Increased expression of cyclooxygenase 2 contributes to aberrant renin production in connexin 40-deficient kidneys

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Wagner C, de Wit C, Gerl M, Kurtz A, Höcherl K. Increased expression of cyclooxygenase 2 contributes to aberrant renin production in connexin 40-deficient kidneys. Am J Physiol Regul Integr Comp Physiol 293: R1781–R1786, 2007. First published September 12, 2007; doi:10.1152/ajpregu.00439.2007.—We previously found that deletion of connexin 40 (Cx40) causes a misdirection of renin-expressing cells from the media layer of afferent arterioles to the perivascular tissue, extraglomerular mesangium, and periglomerular and peritubular interstitium. The mechanisms underlying this aberrant renin expression are unknown. Here, we questioned the relevance of cyclooxygenase-2 (COX-2) activity for aberrant renin expression in Cx40-deficient kidneys. We found that COX-2 mRNA levels were increased three-fold in the renal cortex of Cx40-deficient kidneys relative to wild-type (wt) kidneys. In wt kidneys, COX-2 immunoreactivity was minimally detected in the juxtaglomerular region, but renin expression was frequently associated with COX-2 immunoreactivity in Cx40-deficient kidneys. Treatment with COX-2 inhibitors for 1 wk lowered renin mRNA levels in wt kidneys by about 40%. In Cx40-deficient kidneys, basal renin mRNA levels were increased two-fold relative to wt kidneys, and these elevated mRNA levels were reduced to levels of untreated wt mice by COX-2 inhibitors. In parallel, renin immunoreactive areas were clearly reduced by COX-2 inhibitors such that renin expression vanished and decreased significantly in the periglomerular and peritubular extensions. Notably, COX-2 inhibitor treatment lowered plasma renin concentration (PRC) in wt kidneys by about 40% but did not affect the highly elevated PRC levels in Cx40-deficient mice. These findings suggest that aberrant renin-producing cells in Cx40-deficient kidneys express significant amounts of COX-2, which contribute to renin expression in these cells, in particular, those in the periglomerular and peritubular position. Apparently, these disseminated cells do not contribute to the enhanced renin secretion rates of Cx40-deficient kidneys.

gap junctions; juxtaglomerular cell; prostaglandins

The juxtaglomerular apparatus (JGA) plays an important role in regulating renal function by controlling glomerular blood flow and glomerular filtration, but also for the systemic control of the circulation by renin release from the juxtaglomerular epithelioid cells of the afferent arterioles (22). Numerous gap junctions between the different cells of the JGA suggest an important role of intercellular communication in JGA function (19, 24, 25). In particular, the renin-producing juxtaglomerular cells appear to form gap junctions, not only among each other, but also with neighboring cells, such as endothelial, smooth muscle, and extraglomerular mesangial cells. Gap junctions comprise connexin proteins (23), and in recent years, converging evidence suggests that connexin 40 (Cx40) is the main connexin of renin-producing cells (1, 6, 11, 28). The availability of Cx40-deficient mice (15) has provided initial evidence for the role of Cx40 and gap junctions formed by Cx40 in the general function of renin-producing cells. Cx40-deficient mice have a massive renin phenotype, including the striking dislocation of renin-producing cells (16), elevated renin mRNA levels and significantly elevated plasma renin concentrations, which give rise to severe hypertension (26).

It appears that the physiologically important negative feedback control of renin secretion exerted by blood pressure and ANG II is defective in the absence of Cx40 (26). The reasons for this defect are unknown; they may be due to an interruption of intercellular communication or the mislocation of renin-producing cells.

This mislocation of renin-producing cells results from a shift of renin-producing cells from their normal position in the media layer of afferent arterioles into the glomerular capillary network, perivascular space, extraglomerular region, and the periglomerular and peritubular interstitial tissue (16). Although these aberrant renin-producing cells form numerous secretory granules, the morphology of the cells is quite different (16). Although normal juxtaglomerular cells display a more cubic epithelioid appearance, the aberrant renin-producing cells in Cx40-deficient kidneys appear to be mesenchymal or fibroblast-like cells with processes. These cells can surround glomeruli or tubules in the vicinity of a glomerulus. The nature of these cells is yet unclear as are the mechanisms that trigger their strong expression of renin.

In recent years, evidence has accumulated that indicates two main factors essentially determine renin expression levels in the kidney. One is the sympathetic nervous system, acting through catecholamines via β-adrenergic receptors (10, 13) and the other is the activity of cyclooxygenase 2 (COX-2), which acts as an important permissive factor for renin expression in the kidney (3, 7, 14, 18). This effect of COX-2 is mediated by prostanoids that stimulate renin gene transcription through the cyclic AMP signaling pathway (12). Although COX-2 expression in the JGA of mouse kidneys is weak relative to other species (27), one can assume that the afferent

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arterioles and the macula densa cells of the JGA are capable of expressing COX-2 and producing prostanoids. Because factors determining renin expression levels in aberrant renin-producing cells of Cx40-deficient mice are unknown, we were interested in learning about the role of COX-2 activity in the maintenance of renin expression in Cx40-deficient kidneys. For this purpose, we studied the effects of COX inhibitors on renin expression and secretion from Cx40-deficient kidneys.

MATERIALS AND METHODS

Animal Experiments

All animal experiments were conducted according to the National Institutes of Health guidelines for the care and use of animals in research and were approved by the local ethics committee. All experiments were conducted in 12- to 20-week-old Cx40-deficient mice and age-matched wild-type (wt) controls. Strain background (C57BL/6) of Cx40-deficient and wt animals was considered similar after Cx40-deficient mice were backcrossed eight times into a C57BL/6 background.

Male mice (n = 6 per group) were treated with meclofenamate (10 mg/kg/day) or SC-58236 (5 mg/kg/day) in their drinking water for 7 days. SC-58236 was dissolved in vehicle (final concentration: 0.01% Tween 20 and 0.2% PEG-200). All other animals received equal amounts of vehicle in their drinking water. Meclofenamate and SC-58236 were purchased from Calbiochem (Schwalbach/Ts., Germany).

Immunohistochemistry for Renin and COX-2

After death, kidneys were fixed in methyl-Carnoy solution (60% methanol, 30% chloroform, and 10% glacial acetic acid), as described previously (16). Immunolabeling was performed on 5-μm paraffin sections. After blocking with 3% H2O2 in methanol for 20 min and 10% horse serum and 1% BSA in PBS for 0.5 h at room temperature, sections were incubated with chicken anti-renin IgG (diluted 1:200, Davids Biotechnologie, Regensburg, Germany) or anti-COX-2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C followed by several washing steps and blocking with phenylhydrazine. The sections were incubated with Cy2-conjugated donkey anti-chicken IgG and rhodamine-conjugated donkey anti-mouse IgG fluorescent antibodies (Dianova, Hamburg, Germany) for 2 h and mounted with glycergel (DakoCytomation, Glostrup, Denmark).

Quantification of renin immunoreactive areas was done using the freely available software ImageJ. (W. Rasband, National Institutes of Health, Bethesda, MD). For analysis of the total renin-immunoreactive area per kidney section was related to the number of glomeruli visible on the sections.

Real-time PCR Analysis of COX-1, COX-2, and Renin mRNA

Total RNA in the cortical samples was reverse transcribed into cDNA according to standard protocols as described previously (4, 18). Real-time PCR was performed in a LightCycler (Roche, Mannheim, Germany). All PCR experiments were performed using the LightCycler DNA Master SYBR Green I kit provided by Roche Molecular Biochemicals (Mannheim, Germany). Each reaction (20 μl) contained 2 μl cDNA, 3.0 or 5.0 mM MgCl2, 1 pmol of each primer (COX-1 sense: tttgtgaagggggttc; antisense: gtcgacaacgctgtatg; COX-2 sense: tccagtgacagatgc; antisense: caatcttcacctgg; renin sense: atgagggggtgtctggtggtg; antisense: atgctggggaggtcggactcg; β-actin sense: ccgcttggcagcaggtt; antisense: ggctgggtgggacttgctt), and 2 μl of Fast Starter Mix (containing buffer, dNTPs, SYBR Green dye, and Taq polymerase). The amplification program consisted of 1 cycle of 95°C with 10 min hold (“hot start”) followed by 40 cycles of 15 s at 95°C, 5 s at 60°C, and 20 s at 72°C. Amplification was followed by melting curve analysis to verify the correctness of the amplicon. A negative control with water instead of cDNA was run with every PCR to assess the reaction specificity. To verify the accuracy of the amplification PCR products, we further analyzed the products on an ethidium bromide-stained 2% agarose gel. Data analysis was performed using LightCycler software version 3.5.3. Standard curves were generated using cDNA of the mouse cortex as a template, which was diluted 1:5, 1:10, 1:50, 1:100, and 1:1,000. For each sample, the ratio of the amount of mRNA to β-actin mRNA was calculated.

Determination of Plasma Renin Concentration

To determine the plasma renin concentration, blood samples taken from the tail vein were centrifuged, and the plasma was incubated for 1.5 h at 37°C with plasma from bilaterally nephrectomized male rats as a renin substrate. ANG I (ng/ml × h) was analyzed by radioimmunoassay (Byk & DiaSorin Diagnostics, Dietzenbach, Germany).

If intended, to convert plasma prorenin into active renin, plasma was frozen and thawed three times.

Statistical Analysis

Values are provided as means ± SE. Differences between groups were analyzed by Kruskal-Wallis test. A P value less than 0.05 was considered statistically significant.

RESULTS

As a first measure of the expression of COX-2 in wt and in Cx40-deficient kidneys, we determined the abundance of COX-2 mRNA in the kidney cortex. COX-2 mRNA abundance
was 2.8-fold higher in the cortex of Cx40-deficient kidneys relative to wt kidneys (Fig. 1, top). For comparison, COX-1 mRNA abundance was not different between the two genotypes (Fig. 1, bottom). Coimmunostaining for renin and COX-2 revealed the typical localization of renin in the terminal segments of afferent arterioles in wt kidneys (Fig. 2A); however, faint visible signals for COX-2 in the vicinity of renin expression (Fig. 2C) were observed. In Cx40-deficient kidneys, the tissue pattern of renin expression was strongly altered. The fibroblast-like cells outside the vessel walls strongly expressed renin and often formed impressive plaques of cells that extended into the periglomerular and peritubular interstitium (Fig. 2B). Frequently, but not regularly, COX-2 immunoreactivity became visible in these aberrant renin-expressing cells (Fig. 2D).

To obtain information about the relevance of COX-2 activity for renin expression, wt and Cx40-deficient mice were treated with COX inhibitors for 7 days. We used meclofenamate (10 mg/kg × day) as a commonly used nonselective COX inhibitor and SC-58236 (5 mg/kg × day) as a selective COX-2 inhibitor. We found that both meclofenamate and SC-58236 lowered renal abundance of renin mRNA in wt mice. Meclofenamate was somewhat less effective than SC-58236, which lowered renin mRNA levels by about 50% (Fig. 3). Renin mRNA levels in kidneys of untreated Cx40-deficient mice were about 1.8-fold increased relative to wt kidneys. After treatment with COX-2 inhibitors, renin mRNA levels in Cx40-deficient kidneys were reduced to the range of untreated wt mice (Fig. 3).

Immunohistochemical analysis revealed that renin immunoreactive areas in the kidneys of wt mice were not changed by COX-2 inhibitors (Fig. 4), except that the intensity of renin immunoreactivity was somewhat attenuated. In Cx40-deficient kidneys, however, treatment with COX-2 inhibitors produced a clearly recognizable change in renin expression patterns. It became apparent that renin-expressing cell accumulation was significantly reduced (Fig. 4). In particular, the extension of

Fig. 2. Renin (A and B) and COX-2 (C and D) immunoreactivity in kidney sections of wt (A and C) and Cx40-deficient (B and D) mice. Arrows highlight COX-2 immunoreactivity. Scale bar = 50 μm.
renin expression into the periglomerular and peritubular interstitium was markedly attenuated, if not absent (Fig. 5).

As a measure of overall kidney secretion of active renin, we determined PRC as the plasma renin activity in the presence of excess concentration of renin substrate. We found that PRC values were reduced by about 40% by COX-2 inhibitor treatment in wt mice (Fig. 6). Basal PRC values were strongly increased in Cx40-deficient mice relative to untreated wt controls. Notably, treatment with COX-2 inhibitors did not change PRC values in Cx40-deficient animals. To rule out the possibility that even upon addition of exogenous renin substrate, the substrate concentration limited the enzymatic reaction, we also measured samples with higher concentrations of exogenously added renin substrate. However, also under this condition, no difference in PRC values between Cx40-deficient mice treated with and without COX inhibitors became apparent (data not shown).

We further considered the possibility that the decrease of renin expression by COX-2 inhibitors in Cx40-deficient kidneys was primarily due to a change of enzymatically inactive prorenin, which might have escaped our attention by the measurement of primarily active renin in the plasma. We therefore subjected the plasma samples to three cycles of freezing and thawing to activate prorenin. This procedure led to a nonsignificant increase of renin activity in all plasma samples but did not change the pattern of results depicted in Fig. 6. In particular, there was no difference in plasma renin concentrations after activation between Cx40-deficient mice with or without COX-2 inhibitor treatment.

DISCUSSION

Our findings show that COX-2 inhibition lowers renal renin mRNA levels, and plasma renin concentration in normal mice confirms previous data (3, 14, 18) and suggests that likely COX-2-derived prostanoids relevantly contribute to the basal activity state of the renal renin system. Our data also show that COX-2 activity apparently is also relevant for the expression state of the renin gene in Cx40-deficient kidneys. This observation is of interest, since in Cx40-deficient kidneys, renin-producing cells are different in morphology and location relative to normal kidneys (16). The relevance of COX-2 activity for renin expression in these aberrant cells is underscored by the demonstrable COX-2 immunoreactivity. Though COX-2 gene transcripts can be found in normal mouse kidney cortex, the intensity of COX-2 protein expression is often too low to be detected by immunohistochemistry. One may infer that COX-2 is expressed in afferent arterioles (2) and in the macula densa cells of the distal tubule (8). In fact, under conditions of chronic interruption of the renin-angiotensin-aldosterone system, COX-2 immunoreactivity can appear in the macula densa cells of mouse kidneys (9, 17). The renin-producing cells themselves, however, remain free of COX-2 immunoreactivity in these settings. Similar to wt kidneys, macula densa cells and afferent arterioles also lack demonstrable COX-2 immunoreactivity in Cx40-deficient kidneys. However, in these kidneys, COX-2 immunoreactivity frequently appears in aberrant renin-producing cells. Molecular mechanisms underlying the induction of COX-2 in these cells remain a subject of intense speculation currently and may be related to the p38 MAP kinase pathway as a common general trigger of COX-2 expression in the kidney (8). COX-2 inhibition caused a striking change in the pattern of renin expression in Cx40-deficient kidneys. As noted earlier, the interglomerular pattern of renin expression is rather heterogeneous in Cx40-deficient kidneys.
because the number of renin expression cells in the periglomerular areas differed markedly, ranging from very few cells to enormous plaques infiltrating the periglomerular and peritubular interstitium (16). From our findings, it appears that renin expression in the peripheral extension of those plaques was particularly sensitive to COX-2 inhibition. In Cx40-deficient mice treated with COX-2 inhibitors, the sizes of renin-expressing plaques were clearly reduced, and renin expression remained restricted to the juxtaglomerular core of the plaques. This phenomenon could suggest that COX-2 activity is particularly relevant for renin expression in aberrant periglomerular and periglomerular regions or could simply reflect the fact that the intensity of renin expression is lower in the peripheral expression of renin and thus more sensitive to extinction than regions with a higher intensity of renin expression.

In spite of the clear effect of COX-2 inhibitors on renin mRNA abundance and immunoreactivity in Cx40-deficient kidneys, plasma renin concentrations were unchanged. In accordance with previous data, we found that plasma renin concentrations were about 10-fold elevated, relative to normal values, likely reflecting the defective feedback inhibition of renin secretion by blood pressure and ANG II (26). To narrow down the possible technical limitations that could lead to misinterpretation of the data, we also considered the possibilities of renin substrate limitation in the plasma, as well as differences in plasma prorenin concentrations. However, we did not obtain evidence that those explanations could obscure effects of COX-2 blockers on circulating renin in Cx40-deficient mice. Given that plasma renin concentrations reflect the in vivo secretion rates of renin from the kidneys, one has therefore to infer from these findings that during COX-2 inhibition, the number of renin-expressing cells is reduced, while renin secretion rates are not. This would indicate that renin expression sensitive to COX-2 inhibition is not relevant

Fig. 5. Distribution of renin immunoreactivity in kidneys of Cx40 knockout mice after treatment with vehicle (A and B) or with the COX-2 inhibitor SC-58236 (C and D) for 7 days. Arrows highlight renin immunoreactivity. Scale bar = 50 μm.

Fig. 6. Plasma renin concentration of wt (top) and of Cx40-deficient (bottom) mice after treatment with vehicle or with the cyclooxygenase inhibitors meclofenamate and SC-58236 for 7 days. Data are means ± SE of six animals in each group. * P < 0.05. § P < 0.05 vs. vehicle controls of wt mice.
for systemic secretion. If so, this conclusion might be compatible with our observation that COX-2 inhibition preferentially lowered renin expression in the peripheral interstitial extensions of renin-expressing plaques, which might not be involved (might not participate) in the secretion of renin into the circulation. This could be due to defective secretory exocytotic mechanisms of the cells or may reflect the disability of renin to gain access to the vascular system at this site. In this context, it is still a matter of debate how renin enters the circulation in normal kidneys, since renin-secreting cells in the afferent arterioles are separated from the vessel lumen by the endothelium. Therefore, it has been speculated that renin leaves the kidney via the lymphatic pathway (5, 20); this is still a matter of debate. Another possibility that has been supported by recent findings would be that renin enters the intravascular space via endothelial fenestrations, which are usually seen only in the region of renin-expressing cells (21). This latter hypothesis could explain why renin expression minimally contributes to the overall secretion of renin from the kidneys.

**Perspectives and Significance**

Our findings indicate that aberrant and uncontrolled renin production occurring in the absence of connexin 40 is, in part, due to an enhanced production of COX-2-mediated prostanoids. The obtained results also suggest that plasma renin concentrations do not always reliably reflect renin protein in the kidney, suggesting that the intrarenal activity of the renin system cannot strictly be estimated from PRCs, which could be of clinical relevance. Future research addressing the mechanisms that promote the induction of COX-2 expression in renin-producing cells in the absence of Cx40 must be conducted.

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