormone. In the latter case, an inhibitory or suppressive effect of the Cx32-dependent signaling would be considered. Previous experiments testing the effects of a Cre-mediated loss of Cx43 between ECs (28, 29) have not investigated the effects of the 2K1C procedure, nor tested the expression and release of renin. Evaluating these parameters could therefore help to pinpoint whether the protection against hypertension of the homozygous KI32 mice is exclusively dependent on Cx43 and the endothelium. However, the usefulness of these animals — which were not available for this study — should be carefully evaluated, inasmuch as previous studies have documented variable changes in their blood pressure (28, 29). Furthermore, some of these studies have not reported on the blood pressure of the Tie2-Cre mice (29), which is essential in view of the increasing evidence that the site of integration of a promoter Cre transgene can modulate the expression pattern of the prokaryotic protein, which may lead to undesired or confounding effects (46–49). Also, the endothelial-specific deletion of Cx43 may be associated in the afferent arteriole with a compensatory change in the expression of other connexins, as documented in other deletion studies (50, 51). Eventually, this cell-specific deletion leaves the VSMCs, which significantly contribute to the development and maintenance of hypertension in the whole animal, coupled by Cx43 (52). No study has yet investigated the effects of a selective deletion of this connexin between VSMCs. Thus, an unambiguous testing of which connexin, Cx43 or Cx32, is important for preventing the hypertension in the renin-dependent model we have studied here, awaits the development of a novel mouse line in which the expression of individual connexin isoforms could be rapidly modulated in adult mice, on demand, and in 1 or more selected cell types.

At any rate, in view of the mounting evidence that appropriate levels of specific connexin isoforms are necessary to sustain proper hormone secretion of many endocrine organs (8, 53), it is conceivable that the alteration of the signaling normally ensured by Cx43 channels, either directly or via the interaction of Cx43 with either cytosolic and membrane proteins (54, 55), resulted in disturbed functioning of the renin-producing cells. However, consideration of this possibility should take into account that, at variance with most other types of endocrine cells (8), renin-producing cells in situ are linked to each other, as well as to the ECs of the affer-

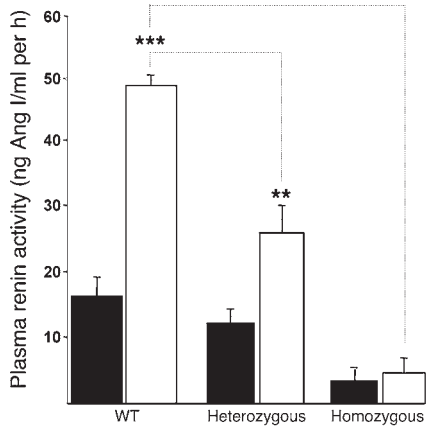

Figure 6
Plasma renin activity was increased in hypertensive but not in normotensive KI32 mice. Four weeks after clipping of a renal artery, plasma renin activity of the hypertensive, WT 2K1C mice (white bar) was higher than that of the normotensive sham-operated controls (black bar). Similar observations were made in heterozygous 2K1C mice. However, after clipping of the renal artery, these animals showed lower renin levels than WT littermates. In contrast, homozygous KI32 mice showed a much lower plasma renin activity (black bar), which was not affected by clipping of the renal artery (white bar). Data are mean ± SEM values of the number of mice indicated in Table 1. White bars, 2K1C mice; black bars, sham-operated mice. $^{**}P < 0.01$; $^{***}P < 0.001$.

ent arteriole, by Cx40 channels, whereas Cx43 is detected between some ECs of the afferent arteriole (23). Therefore, for the loss of Cx43 to account for altered renin expression and secretion, proper signaling would have to be perturbed between the endothelial and the renin-producing cells of the afferent arteriole. This is conceivable, inasmuch as Cx43 and Cx40 can establish cell-to-cell coupling via heteromeric and/or heterotypic channels (56, 57), and is in keeping with the observation that the ECs, which express Cx43, modulate renin secretion (58). The data also imply that neither Cx32, which replaced Cx43 in the homozygous mice but cannot form functional gap junction channels with Cx40, nor Cx40 alone, which was still normally expressed in homozygous KI32 mice (data not shown), can fulfill the same endothelium–renin cell signaling. These considerations do not rule out that other mechanisms, not dependent on the extent of coupling established by the connexin channels of endothelial and/or renin-producing cells, may also play a central role. Specifically, our finding that the transcription of the renin gene is selectively altered in the KI32 mice raises the intriguing possibility that the loss of Cx43, the gain of Cx32, or the replacement of the former by the latter connexin may significantly modulate the expression of the array of genes that contribute to control hypertension. Wide-scale transcriptome studies have certainly indicated that the expression of a number of genes is altered after loss of Cx43 (59–61), a protein that also interacts with a variety of transcription factors (62–64). Full validation of this putative mechanism requires that these studies be now extended to mice featuring a tissue-specific deletion of either Cx43 or Cx32, or a total replacement of the former by the latter connexin.

The synthesis and release of renin by the juxtaglomerular epithelioid cells located in the media layer of the afferent arteriole are influenced by the hydrostatic pressure within the vessel, as well as by the circulating levels of angiotensin II, and the sodium concentration facing the macula densa (65). Cx43 and Cx40 connect the ECs and the VSMCs that sense the hydrodynamic changes within the afferent arteriole (23, 41, 66) with the renin-producing cells which, by interacting with the cells of the macula densa, sense the circulating angiotensin II and the salt content of the distal tubule (67). Therefore, connexin channels are ideally located to transmit the mechanical, hormonal, and ionic inputs that control renin secretion, providing a mechanism for the integration of distinct cell types into an integrated multicellular unit, whose activity is tightly modulated as a function of the physiological demand. Our data show that Cx43 is critical in this network, possibly because of its mechanosensitive properties (68), and cannot be replaced by Cx32 for integrating the multiple signals that control renin secretion after a chronic decrease in blood pressure within the clipped kidney.

In summary, to our knowledge, our data provide the first evidence that Cx43 specifically regulates the secretion of renin-producing cells within the afferent arteriole of the juxtaglomerular apparatus, and that, in this function, it cannot be replaced by

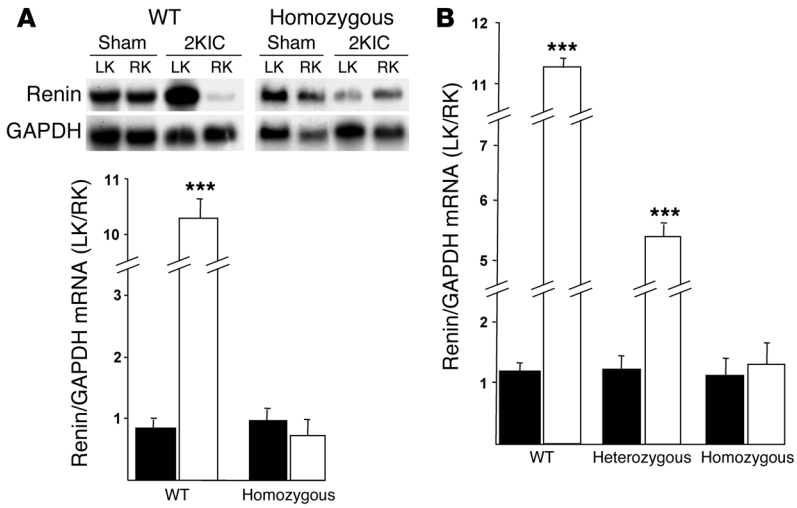

Figure 7
Expression of renin mRNA increased in the clipped kidneys of hypertensive, but not of normotensive, KI32 mice. (**A**) Northern blots revealed that, 4 weeks after surgery, the expression of the renin gene was similar in the left kidney (LK) and right kidney (RK) of WT, sham-operated control mice. In contrast, the renin transcript was markedly increased in the clipped kidney (LK), and decreased in the contralateral kidney (RK), of 2K1C animals. No significant change in the expression of the renin gene was observed in 2K1C homozygous KI32 littermates. As a result of these changes, the ratio of renin mRNA levels between the left (clipped) and the right kidney, as quantified in 4 independent experiments, was increased approximately 10-fold in WT 2K1C mice, and unchanged in homozygous KI32 2K1C littermates. (**B**) Similar changes were evaluated using quantitative RT-PCR. This approach further revealed that the renin expression of heterozygous KI32 2K1C mice was also increased in the clipped kidney, though to a level that was about half that evaluated in WT littermates. $^{***}P < 0.001$.

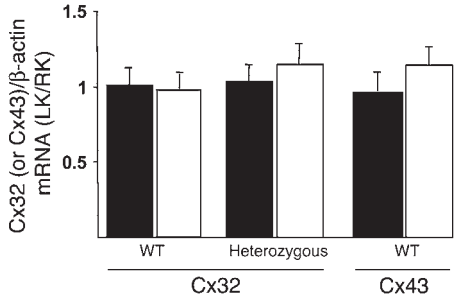

Figure 8
Relative expression of connexin mRNA was unchanged in kidneys of 2K1C mice. Four weeks after surgery, the expression of *Cx32* and *Cx43*, as evaluated by the ratio of the cognate transcripts in the left (clipped) and right kidneys, was unchanged in the 3 mouse genotypes that we compared.

Cx32. They further document that, by impairing renin secretion, the replacement of Cx43 by Cx32 provided for a full protection of the transgenic mice against the enzyme-dependent changes that lead to hypertension in a model of the hypoperfused kidney.

Methods

Transgenic mice and genotype analysis. The development of transgenic mice in which the coding region of the *Cx43* gene is replaced by that of *Cx32* has been previously described (31). $Cx43^{del/+}$ mice, generated by ubiquitous Cre-mediated deletion of the floxed gene elements, carried a *lacZ* gene in place of the *Cx43* coding region (28).

For analysis of KI32 mice, tail tips were digested at 55°C in a buffer containing 400 μg/ml proteinase K. Purified genomic DNA was subsequently analyzed by PCR, using the following primers: Cx43 wild-type allele (381 bp); *Cx43*-3UTR: 5′-CGCCTCATTACTGAGGTTGTTGAG-3′ (sense) and *Cx43*-orf: 5′-CGCAATTACAACAAGCAAGCCAGC-3′ (antisense); Cx43KI32 recombinant allele (564 bp); *Cx43*-3UTR: 5′-CGCCTCATTACTGAGGTTGTTGAG-3′ (sense) and *Cx32*-orf: 5′-GTCTTCACTGTCTTTATGCTCGC-3′ (antisense).

Induction of hypertension. Animals were handled according to institutional guidelines after approval from the State Veterinary Office (Lausanne, Switzerland). Mice were anesthetized by inhalation of 1–2% halothane (Arovet AG) in oxygen. The kidney was exposed through a small flank incision, externalized, and maintained with an ophthalmic forceps. The left renal artery was individualized over a short segment by blunt dissection, and clipped with a U-shaped silver clip of 0.12 mm internal diameter. The kidney was then pushed back into the retroperitoneal cavity. Sham-operated mice were exposed to the same surgical manipulations, except the clipping. After surgery, all mice were kept on a regular diet with free access to water and were housed for 4 weeks at constant temperature and humidity, according to standard light/dark cycles (23, 26, 32). One day before sacrifice, the right carotid artery was exposed through a cervical incision, and isolated by blunt dissection. The vessel was catheterized with a PE-10 tubing (Ethicon) filled with 5% glucose, 300 IU/ml heparin, and a minute amount of 1% Xylocaine. A ligature was tied around the artery to retain the catheter, which was then tunneled subcutaneously to exit at the back of the neck. The skin incision was closed with surgical staples. After 1 day of recovery, the mice were placed in a plastic tube to measure hemodynamic parameters. After a first 1-hour period, during which these parameters reached base-line values, the arterial catheter was connected to a pressure transducer using a computerized data acquisition system (NOTOCORD) that allowed for recording of intra-arterial pressure and heart rate every 20 seconds, for a 15-minute period (32, 44, 45). Once this recording was terminated, 300 μl blood was collected in EDTA-coated tubes and immediately centrifuged at 4°C. Plasma was frozen at –20°C. The animals were then killed by neck dislocation under deep halothane anesthesia, and immediately infused through the left ventricle with 20 ml diethyl pyrocarbonate–supplemented PBS. Hearts and kidneys were then removed, weighed, and rapidly frozen in liquid nitrogen until further processing.

Another series of mice were fed a diet (Indulab AG) containing either 6% NaCl (high-salt group) or 0.3% NaCl (control group), with free access to tap water. After 3 weeks, mean arterial blood pressure was monitored, and the kidneys were then processed as described above.

Plasma renin activity. Plasma renin activity was measured by a microassay based on angiotensin I (Ang I) trapping by an antibody (32, 69). Briefly, 25 μl of plasma was incubated in duplicate for 15 minutes at 37°C in the presence of a fixed amount of a rabbit polyclonal antibody against Ang I. The amounts of Ang I produced during the incubation period were then determined using a sensitive radioimmunoassay with commercially available ^{125}I-angiotensin I (Anawa Trading SA).

RNA isolation, Northern blot analysis, and quantitative RT-PCR. Kidneys and hearts were homogenized in TriPure Isolation Reagent (Roche Diagnostics Corp.), using a Kinematica Polytron blender (Kinematica AG), and total RNA was extracted according to the kit protocol. Fifteen micrograms mRNA was size-fractionated on 1% agarose gels containing 8% formaldehyde (Sigma-Aldrich) and 1× Mops buffer (Sigma-Aldrich). RNAs were transferred overnight to GeneScreen membranes (DuPont) in the presence of 10× SSC. Membranes were UV cross-linked and vacuum-baked for 2 hours at 80°C. After prehybridization, total mRNA levels were determined by hybridization with random-primed (Boehringer Mannheim) cDNA probes, labeled with α^{32}P-dCTP (Amersham Biosciences) and specific for *renin* (32) and the 1.1-kb (HindIII-EcoRI) fragment of *GAPDH* cDNA (23). Hybridizations were performed overnight at 42°C in the presence of 5× saline sodium phosphate EDTA, 50% formamide, 5× Denhardt's solution, 5% SDS, and 100 μg/ml purified salmon sperm DNA. Blots were washed 3 times for 10 minutes at 42°C in 2× SSC containing 1% SDS, and 3 times for 20 minutes in 0.1× SSC containing 1% SDS. Exposure times of all membranes to x-ray film (X-OMAT AR; Kodak) were chosen to optimize the signals, under conditions preventing saturation. To normalize signal levels, each filter was rehybridized with probes for the ubiquitously expressed gene GAPDH.

Quantitative real-time PCR was performed on total RNA treated 30 minutes in the presence of DNase I (DNA-free kit; Ambion Inc.), using a LightCycler Instrument (Roche Diagnostics Corp.) and the QuantiTect SYBR Green PCR kit (QIAGEN). One-microgram aliquots of DNase-treated RNA were reverse-transcribed using ImProm-II Reverse Transcription System (Promega), as described in the kit protocol. The cDNA samples were subjected to serial dilutions in water. Each reaction mixture (20 μl) contained 4 μl cDNA, 10 μl 2× PCR Master Mix (containing HotStarTaq DNA Polymerase, buffer, and deoxynucleotide triphosphates; QIAGEN), and 3 pmol of each primer. The amplification program consisted of 1 cycle of 15 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C, 20 seconds at 50–52°C, and 20 seconds at 72°C. In each run, contamination and specificity of the PCR were checked by inclusion of both a non–reverse-transcribed RNA sample and a sample of 2× PCR Master Mix containing 3 pmol of each primer, respectively. Amplification was followed by melting curve analysis to verify the identity of the amplicon. PCR efficiency was determined by analysis of a dilution series of the RT-PCR reaction, which contained the target gene and served as a reference standard. Analysis of data was performed using version 3.5 of the LightCycler software (Roche Diagnostics Corp.), which generated a best-fit line from the log-linear region of each curve defining the crossing line. The intersection

between the emitted fluorescence and the crossing line defined the crossing point. The concentration of target DNA was calculated by plotting of the crossing point of each sample on the standard curves. cDNAs were amplified using the following primers: mouse *Cx32* (192 bp): 5′-ATACAGGTGTGAATGAGGGAGGATG-3′ (sense) and 5′-TGGAGGGTGTTACAGATGAAAGAGG-3′ (antisense); mouse *GAPDH* (334 bp): 5′-GACTCCACGACATACTCAGC-3′ (sense) and 5′-GTCGGTGTGAACGGATTTGG-3′ (antisense); mouse *Cx43* (255 bp): 5′-GATTGAAGAACACGGCAAGG-3′ (sense) and 5′-AGAGCGAGAGACACCAAGGA-3′ (antisense); mouse *renin* (158 bp): 5′-TCTCTGGGCACTCTTGTTGCTCTG-3′ (sense) and 5′-ATACGTCCCATTCAGCACTGAGCC-3′ (antisense).

Detection of connexins. For light microscopy, kidneys and hearts were excised and cut in fragments that were quickly frozen in 2-methylbutane previously cooled in liquid nitrogen. Tissue fragments were embedded in OCT medium (Miles Inc.) and cryosectioned at about 5 μm thickness. Sections were rinsed in PBS, incubated 30 minutes in a buffer containing 0.5% BSA, and then exposed for 20 hours to polyclonal antibodies against either Cx43 (Zymed Laboratories Inc.; diluted 1:500 in PBS) or Cx32 (Santa Cruz Biotechnology Inc.; diluted 1:200 in PBS). Primary antibodies were detected using secondary antibodies labeled with FITC or rhodamine (Invitrogen Corp.) and directed against rabbit or goat IgG, whichever was applicable. Sections were then rinsed in PBS, stained with Evans blue (only when fluoresceinated secondary antibodies were used), viewed with an Axiophot microscope (Zeiss), and photographed on Kodak T-MAX 400 film or recorded with a digital camera (AxioCam; Zeiss). Double immunofluorescence labeling was performed according to the same protocol, except that heart sections were exposed to a mixture of the anti-Cx43 and the anti-Cx32 antibodies. Controls included (a) double staining of tissues from control C57BL/6 mice, which are known to lack either 1 (liver for Cx43; heart for Cx32) or the 2 of the connexins under study (skeletal muscle), (b) single immunolabeling of 1 connexin species, and (c) incubations of sections with only the secondary, fluorochrome-labeled antibodies. For 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal) staining, sections were processed as previously described (70).

For immuno-electron microscopy, kidney fragments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, washed in 0.1 M phosphate buffer, embedded in 12% gelatine, cooled on ice, infused with 2.3 M sucrose, frozen in liquid nitrogen, and sectioned with an EM FCS cryoultramicrotome (Leica). Ultrathin sections were mounted on Parlodion-coated (Electron Microscopy Sciences) copper grids. The sections were processed as in previously described protocols (71), which, in these experiments, included a 1-hour exposure to the anti-Cx32 antibodies mentioned above (1:100), and a 20-minute exposure to 10-nm protein A–coated gold particles. Cryosections were screened and photographed in a CM10 electron microscope (Philips). Negative controls were run by exposure of the sections to only the protein A–coated gold particles and resulted in a minimal, inconsistent staining of the tissues (not shown).

Detection of renin. For the in situ hybridization of the renin transcript, kidneys were cryostat-sectioned at 12 mm thickness. Sections were fixed 10 minutes with 4% paraformaldehyde in PBS, rinsed in diethyl pyrocarbonate, and hybridized as previously described (23, 72) with a probe corresponding to the same fragment used for Northern blot analysis of *renin* (32). Briefly, hybridization with digoxigenin-labeled antisense riboprobes was carried out for 40 hours at 58°C in 5× SSC and 50% formamide. Sections were washed 30 minutes at room temperature in 2× SSC, 1 hour at 65°C in 2× SSC, and 1 hour at 65°C in 0.1× SSC and then stained for alkaline phosphatase. Stained sections were dehydrated and mounted with Eukitt (Kindler O. Co.). The specificity of hybridization was ascertained under the very same conditions, using a sense renin probe that had the same length, GC content, and specific activity as the corresponding antisense probe.

For the localization of the hormone, kidney sections were immunolabeled as described above for connexins, using mouse monoclonal 2D12 (a generous gift of P. Corvol, Collège de France, Paris, France; diluted 1:200).

Statistics. Densitometric analysis of mRNA signals was performed by scanning autoradiograms with an apparatus (Molecular Dynamics) that integrates areas and corrects for background. Signals of specific transcripts were related to the corresponding GAPDH signals. Data were expressed as mean ± SEM. Mean blood pressure, heart rate, body weight, plasma renin activity, cardiac weight index, kidney index, and relative mRNA levels were compared using ANOVA and Fisher's least significant difference (Fisher's protected LSD) tests. Statistical significance was defined at values of $P < 0.05$, $P < 0.01$, and $P < 0.001$.

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