Increased Expression of Renal Cyclooxygenase-2 and Neuronal Nitric Oxide Synthase in Hypertensive Cx40-Deficient Mice

Nathalie Krattinger^a Florian Alonso^a Alessandro Capponi^b Lucia Mazzolai^a
Pascal Nicod^a Paolo Meda^c Jacques-Antoine Haefliger^a

^aDepartment of Medicine, University Hospital, Lausanne, ^bDepartment of Internal Medicine, University of Geneva, School of Medicine, HUG, and ^cDepartment of Cell Physiology and Metabolism, University of Geneva, School of Medicine, CMU, Geneva, Switzerland

Key Words

Gap junctions · Connexins · Transgenic mice · Kidney · Hypertension · Secretion

Abstract

Cx40-deficient mice (Cx40^{-/-}) are hypertensive due to increased renin secretion. We evaluated the renal expression of neuronal nitric oxide synthase (nNOS) and cyclooxygenases COX-1 and COX-2, three macula densa enzymes. The levels of nNOS were increased in kidneys of Cx40^{-/-} mice, as well as in those of wild-type (WT) mice subjected to the two-kidney one-clip model of hypertension. In contrast, the levels of COX-2 expression were only increased in the hypoperfused kidney of Cx40^{-/-} mice. Treatment with indomethacin lowered blood pressure and renin mRNA in Cx40^{-/-} mice without affecting renin levels, indicating that changes in COX-2 do not cause the altered secretion of renin. Suppression of NOS activity by N^G-nitro-L-arginine methyl ester (L-NAME) decreased renin levels in Cx40^{-/-} animals, indicating that NO regulates renin expression in the absence of Cx40. Treatment with candesartan normalized blood pressure in Cx40^{-/-} mice, and decreased the levels of both COX-2 and nNOS. After a treatment combining candesartan and L-NAME, the blood pressure of Cx40^{-/-} mice was higher than that of WT mice, showing that NO may counterbalance the vasoconstrictor effects of angiotensin II in Cx40^{-/-} mice.

These data document that renal COX-2 and nNOS are differentially regulated due to the elevation of renin-dependent blood pressure in mice lacking Cx40.

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Introduction

Prostaglandins affect the function of macula densa and renin cells in hypertension [1–4]. The renal cyclooxygenases COX-1 and COX-2 are the rate-limiting enzymes in their synthesis [2]. COX-1 is constitutively expressed in smooth muscle of renal vessels and in the collecting ducts of the renal medulla [5, 6], whereas COX-2 is mainly localized in the macula densa, the thick ascending limb of the loop of Henle and the interstitial cells of the medulla [6, 7]. Due to this distribution, COX-2 triggers the formation of prostanoids, which promote the expression and secretion of renin by the cells of the neighboring juxtaglomerular apparatus (JGA) [8]. Accordingly, the expression of COX-2 is increased in the renal cortex under several conditions of increased renin production [5, 9–12], indicating a tight coordination between the expression of the two enzymes. Nitric oxide (NO) is another

N. Krattinger and F. Alonso contributed equally to this work.

er tonic stimulator of renin expression which, under physiological conditions, is counteracted by angiotensin II [13] and contributes to regulating the macula densa [14]. Under a variety of conditions, the expression of neuronal nitric oxide synthase (nNOS) and COX-2 changes in parallel with that of renin even though the expression of the nNOS and COX-2 genes may not be interdependently regulated [15–19].

Recent studies have shown that the signaling due to connexins (Cx) is an important mechanism in the multifactorial coordination of JGA cells and renin secretion [20–28]. The renin-secreting cells (RSCs) of the JGA form gap junction channels made of Cx40 and Cx37 [23, 29, 30]. We and others recently demonstrated that mice lacking Cx40 [31–34] feature excessive renin production and secretion, leading to sustained activation of the renin-angiotensin system [35–38]. Cx40 is ideally located to detect blood-borne signals and to propagate them across renal vessels and the RSCs of the JGA [24], thus permitting the integration of the mechanical, hormonal and ionic inputs that control blood pressure [20, 38].

So far, however, no study has investigated whether and how Cx40-dependent signaling may interact with signaling dependent on prostaglandins and/or NO, which play a role in the *in vivo* control of hypertension. To address this question, we compared the renal expression of COX-1, COX-2 and nNOS in the kidneys of hypertensive Cx40 null mice and their control littermates, and tested the effects of drugs blocking the activity of these key enzymes.

Methods

Knock-Out Mice

Breeding pairs of Cx40^{−/−} mice [31] originally provided by Dr. K. Willecke were crossed with control C57Bl6 partners. Identification of the wild-type (Cx40^{+/+}), and homozygous Cx40^{−/−} mice was performed by PCR of genomic DNA, using the following primers: for the Cx40 WT allele (314 bp), 5′-GGGAGATGAG-CAGGCCGACTTCCGGTGCG-3′ (sense) and 5′-GTAGGGT-GCCCTGGAGGACAATCTTCCC-3′ (antisense); for the Cx40 recombinant allele (494 bp), 5′-GGATCGGCCATTGAACAA-GATGGATTGCAC-3′ (sense) and 5′-CTGATGCTCTTCGTC-CAGATCATCCTGATCG-3′ (antisense). Three- to four-month-old mice, from litters obtained after at least 10 backcrosses into the C57/Bl6 background, were used in all the experiments [31, 37].

Animal Experiments

Mouse care, surgery and euthanasia procedures were approved by our institutional committee for animal experiments and State of Vaud Veterinary Office. Groups of animals were submitted to

one of the following experiments: (1) hypertension was induced for 4 weeks by the two-kidney, one-clip (2K1C) procedure [30, 37]; (2) the renin-angiotensin system was blocked by adding 3 mg/kg·day of the AT₁-receptor antagonist candesartan (Cilexetil®; AstraZeneca) to the drinking water for 2 weeks [37]; (3) the function of the macula densa was modulated by feeding a diet (Kliba, Switzerland) containing either 0.03% (low-salt group), 4% (high-salt group) or 0.3% NaCl (control group) for 3 weeks [11]. All mice had free access to tap water. (4) To inhibit prostaglandins, mice were injected intraperitoneally with 3 mg/kg·day the nonselective COX inhibitor indomethacin (Indocin®; Merck) for 3 successive days. Control mice were similarly injected with 0.9% NaCl. Animals were studied at the end of the 3-day period. (5) To inhibit NO, the mice received 80 mg/kg·day L-NAME (Sigma Chemical Co.) in the drinking water for 3 weeks [39]. The L-NAME solution was made fresh every 3 days; (6) L-NAME was also added in drinking water in combination with candesartan. In this case, 2 weeks after treatment with 3 mg/kg·day candesartan, when the blood pressure of Cx40^{−/−} mice had returned to control values, 80 mg/kg·day L-NAME were added to the candesartan solution for another 2 weeks [40].

Mean blood pressure was measured by an invasive method [30, 37]. One day before sacrifice, a catheter connected to a pressure transducer was inserted into the right carotid artery of anesthetized mice. The next day, a computerized data acquisition system was used to record the heart rate every 20 s as well as the diastolic and systolic intra-arterial pressures of the conscious mice [30, 41]. After a 30-min recording period, 300 µl blood was collected in EDTA-coated tubes and centrifuged at 4°C. Plasma was stored at −70°C until use. In some experiments, mean blood pressure and pulse rate were also monitored in mice pretrained for 7 days, by a noninvasive computerized tail cuff method (BP-2000; Visitech Systems). Data are shown as the mean of the diastolic and systolic values.

Measurements of Plasma Renin Activity

Plasma renin activity was measured by a radioimmunoassay based on angiotensin I trapping by an antibody [30, 37].

RNA Analysis

Kidneys were homogenized in Tripure Isolation Reagent (Roche, Switzerland), using a Kinametic polytron blender (Kinametica, Switzerland). RNA isolation was performed as described [30]. Quantitative real-time PCR was performed on total RNA treated for 30 min in the presence of DNase I (DNA-free kit; Ambion, Cambridge, UK), using a LightCycler Instrument (Roche Diagnostics GmbH, Mannheim, Germany) and the QuantiTect SYBR Green PCR Kit (Qiagen, Basel, Switzerland) [30]. Negative controls included amplification of distilled water, and RNA samples that had not been reverse transcribed. Analysis of data was performed using the 3.5 version of the LightCycler software (Roche Diagnostics GmbH). cDNAs were amplified using the following mouse primers: for COX-2 (239 bp): 5′-ACAACAACCTC-CATCCTCCTG-3′ (sense) and 5′-AGCCATTTCCTTCTCTCC-TG-3′ (antisense); for COX-1 (209 bp): 5′-GTGGCTATTTCC-TGCAGTC-3′ (sense) and 5′-CAGTGCCTCAACCCCATAGT-3′ (antisense); for renin (158 bp): 5′-TCTCTGGGCACTCTTGT-TGCTCTG-3′ (sense) and 5′-ATACGTCCCATTGAGCACTGA-GCC-3′ (antisense); for nNOS (222 bp): 5′-CAACAGC-GTCTCCTCCTATTCT-3′ (sense) and 5′-TCATCTCCCTCCCT-

CATCTT-3' (antisense); for GAPDH (334 pb): 5'-GACTCCAC-GACATACTCAGC-3' (sense) and 5'-GTCGGTGTGAACGGA-TTTGG-3' (antisense).

Immunodetection of COX and nNOS

Kidney fragments were frozen in 2-methylbutane previously cooled in liquid nitrogen. Cryostat sections were immunostained [30] using polyclonal antibodies against either COX-2 (160126; Cayman Chemical Co.), or nNOS (610310; BD Transduction Laboratories) both diluted 1:100. Binding sites of COX-2 and nNOS antibodies were visualized using an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories, Inc., Burlingame, Calif., USA), diluted 1:50 [42]. After rinsing in PBS, the product of the peroxidase reaction was visualized using 0.1% 3,3'-diaminobenzidine tetrahydrochloride dehydrate, dissolved in PBS containing 0.3% H₂O₂. Reactions were stopped after 10–15 min to maximize signal intensity.

Western Blotting

Four mice per group were sacrificed and immediately infused with 15 ml PBS. The kidneys were excised, rapidly placed into liquid nitrogen, reduced to powder, and stored at –80°C. Aliquots of 50 mg kidney powder were homogenized by sonication in 1 ml Passive Lysis Buffer (Promega). Total protein content was determined by the DC protein assay reagent kit (Bio-Rad Laboratories). Protein aliquots (30 µg) were fractionated by electrophoresis in a 12.5% polyacrylamide gel, and transferred onto Immobilon-P membranes (Millipore Co.) at 75 V. The following primary antibodies were used: rabbit polyclonal antibodies against COX-2 (160126; Cayman Chemical Co.) diluted 1:100, rabbit polyclonal antibodies against nNOS (160870; Cayman Chemical Co.) diluted 1:100; mouse monoclonal antibodies against α-tubulin (T5168; Sigma) diluted 1:2,000. The secondary antibodies were sheep anti-rabbit immunoglobulin coupled to alkaline phosphatase (Bio-Rad Laboratories) for COX and nNOS detection, and rabbit anti-mouse immunoglobulin coupled to alkaline phosphatase (Bio-Rad Laboratories) for tubulin detection. Bands were developed using enhanced chemiluminescence (Amersham Biosciences). The densitometric levels of immunolabeled proteins were normalized relative to those of tubulin [31, 37].

Statistical Analysis

Results are presented as mean ± SEM. Statistical comparisons between groups were performed by a one- or two-way analysis of variance, followed by a Newman-Keuls test, whichever was appropriate. Statistical significance was defined at a value of $p < 0.05$ or less.

Results

Cx40–/– Mice Are Hypertensive due to Activation of the Renin-Angiotensin System

Cx40–/– mice featured a 40% increase ($p < 0.001$) in blood pressure (table 1), and a 2-fold increase ($p < 0.05$) in kidney expression of renin mRNA (table 2) which resulted in a 20-fold elevation ($p < 0.001$) of the circulating

Table 1. Characteristics of mice and plasma renin activity levels at the end of the experiment

Mice/Groups	n	Body weight, g	MBP mm Hg	PRA ng ANG I/ml·h
WT				
Normal salt	10	28.5 ± 1.7	103 ± 3	2.00 ± 0.80
Candesartan	10	30.2 ± 1.0	107 ± 3	36.0 ± 8.00***
Sham	9	30.5 ± 0.6	104 ± 4	1.80 ± 0.90
2K1C	9	28.8 ± 0.8	142 ± 6**	14.0 ± 4.00***
Low salt	9	25.4 ± 0.9	105 ± 5	8.00 ± 1.00*
High salt	9	26.0 ± 0.9	104 ± 4	0.20 ± 0.02*
Indomethacin	6	28.2 ± 1.0	98 ± 4	5.00 ± 1.55
L-NAME	6	27.5 ± 0.9	133 ± 2**	3.20 ± 0.90
Cx40–/–				
Normal salt	10	25.9 ± 1.0	154 ± 3 ⁺⁺	46.0 ± 8.00 ⁺⁺
Candesartan	12	31.6 ± 2.5	117 ± 11**	43.5 ± 4.00
Sham	11	26.3 ± 1.6	150 ± 3	52.0 ± 13.0 ⁺⁺
2K1C	11	28.2 ± 0.6	158 ± 5	51.0 ± 10.0 ⁺⁺
Low salt	10	26.0 ± 2.8	151 ± 11	60.0 ± 13.0 ⁺⁺
High salt	11	27.2 ± 1.3	154 ± 4	31.0 ± 8.00* ⁺⁺
Indomethacin	6	30.2 ± 1.2	137 ± 4 ^{*,+}	42.2 ± 9.00 ⁺⁺
L-NAME	5	25.8 ± 1.2	167 ± 5**	34.2 ± 8.00 ^{*,++}

n = Number of mice; sham = sham-operated mice; MBP = mean blood pressure; PRA = plasma renin activity. Values are expressed as mean ± SEM.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus WT or Cx40–/– untreated or sham-operated animals; ⁺ $p < 0.001$, ⁺⁺ $p < 0.001$, versus the respective WT animals.

levels of the enzyme (table 1). Treatment with candesartan reduced ($p < 0.001$) the blood pressure of Cx40–/– mice to the levels observed in control littermates (table 1), without altering the levels of plasma renin (table 1) and renin mRNA (table 2). In contrast, the same treatment markedly increased ($p < 0.001$) the renin levels of control mice (table 2).

Renin Secretion Is Dysregulated in Cx40–/– Mice

To evaluate the functioning of RSCs, mice were submitted to 2K1C surgery [30, 37]. Four weeks later, blood pressure was increased ($p < 0.01$) in WT mice, which showed elevated levels ($p < 0.001$) of circulating renin as a result of increased ($p < 0.05$) expression of the enzyme in the clipped kidney (table 1). Under the same conditions, the blood pressure and circulating levels of renin were unchanged in Cx40–/– mice (table 1), which also showed unchanged levels of renin mRNA (table 2).

Table 2. Renin mRNA levels at the end of the experiment

Mice/Groups	n	Renin mRNA levels	
		LK	RK
WT			
Normal salt	10	1.01 ± 0.05	0.31 ± 0.08**
Candesartan	10	2.26 ± 0.18*	
Sham	9	1.15 ± 0.05	
2K1C	9	1.73 ± 0.19*	
Low salt	9	1.60 ± 0.13*	
High salt	9	0.80 ± 0.07*	
L-NAME	10	0.48 ± 0.04**	
Candesartan + L-NAME	10	1.10 ± 0.05	
Cx40-/-			
Normal salt	10	1.83 ± 0.10 ⁺	0.79 ± 0.17*, ⁺
Candesartan	12	2.01 ± 0.22	
Sham	11	1.68 ± 0.11 ⁺	
2K1C	11	1.99 ± 0.38*, ⁺	
Low salt	10	3.47 ± 0.15*, ⁺⁺	
High salt	11	1.42 ± 0.11*, ⁺	
L-NAME	5	0.72 ± 0.04*, ⁺⁺	
Candesartan + L-NAME	11	1.41 ± 0.09 ⁺	

n = Number of mice; sham = sham-operated mice. Values are expressed as mean ± SEM.

* p < 0.05, ** p < 0.01, versus WT or Cx40^{-/-} untreated or sham-operated animals; ⁺ p < 0.05, ⁺⁺ p < 0.01, versus the respective WT animals.

Expression of COX-2 but Not COX-1 Is Altered in Cx40^{-/-}

The levels of COX-1 mRNA were similar in Cx40^{-/-} and control littermates, and were not modified by the 2K1C procedure or after treatment with candesartan (fig. 1A). In contrast, the transcript levels of COX-2 were markedly (400%) higher (p < 0.001) in Cx40^{-/-} than in WT littermates (fig. 1B). During 2K1C-induced hypertension, COX-2 mRNA levels increased in the clipped kidney, but not in the contralateral kidney of WT mice. In contrast, COX-2 mRNA levels were not modified in the clipped kidney of Cx40^{-/-} mice, and were reduced to control levels in the contralateral kidney (fig. 1B). Treatment with candesartan slightly increased (p < 0.05) COX-2 mRNA levels in WT mice, but decreased (p < 0.01) these levels by 50% in the kidneys of Cx40^{-/-} animals (fig. 1B). Immunolabeling revealed that cells of the macula densa were more intensively stained for COX-2 in Cx40^{-/-} than WT mice (fig. 1C). Western blot analysis confirmed that the former animals had 4 times more renal COX-2 than WT mice (fig. 1D). Under the conditions we tested, the

levels of this protein changed (fig. 1D) in parallel with those of its transcript (fig. 1B).

After 3 weeks of salt restriction (0.03% NaCl) or salt intake (4% NaCl), blood pressure was unchanged both in WT and Cx40^{-/-} mice (table 1). The levels of renin circulating in the latter animals were significantly (p < 0.001) reduced by the high-salt diet (as in controls), but were still 15-fold higher than those measured in WT mice fed a normal salt diet (table 1). The decrease in plasma renin was paralleled by a downregulation of the renin transcript both in controls and in Cx40^{-/-} littermates (table 2). Under these conditions, no change in COX-1 expression was observed in either type of mouse (fig. 2A). In contrast, COX-2 expression changed with that of renin in WT mice, i.e. it was increased (p < 0.05) in the mice fed a low-salt diet and decreased (p < 0.05) in those receiving a high-salt diet. These changes were not seen in Cx40^{-/-} mice (fig. 2B).

Blockade of Prostaglandin Production Reduces the Blood Pressure of Cx40^{-/-} Mice

In view of the parallel increase in COX-2 and renin expression in Cx40^{-/-} mice, we investigated the role of prostaglandins in renin production. For this purpose, mice were treated with the nonselective COX inhibitor indomethacin. The drug slightly lowered (p < 0.01) the blood pressure of Cx40^{-/-} mice but not of WT mice (fig. 3A). Exposure to indomethacin did not affect renin expression in control animals, but markedly reduced it to control levels in Cx40^{-/-} mice (fig. 3B). However, no parallel decrease in plasma renin levels was observed (table 1). Indomethacin also increased COX-2 expression (p < 0.001) and COX-1 (p < 0.05) levels both in WT and their Cx40^{-/-} littermates (fig. 3C, D).

nNOS Expression Is Increased in Cx40^{-/-} Mice

In the kidneys of Cx40^{-/-} mice, the levels of nNOS were about 2-fold higher (p < 0.01) than in those of their WT littermates, whether evaluated at the transcript (fig. 4A) or protein level (fig. 4B). Four weeks after 2K1C surgery, nNOS mRNA levels were increased about 2-fold both in the hypoperfused and the contralateral kidney of WT animals (fig. 4A), a change which was significantly smaller (p < 0.01) than that seen in Cx40^{-/-} mice. Candesartan did not alter nNOS expression in WT mice, but decreased that in Cx40^{-/-} mice to control levels (fig. 4A). In both Cx40^{-/-} and WT mice, immunolabeling revealed nNOS in macula densa cells; immunolabeling intensity was somewhat higher in Cx40^{-/-} than in WT mice (fig. 4C).

Fig. 1. COX-2 expression is increased in the kidneys of Cx40^{-/-} mice. LK = Left kidney; RK = right kidney. **A** WT (open bars) and Cx40^{-/-} mice (solid bars) expressed similar COX-1 levels under all the conditions tested. **B** In contrast, COX-2 expression was higher in Cx40^{-/-} than in their WT littermates, and was differentially regulated in the two types of mice. **C** The intensity of COX-2 immunostaining in the macula densa (md) was higher in

Cx40^{-/-} than WT mice. gl = Glomerulus. Bar: 150 μm. **D** Western blot showed that the levels of COX-2 protein were 4-fold higher in Cx40^{-/-} than in WT mice, and changed under different experimental conditions as the cognate transcript. Data represent mean ± SEM values of the number of mice specified in table 1. ** p < 0.01, *** p < 0.001, compared to WT; ° p < 0.05, °° p < 0.01, °°° p < 0.001, compared to controls.

Fig. 2. Salt diets reveal an abnormal regulation of COX-2 expression in Cx40^{-/-} mice. **A** Different salt loads did not change COX-1 expression in either WT (open bars) or Cx40^{-/-} mice (solid bars). **B** In contrast, a low-salt diet (LS) increased COX-2 expression in WT mice, whereas a high-salt diet (HS) decreased this expression. These alterations were not observed in Cx40^{-/-} animals. Data are mean $\pm$ SEM values of the number of mice specified in table 1. ** $p < 0.01$, *** $p < 0.001$, compared to WT; ° $p < 0.05$ compared to control.

Fig. 3. Inhibition of COXs by indomethacin reduced the blood pressure of Cx40^{-/-} mice. **A** Indomethacin, a nonselective inhibitor of COXs, reduced blood pressure in Cx40^{-/-} mice (n = 6; solid bars) but not in control WT mice (n = 6; open bars). **B** After the indomethacin treatment, the renin mRNA levels of Cx40^{-/-} mice had decreased to the values observed in WT mice. These alterations were not observed in WT mice. **C, D** COX-1 and COX-2 mRNA levels were increased both in WT and Cx40^{-/-} mice after treatment with indomethacin. ** $p < 0.01$, *** $p < 0.001$, compared to WT; ° $p < 0.05$, °° $p < 0.01$, °°° $p < 0.001$, compared to controls.

Fig. 4. Cx40^{-/-} mice feature increased expression of nNOS and abnormal regulation of this enzyme. **A, B** Renal nNOS mRNA (**A**) and protein (**B**) levels were higher in Cx40^{-/-} mice (n = 10; solid bars) than in their WT littermates (n = 10; open bars). Treatment with candesartan decreased nNOS mRNA levels in the kidneys of Cx40^{-/-} mice but not in those of WT controls. In both mouse genotypes, nNOS mRNA levels were increased during 2K1C-induced hypertension. Data are mean $\pm$ SEM values of the number of mice specified in table 2. * p < 0.05, ** p < 0.01, compared to WT; ^{oo} p < 0.01 compared to control. **C** nNOS immunolabeling showed nNOS expression in macula densa (md) cells. The intensity of the labeling appeared slightly higher in the kidneys of Cx40^{-/-} mice than in those of WT controls. gl = Glomerulus. Bar: 150 μ m.

Blockade of NOS Production Increases Hypertension in Cx40^{-/-} Mice

Three weeks after administration of L-NAME, blood pressure increased (p < 0.001) both in WT and Cx40^{-/-} mice (fig. 5A) in parallel with a decrease in renin mRNA (table 2), COX-2 (fig. 5B), and nNOS mRNA levels (fig. 5C). Plasma renin levels (table 1) were only affected in Cx40^{-/-} mice. To assess the contribution of NO, we associated candesartan and L-NAME. To this end, when blood pressure of Cx40^{-/-} mice had returned to control values after a 2-week treatment with candesartan, L-NAME was added to the candesartan solution for another 2 weeks. This association increased the blood pressure

of WT mice and Cx40^{-/-} mice (p < 0.01) over the values observed after treatment with candesartan alone (fig. 5A). However, this increased blood pressure was lower (p < 0.05) than that observed in untreated Cx40^{-/-} animals (fig. 5A). Compared to candesartan alone, the candesartan plus L-NAME treatment decreased renin mRNA to the levels observed in controls (table 2); it did not change the expression of renin and nNOS, and marginally decreased (p < 0.05) COX-2 expression in WT mice (fig. 5B, C). In contrast, it decreased renin (p < 0.05) and COX-2 (p < 0.01), but not nNOS mRNA levels in Cx40^{-/-} mice (fig. 5B, C; table 2).

Fig. 5. Inhibition of NOS increased hypertension in Cx40^{-/-} mice. **A** Treatment with L-NAME increased blood pressure in both WT (n = 10; open bars) and Cx40^{-/-} mice (n = 10; solid bars). This change was milder in both types of mice when L-NAME was associated with candesartan. **B** Both L-NAME and candesartan/L-NAME treatments reduced the expression of COX-2 in WT and Cx40^{-/-} mice. **C** nNOS expression was decreased in WT and Cx40^{-/-} mice after the L-NAME treatment, but not when the drug was associated with candesartan. Data are mean $\pm$ SEM values. ** p < 0.01, *** p < 0.001, compared to respective WT; ° p < 0.05, °° p < 0.01, °°° p < 0.001, compared to respective control; §§ p < 0.01 compared to treated animals.

Discussion

Recent studies have demonstrated that the hypertension of Cx40^{-/-} mice is mostly due to increased numbers and function of the RSCs, which activate the renin-angiotensin system [36, 37]. In the absence of Cx40, the regulation of this system is altered both under a low-salt regimen and under conditions of changed renal blood flow, two situations sensed by different cell types of the JGA [21, 22]. In the present study, we investigated the mechanism whereby loss of Cx40 leads to excessive renin secretion

by characterizing the involvement of the COXs and nNOS, the key enzymes of the macula densa, which are known to participate in the control of RSCs [13].

We first demonstrate that COX-2 is increased in Cx40^{-/-} mice, up to the levels observed in the hypoperfused kidney of 2K1C hypertensive mice. These findings indicate that prostaglandins play an adaptive role in the vascular response to hypertension in Cx40^{-/-} mice, and stress the parallel regulation of the renin and COX-2 genes, which accounts for the regulation of RSCs by the macula densa cells [3, 10, 43]. Since Cx40^{-/-} mice have

lost the normal downregulating control of renin secretion in the presence of increased blood pressure [36] due to a still undetermined mechanism, we investigated the participation of prostaglandins, under the conditions of a pharmacologic blockade of COX activity. Inhibition of COX-2 by indomethacin did not change the levels of renin mRNAs in WT animals, suggesting that prostaglandins do not contribute to the basal regulation of the renin system. In contrast, the renin mRNA levels, which were about two-fold higher in the kidneys of untreated Cx40^{-/-} mice, were normalized after indomethacin treatment. These observations are comparable to those of a recent report documenting that treatment of Cx40^{-/-} mice with the COX-2 inhibitors meclofenamate and SC-58236 reduces renin expression to the control levels measured in untreated WT mice [44]. This study further showed that the COX-2 inhibitors markedly reduce the levels of the renin protein in the kidneys of Cx40^{-/-} but not WT mice [44]. Our measurements together with those of Wagner [44] show that indomethacin does not change the levels of circulating renin in either WT or Cx40^{-/-} mice, consistent with the finding that COX-2 is particularly relevant for the ectopic expression of renin in periglomerular regions of Cx40^{-/-} mice [35], and not for renin secretion into the systemic circulation [44].

Treatment with candesartan increased the renal expression of COX-2 in WT mice, but decreased it in their Cx40^{-/-} littermates, suggesting that the activation of macula densa cells by angiotensin II triggers the production of COX-derived prostanoids. In turn, prostanoids are expected to elicit a systemic vasodilatation, which presumably compensates the large increase in blood pressure resulting from excessive RSC function [1]. Strikingly, different salt diets, that affect renin and COX-2 expression in parallel in normal mice [5, 18], did not modify COX-2 expression in Cx40^{-/-} mice, demonstrating that these animals do not properly regulate the expression of this cyclooxygenase in response to high angiotensin levels.

The renin-angiotensin system also contributes to the regulation of NOSs [45, 46]. We have found that hypertensive mice featuring a selective increase in renin gene expression (table 2) also featured a significant increase in renal nNOS expression (fig. 4A). This change was observed in WT and Cx40^{-/-} mice, and both in the clipped kidney, which was exposed to decreased blood pressure, and the contralateral kidney, which was subjected to higher systemic blood pressure. The upregulation in the nNOS gene in both kidneys of the 2K1C mice suggests that circulating angiotensin II plays a role in local renal

nNOS gene expression, and that the elevated blood pressure per se is not the primary factor responsible for the control of renal nNOS expression.

In the kidneys of Cx40^{-/-} mice, we found an increase in the expression of the nNOS isoform, which colocalizes with COX-2 in macula densa cells, as reported in WT animals [14, 47]. Pharmacological inhibition of NOS activity demonstrated that NO acts as a tonic stimulator of both renin and COX-2 expression in the kidney of both normal [13, 14] and Cx40^{-/-} mice. Thus, after treatment with L-NAME, Cx40^{-/-} mice showed a markedly reduced renin mRNA and a milder decrease in circulating renin levels. The data imply that nNOS stimulates renin expression in Cx40^{-/-} and that the NO generated by nNOS or systemic eNOS activity [48] counteracts the excess of circulating angiotensin II in Cx40^{-/-} mice to prevent a further elevation of arterial pressure. The increased NO levels observed in Cx40 null mice therefore appear to participate in the adaptations of renal vessel to hypertension as observed in other models [49–51].

This conclusion fits with the antagonistic effects of angiotensin II and NO in the JGA, [52, 53], as well as with the crosstalk between these two regulators. Thus, it has been shown that angiotensin II induces the release of NO, which antagonized the contractile effect of angiotensin II on the afferent arteriole at the level of the JGA and its sensitizing effect on the tubuloglomerular-feedback mechanism [54]. This complex feedback is mediated by AT1 receptors on the angiotensin side and by cGMP levels on the NO side [55, 56]. As a result, local NO production in response to an increased perfusion pressure inhibits renin release.

In summary, our findings demonstrate that the renal levels of COX-2 and nNOS are increased in mice lacking Cx40. Conversely, the reduction of prostaglandin synthesis by indomethacin was associated with a significant decrease in the blood pressure of Cx40^{-/-} mice which was paralleled by a decrease in the renal expression of renin but was not associated with a change in the circulating levels of the enzyme. These findings show that prostaglandins play an adaptive role in the response to hypertension in Cx40^{-/-} mice, but are probably not involved in the mediation of the MD signaling that modulates renin secretion. In contrast, changes in nNOS levels may participate with those of eNOS to counteract the effects of the large excess of circulating angiotensin II in Cx40^{-/-} animals as a result of abnormal renin production.

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