Renal Cyclooxygenase-2
Evidence for Recruitment of Thick Ascending Limb of Henle Cells in Microdissected Nephron Segments

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Abstract—Prostaglandins participate in the regulation of sodium and water renal excretion. They are synthesized by cyclooxygenases (COX): the constitutive isoform and the enzyme regulated by physiological stimuli (COX-2). Our previous immunohistochemical studies have demonstrated the presence of COX-2 in a subset of thick ascending limb (TAL) of Henle cells and its induction during the postnatal period and after adrenalectomy. Previous results suggested that this induction phenomenon proceeds by recruitment of TAL cells from the cortex to the outer medulla. The present work aimed to specifically address these preliminary observations by using immunohistochemical techniques in single microdissected nephron segments. Normal adult rats, adrenalectomized rats, adrenalectomized rats on dexamethasone and 5, 10, and 15 days postnatal age were used (Sprague-Dawley rats, n = 5 each group). Glomeruli and different segments of nephron were microdissected from collagenase-treated kidney tissue. Tubules were immunostained with specific antibodies against COX-2. We confirmed that COX-2 was localized exclusively in TAL segments; it was induced after adrenalectomy and during postnatal age, peaking at 15 days after birth. We provided morphological evidence that the induction of COX-2 along TAL proceeded in a defined pattern by recruitment of cells from the cortical portion close to the glomeruli toward the outer medulla. No COX-2 was observed in the post–macula densa portion of the segments. Our results provide the anatomical basis for the contribution of COX-2 in physiological mechanisms such as renin secretion, tubuloglomerular feedback, and the interaction with neuronal NO synthase at the juxtaglomerular apparatus. (Hypertension. 2001;38[part 2]:630-634.)

Key Words: cyclooxygenase ■ kidney ■ rat ■ immuno histochemistry ■ glucocorticoids ■ prostaglandins

In the kidney, eicosanoids act as paracrine agents that modulate kinins actions and vasoactive hormones and regulate several aspects of renal function: renal blood flow and hemodynamics1 and transepithelial NaCl transport in several nephron segments.2 Prostaglandin production depends on activation of the PGG2/H2 synthase, also known as cyclooxygenase (COX). Two distinct isoforms of this enzyme have been described: the constitutive isoform (COX-1) and the regulated isoform (COX-2). In the kidney, COX-1 is found in arterial vascular endothelial cells, medullary and cortical collecting ducts, and medullary interstitial cells.3 The COX-2 isoform is commonly known as an inductive enzyme that expresses in conditions such as inflammation and tissue damage.4 Previously, our laboratory demonstrated the presence of COX-2 in thick ascending limb (TAL) of Henle cells in normal adult rat,5 its regulation during the postnatal development,6 and its regulation by glucocorticoid.7 There is also evidence that COX-2 is regulated by dietary salt intake,8,9 angiotensin II,10 corticosteroid,11 and furosemide.12 Furthermore, COX-2–derived prostaglandins participate in renin secretion,13,14 tubuloglomerular feedback,15 and sodium handling.10

Our studies during postnatal development in rats and in adrenalectomized adult rats have suggested a recruitment of TAL cells from the cortex to medulla.6,7 However, the immunohistochemical techniques used on tissue slices in these studies did not allow precise analysis of the axial distribution of the enzyme along nephron segments owing to the low probability of obtaining a complete tubule along its longitudinal axis.

Taking advantage of the fact that microdissection of nephron segments allows the study of long nephron segments, the aim of the present work was to specifically test the hypothesis that the increase in COX-2 protein observed along postnatal development and during adrenalectomy corresponded to a recruitment phenomenon.

Methods

Experiments were carried out on male Sprague-Dawley rats maintained with free access to tap water and fed with normal rat chow. The animals were maintained at the Catholic University animal care...
facilities, and the experimental procedures were in accordance with institutional and international standards for humane care and use of laboratory animals (Animal Welfare Assurance No. A5427-01, Office for Protection from Research Risks, Division of Animal Welfare, PHS, NIH).

For postnatal development studies, rats were studied at the age of 5, 10, and 15 days (n = 5 for each age) as previously described.6 The adenectomy studies were done as described.2 Briefly, normal adult, adrenalectomized (ADX), and ADX + dexamethasone (ADX + Dex) rats were used (n = 5 in each group). The adenectomy was performed under anesthesia (25 mg/kg and xylazine 2.5 mg/kg IP) in adult rats (180 to 200 g, n = 5 in each group). After the surgery, the ADX rats were maintained on normal rat chow with 0.9% NaCl in their drinking water. The experiments were performed 5 days after adenectomy. The ADX + Dex group was treated with dexamethasone (0.5 mg · kg$^{-1}$ · d$^{-1}$ SC for 2 days). Dexamethasone (5 mg/mL Oradexon, NV Organon Oss Holland) was diluted just before the administration in sterile saline solution.

Microdissection

For the microdissection, the rats were anesthetized with pentobarbital sodium (60 mg/kg IP), the left renal artery was cannulated, and the kidney was perfused initially with 10 mL standard solution ([in mmol/L] NaCl 127, KCl 5, MgSO$_4$ 0.8, Na$_2$HPO$_4$ 0.33, KH$_2$PO$_4$ 0.44, MgCl$_2$, 1, NaHCO$_3$ 4, CaCl$_2$ 2, d-glucose 5, Na acetate 10, and HEPES 20, pH 7.4, and 1 mg/mL bovine serum albumin) and then with 5 mL of the same solution containing 1.4 mg/mL collagenase A. After removal, the kidney was decapsulated and longitudinally sliced. Thin pyramids were cut out and incubated in standard solution with collagenase A (1 mg/mL, 10 to 15 minutes, 30°C). After washing, the pyramids were placed in buffered standard saline solution and microdissection was performed under a stereomicroscope at 1°C to 4°C or at room temperature in standard solution for about 2 hours for each kidney. Glomeruli and different segments of nephron were isolated as previously described.16,17 The segments were (1) proximal convoluted tubule, (2) pars recta, (3) TAL, (4) medullary TAL, (5) cortical TAL (cTAL), (6) cortical collecting duct, and (7) medullary collecting duct. Because of technical problems, the kidneys from 5- and 10-day-old postnatal rats were not perfused before microdissection.

Immunohistochemistry

Glomeruli and tubular segments were attached to glass slides and fixed in Bouin’s solution. Microdissected tubules and glomeruli were immunostained by use of the peroxidase-antiperoxidase method with specific antibodies against COX-2 as previously described.5–7 Briefly, the microdissected segments were rinsed with 0.05 mol/L Tris-phosphate-saline buffer, pH 7.6. After the samples were rinsed with Tris-phosphate-saline buffer, glomeruli and tubular segments were incubated with the primary antisera overnight at 22°C, followed by 30 minutes of incubation at 22°C with the secondary antibody (1:20) and then the peroxidase-antiperoxidase complex (1:150) for 30 minutes at 22°C. The immunoperoxidase reaction was visualized by incubating the sections in 0.1% (wt/vol) 3,3’-diaminobenzidine and 0.03% (vol/vol) hydrogen peroxide. The immunostained samples were dehydrated and protected with coverslips without hematoxylin counterstaining, and then they were observed and photographed on a Nikon Optiphot microscope with a Nikon Microflex UFX IIA photographic system (Nikon Corporation).

Controls for the immunostaining procedure were prepared by preadsorption of the primary antisera with the COX-2 carboxy-terminal peptides or by omission of the first antibody and by replacement of primary antisera with normal or preimmune rabbit serum.

Results

COX-2 was exclusively found in TAL segments in all the experimental groups studied. No staining for COX-2 was observed in other microdissected segments, namely proximal tubules, cortical or medullary collecting ducts, and thin descending tubules.

In control adult rats, expression of COX-2 protein was observed in few cells and restricted mainly to a region near the macula densa; its distribution was not uniform within the TAL segment because COX-2–positive cells were intermingled with COX-2–negative TAL cells (Figure, part a).

In ADX animals, the number of COX-2–positive cells dramatically increased along the axis of the TAL segment toward the medulla (Figure, part b). This increased number of cells expressing COX-2 protein in ADX animals was reversed after treatment with dexamethasone (Figure, part c). In control intact rats treated with dexamethasone, COX-2 protein expression was similar to that of untreated control rats (not shown). During postnatal development, at day 5, a small number of COX-2–positive cells were observed close to the macula densa and dispersed at variable distance from the glomeruli (Figure, part d). The number of COX-2–positive cells increased at day 10 after birth (Figure, part e), with a further increase in the number of TAL cells expressing COX-2 protein at day 15 (Figure, part f). The increase in the number of TAL cells expressing COX-2 protein (in adrenalectomized rats at days 10 and 15) was associated with a stronger intensity of the COX-2 staining, suggesting that more COX-2 protein was being expressed in the individual cells, although no microdensitometric analysis was performed to confirm this observation. Even in conditions of maximum expression of COX-2 protein along TAL segments in adrenalectomized and 15-day-old rats, the enzyme was present before or close to the macula densa; it was never observed past the macula densa segment (Figure, part f).

The overall result showed that under stimulation, we observed a recruitment of TAL cells expressing COX-2 protein that proceed from close to the glomeruli toward more medullary portions at $\approx$250 μm from the macula densa (Figure, part f).

Discussion

Since the first publication of the nephron segment microdissection method by Burg et al16 in 1966, numerous authors have used this technique to localize and analyze different mechanisms responsible for salt and water regulation by the kidney. The possibility of isolating each segment and the miniaturization of measurement methods have contributed to the knowledge of numerous cell hormonal mechanisms and enzyme activities16 in the various tubular segments ($\approx$12) that constitute the nephron. In the present work, we have used the immunohistochemical methods applied to isolated tubules to further characterize and test our hypothesis on the basis of our previous observations suggesting the recruitment of cells expressing COX-2 during the postnatal development6 and after adenectomy.7

To the best of our knowledge, few or no immunohistochemical studies have been performed on microdissected tubular segments. The microdissection and isolation of glomeruli and nephron segments are laborious and time consuming; however, compared with the use of conventional tissue sections in immunohistochemistry, these techniques provide some unique advantages. For a qualified investigator, easier
and more precise identification of tubular segments is possible. The use of specific markers is not required to identify the tubular segments (as in tissue sections). Microdissection and isolation allow a more precise and more complete study of the distribution of specifically stained cells along segments. Indeed, it is possible to microdissect long tubular fragments (≥300 µm and possibly 1000 µm, depending on the segment). In tissue sections, the probability of obtaining a long fragment cut along the longitudinal axis is very low. Finally, with these techniques, the spatial relationship between tubular segments and their enzymes and glomeruli can be better visualized, providing a better morphological basis for understanding its action in a paracrine fashion.

Immunohistochemical staining along the microdissected TAL was in agreement with our previous observations that COX-2 protein expression was restricted to a subset of TAL cells; however, the sensitivity of the method does not absolutely exclude the possibility of the presence in other seg-
ments. Other authors have described the presence of COX-2 in inner medullary collecting ducts and papillary interstitial cells. In the present study, microdissected segments do not contain papillary interstitial cells, and the low levels of COX-2 described in the inner medullary collecting duct (macula densa COX-2-containing segment and cTAL contain 100- and 10-fold more COX-2 than inner medullary collecting duct) were below the level of detection by immunohistochemistry.

These cTAL COX-2–positive cells were few and scattered in normal rats as previously described in tissue sections. Several authors have described the presence of COX-2 in cTAL cells interspersed with COX-2–negative cells, suggesting a subpopulation on cells in cTAL. The possibility that the uneven distribution of COX-2 in the cTAL could correspond to the smooth and rough surface subtypes of TAL cells is under study in our laboratory by immunoelectron microscopy.

It is interesting to note that COX-2–positive cells were close to but not in the macula densa. Thus, the present results in microdissected nephron segments from neonates are in agreement with previous works in kidney sections from neonate rats in which COX-2 was not observed in the macula densa cells themselves but in the cTAL cells in the proximity of the macula densa.

COX-2 protein expression did not go beyond the macula densa; it was restricted to the pre–macula densa segment of TAL. This observation became evident only in the microdissected glomeruli with their corresponding attached TAL and post–macula densa segment; it was not evident in our previous work on tissue section. COX-2 close to and before the macula densa would be strategically located to contribute via COX-2–derived prostaglandins to the flow-dependent regulation of tubuloglomerular feedback mechanism previously described by Ichihara et al. Since the work of Smith and Bell, it is believed that the cTAL segment does not contain COX-1. Further work did not detect COX-1 protein or mRNA in this segment. In our laboratory, although COX-1 was readily detected in collecting ducts, no COX-1 protein was observed in TAL segments, neither in tissue sections nor in microdissected segments. Our results also show that the increase in COX-2 protein expression observed from 5 to 15 days after birth consisted of a progressive recruitment of TAL cells from the macula densa region toward the medulla. COX-2 expression returned to basal level in 60-day-old rats. Administration of dexamethasone in neonates decreased COX-2 expression as visualized in kidney sections immunostained for COX-2 (Lira et al, unpublished data). Experiments using microdissected nephron segments from neonatal rats treated with dexamethasone are still in progress in our laboratory.

In adult rats, adrenalectomy also induced a similar recruitment of COX-2–expressing TAL cells from the macula densa region toward the medulla. Regarding the physiological relevance, we hypothesize that COX-2 is tonically inhibited by glucocorticoids in adults, an effect that is unmasked by adrenalectomy. On the other hand, the low levels of endogenous glucocorticoids in neonatal rats allow the abundant expression of COX-2 during that period. As endogenous glucocorticoids rise in the postnatal stage, the expression of COX-2 decreases. This postnatal upregulation of COX-2 seems necessary for the maturation of the kidney in the final phases of nephrogenesis. Thus, the COX-2 knockout mice having apparently normal kidneys at birth, but by day 10 of postnatal life, outer cortical nephrogenesis is impaired with loss of tubular mass, arrested glomerular growth, and cystic formation. A similar effect is observed with the pharmacological inhibition of COX-2, resulting in impaired renal cortical development and glomerulogenesis.

Other paracrine systems changing in concert with COX-2 during the postnatal development are the vasodilatory kallikrein-bradykinin system and neuronal NO synthase, both located close to or within the juxtaglomerular apparatus. These systems are functionally related because NO regulates COX-2 and COX-2–dependent prostanoids contribute to the renal vasodilatation induced by bradykinin. On the other hand, the renal renin-angiotensin system, which is upregulated in the newborn kidney, undergoes a progressive decline in activity during the first days. The increasing values of kallikrein-bradykinin, NO, and COX-2, together with the decreasing values of renin, may be important for the decline in vascular resistance and increase in blood flow occurring in the kidney after birth.

In conclusion, the application of immunohistochemical techniques to single microdissected TAL allowed the study of the axial distribution of COX-2 protein expression under induction conditions: during postnatal development and after adrenalectomy. The results showed that the recruitment of COX-2–expressing cells proceeds in a defined pattern from the cortex to outer medulla TAL.

Additional work is required to elucidate whether the TAL segments reaching the medulla correspond to juxtedudillary nephrons. This will be done in future work in which nephron segments will be dissected from the different nephron subtypes (cortical, midcortical, juxtedudillary). This information could provide additional insights into the contribution of COX-2 to renal function.

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References


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