Bradykinin Regulates Cyclooxygenase-2 in Rat Renal Thick Ascending Limb Cells

Jorge A. Rodriguez, Carlos P. Vio, Paulina L. Pedraza, John C. McGiff, Nicholas R. Ferreri

Abstract—Cyclooxygenase-2 (COX-2) is constitutively expressed in a subset of thick ascending limb cells in the cortex and medulla and increases when the renin-angiotensin and kallikrein-kinin systems are activated. Although the contribution of angiotensin II to the regulation of COX-2 is known, the effects of bradykinin on COX-2 expression have not been determined in this nephron segment. We evaluated expression of B2 bradykinin receptors in thick ascending limb cells containing COX-2 and the effect of bradykinin on COX-2 expression in primary cultured medullary thick ascending cells. The presence of B2 receptors was studied in renal sections by immunohistochemistry with antibodies against B2, COX-2, and Tamm-Horsfall glycoprotein. B2 receptors were detected on the apical and basolateral portion of the thick ascending cells. These cells also contained COX-2, suggesting that COX-2 expression may be regulated via B2 receptor. Incubation of cultured medullary thick ascending cells with bradykinin (10^{-7} to 10^{-5} mol/L) induced a significant increase on COX-2 protein expression. Maximal expression of COX-2 was observed 4 hours after exposure to bradykinin (10^{-7} mol/L), effect abolished by a B2 receptor antagonist (HOE-140; 10^{-6} mol/L). Prostaglandin E_{2} production increased when these cells were challenged with bradykinin for 4 hours, indicating that COX-2 was enzymatically active. We have demonstrated (1) the presence of B2 receptors in thick ascending limb cells expressing COX-2 and (2) the stimulatory effect of bradykinin on COX-2 protein expression, via B2 receptors, in cultured medullary thick ascending cells. We suggest that bradykinin can affect ion transport in the thick ascending limb via a COX-2–mediated mechanism.

Key Words: cyclooxygenase ■ bradykinin ■ immunohistochemistry ■ rats ■ kidney ■ receptors, bradykinin ■ prostaglandins

Prostaglandins participate in the regulation of the renal circulation and tubular ion transport as well as renin secretion, the latter via cyclooxygenase-2 (COX-2)–dependent mechanism. The constitutive enzyme cyclooxygenase-1 (COX-1) has been identified in the collecting tubules and the renal vasculature. Immunohistochemical localization studies have detected COX-2 in the perimacula densa region and a subset of thick ascending limb (TAL) epithelial cells located in the cortex (cTAL) and outer medulla (mTAL) as well as in interstitial medullary cells. COX-2 expression in the kidney under basal conditions suggests that COX-2 may have a physiological role. Expression of the COX-2 isoform is increased in the kidney during development, adrenalectomy, and low-salt diet; it is decreased by glucocorticoids.

The kallikrein-kinin system (KKS) contributes to the regulation of renal blood flow and salt and water excretion. The renal effects of bradykinin under physiological conditions are mediated by the B2 receptor, whereas the B1 receptor is expressed in pathological states such as inflammation. Activation of the renin-angiotensin and KKS by administration of a low-sodium diet or angiotensin-converting enzyme inhibition increases renal COX-2 expression. The effects of angiotensin II (Ang II) on expression of COX-2 in the TAL segment via activation of Ang II type-1 receptors have been well documented, whereas the effects of bradykinin on renal COX-2 are unknown despite the identification of B2 receptors in this nephron segment.

A biphasic response of the mTAL segment to Ang II, having a time-related dependency, has been characterized; namely, the initial response (0 to 4 hours) to Ang II is mediated by 20-HETE, the principal product of ω hydroxylation, a cytochrome P450 (CYP450) enzyme, whereas after 4-hour exposure to Ang II, COX-2 is expressed and prostaglandin E_{2} (PGE_{2}) is produced in response to challenge by the peptide. We hypothesize that bradykinin, which has been linked to arachidonate product formation by both renal COX and CYP450, also regulates COX-2 expression in the TAL segment. Indeed, Grider et al have identified an early response by the rat mTAL to bradykinin similar to that of Ang II, namely receptor-mediated activation of a CYP450–dependent mechanism that inhibits NaCl reabsorption by the

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mTAL. Blockade of the bradykinin B2 receptor inhibited this effect of the peptide on the mTAL. Accordingly, we have addressed whether (1) bradykinin B2 receptors colocalize with TAL cells expressing COX-2, or (2) bradykinin acts through B2 receptors to stimulate COX-2 expression in mTAL cells.

Methods

Animals

The study was performed on adult male Sprague-Dawley rats (220 to 250 g for immunohistochemistry; 100 to 115 g for cell culture; n=26) maintained with free access to water and fed normal rat chow. The animals were purchased from and maintained at the Catholic University animal care facilities; experimental procedures were conducted in accordance with institutional and international guidelines for the welfare of animals (Animal Welfare Assurance #A5427–01, Office of Laboratory Animal Welfare, PHS, NIH). Surgery was performed under anesthesia with ketamine: xylazine –#A5427 maintained with free access to water and fed normal rat chow. The studies were performed on adult male Sprague-Dawley rats (220 to 250 g for immunohistochemistry; 100 to 115 g for cell culture; n=26) maintained with free access to water and fed normal rat chow. The animals were purchased from and maintained at the Catholic University animal care facilities; experimental procedures were conducted in accordance with institutional and international guidelines for the welfare of animals (Animal Welfare Assurance #A5427–01, Office of Laboratory Animal Welfare, PHS, NIH). Surgery was performed under anesthesia with ketamine: xylazine –#A5427 maintained with free access to water and fed normal rat chow.

Antisera Chemicals and Peptides

Specific antibodies against murine COX-2 and against the bradykinin B2 receptor were used as previously described. Anti-serum against Tamm-Horsfall glycoprotein, secondary antibodies, and peroxidase-antiperoxidase complexes were purchased from ICN Pharmaceuticals (Aurora, Ohio). Tissue culture media was purchased from Life Technologies (Grand Island, NY); bradykinin and the B1 receptor antagonist Des-Arg-Leu8-bradykinin and chemicals were purchased from Sigma (St. Louis, Mo). The B2 receptor antagonist HOE-140 was a gift from Hoechst (Frankfurt, Germany).

Tissue Processing and Immunohistochemistry

Renal slices including cortex, medulla, and papilla were fixed in Bouin’s fixative and processed as described. The localization of B2 receptor and COX-2 protein was evaluated by immunohistochemistry, and the cellular origin of B2 receptors was assessed by double immunolabeling for B2 receptors and Tamm-Horsfall glycoprotein (a specific marker for TAL cells) in the same tissue sections. The double immunolabeling was performed as previously described. Briefly, the first antibody immunostaining was developed with diaminobenzidine-hydrogen peroxide to give a brown color, whereas the following primary antibody, immunostaining was developed with Vector SG substrate to give a blue color. Appropriate immunostaining controls were done as described. The sections were observed and photographed on a Nikon Eclipse 600 microscope with a Nikon DXM1200 digital camera.

Isolation of mTAL Cells

The isolation and characterization of mTAL cells were performed as previously described. Briefly, kidneys were removed, and the outer medulla was excised and incubated in collagenase solution. The supernatant of the suspension was collected, spun, resuspended in Hank’s solution, and filtered through a 52-µm nylon mesh membrane. The tubules retained on the mesh were resuspended, and the cells were cultured. Cells at 80% confluence were made quiescent with either a rabbit anti-COX-2 polyclonal antibody (Cayman Chemicals, Ann Arbor, Mich) or rabbit anti-extracellular signal regulated kinase (ERK)2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, Calif), and incubated horseradish peroxidase-conjugated secondary antibody. Enhanced chemiluminescence was used to evaluate protein expression. The blots were scanned, and densitometric analysis was performed using the public domain NIH Image program v1.61 (US National Institutes of Health, http://rsb.info.nih.gov/nih-image). The expression of ERK2 was used to correct for variation in sample loading.

PGE2 ELISA

Quiescent mTAL cells were incubated with bradykinin (10⁻⁷mol/L) in RPMI 1640 containing 0.5% serum for varying times. Cell-free supernatants were assayed for PGE₂ by ELISA (Neogen) according to the protocol provided by the manufacturer.

Statistical Analysis

The differences were assessed with the nonparametric test of Mann-Whitney. Data are presented as mean±SEM; P<0.05 was considered significant.

Results

Localization of COX-2 in cTAL and mTAL Segments

COX-2 protein expression was observed in small and scattered groups of cells belonging to the TAL located in the...
cortex and outer medulla (Figure 1a and 1b). The cells displaying COX-2 were heavily stained, compared with unstained neighboring tubular cells. The schematic diagram in Figure 1 indicates the relationship between the localization of an arcuate artery and a juxtamedullary nephron as shown in Figure 1b. Because the arcuate artery defines the boundaries between the cortex and the outer medulla, the TAL segment containing COX-2 in the medulla corresponds to juxtamedullary nephrons, whereas those present in the cortex belong to cortical nephrons (Figure 1a and 1b). No immunostaining was observed in glomeruli, proximal tubules, or collecting ducts.

Colocalization of B2 Receptors and COX-2 in the TAL

The immunostained renal tissue revealed the presence of B2 receptors in the TAL, connecting tubule, and collecting duct. The presence of B2 receptors was observed in the apical and in the basolateral portions of TAL cells (Figure 2). Double label immunolocalization confirmed the expression of B2 receptors in TAL as demonstrated by Tamm-Horsfall glycoprotein with the bradykinin B2 receptors (Figure 3a and 3b). COX-2 positive cells also expressed the B2 receptor on both cellular sides (Figure 3c and 3d) although B2 receptors were detected on COX-2 positive as well as negative cells. These data indicate that in subsets of TAL cells, a close association exists between components of the KKS and prostaglandin systems. Nearly all mTAL cells in culture expressed Tamm-Horsfall glycoprotein (95%) (Figure 3e and 3f); however, only few mTAL cells (≈5%) expressed COX-2 displayed in the

Figure 2. Immunohistochemical localization of B2 receptors in renal sections. The B2 receptor was observed in the thick ascending limb. Higher magnification shows the subcellular distribution of B2 receptors in the apical and basolateral sides (arrows). Scale bar=100 μm.

Figure 3. Localization of B2 receptors and colocalization with COX-2 along the TAL. Using double label immunohistochemistry, the presence of B2 receptors (brown) was observed in TAL cells identified by the presence of Tamm-Horsfall glycoprotein (blue; a, b). Cells immunoreactive for COX-2 (blue) expressed bradykinin B2 receptors (brown; c, d). mTAL cells in culture were identified by the presence of Tamm-Horsfall glycoprotein (e). Some of the cells display immunoreactivity to COX-2 within the cytoplasm (brown arrow; f). Scale bar=50 μm.
cytoplasmatic and perinuclear regions (Figure 3f). No staining was observed with immunostaining controls.

**Bradykinin Increases COX-2 Expression in mTAL Cells**

Western blot analysis of cellular extracts was performed to determine the effects of bradykinin on COX-2 expression by cultured mTAL cells. COX-2 protein increased significantly after exposure to concentrations of bradykinin of $10^{-7}$ mol/L (Figure 4). The nearly 2-fold increase in COX-2 expression was transient, peaked at 4 hours, and gradually decreased from 8 to 48 hours (Figure 5). Thus, bradykinin increased COX-2 expression by mTAL cells in a time- and concentration-dependent manner.

Preincubation with a selective B2 receptor antagonist (HOE-140, $10^{-6}$ mol/L), but not a B1 receptor antagonist (Des-Arg-Leu8-bradykinin; $10^{-6}$ mol/L), abolished the stimulatory effect of bradykinin on COX-2 protein expression (Figure 6). To evaluate if the COX-2 protein induced by bradykinin was enzymatically active, mTAL cells were challenged with bradykinin ($10^{-7}$ mol/L) and PGE$_2$ production was measured by ELISA. Bradykinin caused a transient increase in PGE$_2$ synthesis (1.7-fold of control, 10.3±0.8 pg/µg protein) at 4 hours (Figure 7).
Discussion

We have demonstrated that COX-2 protein is constitutively expressed in a small percentage (≈5%) of cTAL and mTAL cells in vivo as well as in cultured mTAL cells. B2 receptors were detected in the collecting ducts and connecting tubules, and to the apical and basolateral portions of TAL cells. Colocalization studies revealed that subsets of TAL cells can be identified based on the differential expression of B2 and COX-2. Namely, TAL cells expressing B2 can be sorted as either B2(+)COX-2(+) or B2(+)COX-2(−). Human podocytes also have been described to express COX-2 and B2 receptors. Bradykinin, via a B2-dependent mechanism, increased COX-2 protein expression that was enzymatically active as it metabolized endogenous arachidonic acid to PGE₂. Bradykinin also induced COX-2 expression in human airways and pulmonary artery smooth muscle cells and potentiated COX-2 mRNA accumulation in fibroblasts, human endothelial cells, and leptomeninges. It is important to note that, although the in vivo expression of COX-2 in the cTAL has been well described, the present study also demonstrates the expression of COX-2 in mTAL cells in vivo. A recent publication also has demonstrated constitutive in vivo expression of COX-2 in the mTAL.

The immunohistochemical results are in agreement with previous observations restricting COX-2 to a subset of TAL cells. The heterogeneous expression of COX-2 in this nephron segment is consistent with the existence of a subpopulation of TAL cells. TAL heterogeneity, reported by Allen and Tisher, described rough and smooth surface subtypes of cells. More recently, differential expression of renal outer medullary K⁺ (ROMK) channels and H⁺-K⁺-ATPase were demonstrated in this nephron segment. However, the complete phenotype of TAL subsets and the potential role for functional diversity have not been clarified. Bradykinin B2 receptors were expressed on both apical and basolateral sides of TAL cells of normal rats. In addition to the TAL, B2 receptors are expressed in connecting tubules and collecting ducts, providing an anatomical basis for involvement of this tubular segment in the renal actions of the KKS and its contribution to the regulation of salt and water excretion. In the present study, the localizaton of B2 receptors along the TAL was performed by a colocalization method to identify the Tamm-Horsfall glycoprotein, a specific marker of the TAL. Expression of B2 receptors was detected in apical and basolateral membranes of TAL cells more apically concentrated, whereas previous reports described similar receptor expression on both sides. Expression of the B2 receptor in both cell sides may explain the asymmetric function of bradykinin described in different nephron segments. Bradykinin when added only to the apical surface of the collecting duct cells caused PGE₂ release, whereas bradykinin added to the basolateral side significantly inhibited chloride transport in mTAL. We conclude that the renal effects of bradykinin are dependent, at least in part, to activation of B2 receptors in TAL cells.

COX-2 is not restricted to cortical nephrons as it is also expressed in juxtamedullary nephrons that extend into the medulla. Owing the important contribution of juxtamedullary nephrons to ion transport and to the osmolar gradient required for concentrating urine, we focused on the mTAL cells in culture where we demonstrated bradykinin-inducible, enzymatically active COX-2 protein. COX-2 activity was demonstrated by increased PGE₂, the main prostaglandin secreted in TAL, in the nephron segment, where its specific synthase is localized. We have shown that Ang II on stimulating tumor necrosis factor production by the mTAL can cause COX-2 expression after a delay of 3 to 4 hours. The immediate response to Ang II of freshly isolated mTAL tubules is production of 20-HETE via ω hydroxylase present in mTAL segments in situ. A similar effect to Ang II has been reported for bradykinin; namely, bradykinin (like Ang II) inhibits mTAL transport function by stimulating production of 20-HETE. It should be noted that cultured mTAL cells lose their capacity to express CYP450 enzyme activity; the latter is superceded by COX-2 when cells are cultured. Activation of the calcium-sensing receptor also increased COX-2 expression in mTAL cells. Moreover, recruitment of COX-2 containing cells in the mTAL has been reported to occur during development or in response to decreased secretion of corticosteroids by adrenalectomy. In each instance, COX-2 expression is observed in an orderly manner proceeding from cortex to outer medulla, indicating that mTAL cells, in addition to cTAL cells, retain the capacity to express COX-2.

The B1 receptor is inducible and is expressed in pathological conditions, whereas the B2 receptor is constitutive and participates in physiological functions of the kidney. The stimulatory effect of bradykinin on COX-2 expression was abolished by a B2 receptor antagonist. The effect of B2 but not B1 receptors on COX-2 regulation is consistent with significant contributions of COX-2–derived prostaglandins to transport functions of the TAL segment under physiological conditions. The regulation of COX-2 expression by bradykinin in the TAL segment may help to explain the effects of bradykinin on salt and water regulation. Thus, it required inhibition of both isoforms of COX (COX-1 and COX-2) in addition to inhibition of nitric-oxide synthase to prevent the excretory actions of bradykinin, suggesting that the natriuretic action of bradykinin requires the participation of both COX-1 and COX-2 as well as NO.

Perspectives

This study suggests a potential role for bradykinin on transport function in a key tubular segment, the mTAL, by virtue of controlling expression and activity of COX-2. We propose that the production of PGE₂ by the TAL via induction of COX-2 in response to kinin stimulation of B2 receptors contributes to regulation of extracellular fluid volume and blood pressure.

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