Immunolocalization of a microsomal prostaglandin E synthase in rabbit kidney

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PGE₂ is by far the major prostanoid synthesized in the kidney and is an important paracrine regulator of salt and water homeostasis (1–5, 8, 13, 14, 24). PGE₂ regulates tubular transport of Na⁺ and water, resulting in natriuresis and diuresis (1, 24). As a potent vasodilator, it helps to maintain glomerular filtration and medullary blood flow in conditions associated with decreased renal perfusion (1, 13, 24) and/or high renin levels (13, 14, 24). Another important tubulovascular function is PGE₂ production by macula densa (MD) cells (22, 23) in signaling low distal tubular NaCl concentration-induced renin release (25). Because PGE₂ acts only in the immediate vicinity of its site of generation, it is very important to know which nephron segments synthesize this autacoid. Previous enzyme immunoassay measurements, using microdissected tubule segments (1, 8) from rabbit kidney, detected large amounts of PGE₂ synthesized in the descending thin limb and the collecting duct system. However, these assays were not able to analyze short tubular segments like the MD and could not identify the exact cell type(s) of tubules, vascular or interstitial structures where PGE₂ may be synthesized. Localization of the synthetic machinery necessary for PGE₂ production could provide further insights into where this autacoid is produced.

To date, cyclooxygenase (COX) activity has been considered the key step in prostaglandin synthesis and the localization of two isoforms, COX-1 and COX-2, has been well characterized in the kidney (4, 9, 10, 13, 14, 26). However, metabolism of arachidonate by either COX-1 or COX-2 yields only the unstable intermediary PGH₂ (2, 3, 14, 16, 18, 19). The subsequent fate of PGH₂ is dictated by coexpression of a prostaglandin synthase, the other key enzyme of prostaglandin synthesis, which is capable of converting PGH₂ to one of the prostanoid end products including PGE₂, PGF₂α, PGD₂, PGI₂ and TXA₂ (2, 3, 14, 27). Recently, the membrane-associated PGE synthase (mPGES), a terminal enzyme of PGE₂ biosynthesis, has been cloned (16, 18). Renal mRNA expression for mPGES was recently described in microdissected tubular segments of the rat kidney and in the mouse (12, 27). Also, very recent work described the site-specific expression of key enzymes for prostaglandin synthesis, including mPGES, in rodent kidney (6). However, to date, there is no information on the renal regulation of mPGES protein and its localization in the rabbit kidney. We therefore analyzed, in detail, the sites of mPGES protein expression in the rabbit kidney and its regulation in the MD using immunofluorescence.

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MATERIALS AND METHODS

Salt Diet and Captopril

Separate groups of New Zealand White rabbits (0.5–1.0 kg, Myrtle) were fed standard (8630 Harlan Teklad, Madison, WI, 0.3% NaCl), low-salt (TD 90188, 0.01% NaCl), or high-salt (TD 98164, 7.7% NaCl) rabbit chow for a minimum of 1 wk. A separate group of rabbits received captopril (Sigma, St. Louis, MO) in the drinking water (500 mg/l) for 7 days.

Immunohistochemistry

Fixation and preparation of tissue for immunohistochemistry. Five-hundred-gram female New Zealand White rabbits were anesthetized with pentobarbital sodium, and the kidneys were perfusion-fixed by first inserting a cannula into the descending aorta distal to the renal arteries. The kidneys were then perfused retrograde with PBS, pH 7.4, at 37°C to remove blood, followed by 4% paraformaldehyde in Dulbecco’s modified Eagle’s/F-12 medium. The kidneys were then removed, and coronal kidney sections were incubated overnight at 4°C in 4% paraformaldehyde and then embedded in paraffin.

Sectioning and immunolabeling. Subsequently, 4-μm-thick sections of the paraffin block were deparaffinized with toluene, washed in graded ethanol, and rehydrated in PBS. Sections were subjected to microwave antigen retrieval before staining and blocked with PBS-Tween for 20 min containing 2% goat serum to lower background fluorescence. Subsequent blocking with goat anti-rabbit Fab IgG (1:100, Jackson ImmunoResearch Laboratories, West Grove, PA) was carried out for 40 min to reduce nonspecific binding when a rabbit polyclonal antibody (anti-mPGES) was used on rabbit tissue. After subsequent washings in PBS, tissues were treated with the affinity-purified rabbit polyclonal mPGES antibody (1:50, Cayman Chemical, Ann Arbor, MI). After being washed, there was a 40-min incubation with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR). Sections were mounted with VectaShield media, containing 4,6-diamino-2-phenylindole (Vector Laboratories). Tissue sections were examined with an Olympus IX70-inverted epi-fluorescence microscope using a UPlanS 40 ×1.3 objective. Images were captured using a SenSys digital camera and IPLab Spectrum software equipped with power microtome (Signal Analytics).

Double labeling mPGES with aquaporin-1 or -2. Some of the kidney sections were double labeled with anti-mPGES and anti-aquaporin (AQP)-1 or AQP2 antibodies. After blocking for 20 min with 2% donkey serum in PBS-Tween, there was a subsequent blocking with goat anti-rabbit Fab IgG (1:100, Jackson ImmunoResearch Laboratories) for 40 min to reduce nonspecific binding. After being subsequently washed with PBS-Tween, tissues were treated with either a goat polyclonal AQP1 or AQP2 antibody (L-19 and C-17, respectively, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The tissue was then washed and subsequently incubated with an Alexa Fluor 594-conjugated donkey anti-goat IgG (1:500, Molecular Probes) for 40 min. Sections were then washed and subsequently incubated with affinity-purified mPGES polyclonal antibody (1:50, Cayman Chemical) for 1 h. After being washed, sections were incubated for 40 min with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500, Molecular Probes). Sections were washed and mounted with VectaShield media, containing DAPI for nuclear staining (Vector Laboratories). Tissue sections were examined as described earlier.

COX-2 immunofluorescence. Rabbit kidney sections were prepared as described above. For labeling, tissues were treated with either a goat polyclonal COX-2 antibody (C-20, 1:100, Santa Cruz Biotechnology) overnight or with the affinity-purified rabbit polyclonal mPGES antibody (1:50, Cayman Chemical). After being washed, there was a 40-min incubation with Alexa 594-conjugated donkey anti-goat IgG (1:500, Molecular Probes) for COX-2 sections or with Alexa 488-conjugated goat anti-rabbit IgG (1:500, Molecular Probes) for mPGES sections.

Immunolabeling controls. The following controls were performed: 1) adsorption controls made by incubation with affinity-purified mPGES polyclonal antibody (10 μg/ml) previously reacted with purified mPGES protein (100 μg/ml) that was used for immunization (Cayman Chemical) and 2) incubation without the use of primary antibodies. All controls revealed an absence of labeling.

RESULTS

Localisation of mPGES in the Renal Cortex

Overall expression of the mPGES protein in the cortex (Fig. 1A) was much lower than in the medulla (Fig. 1B) and was confined to only short or isolated tubule segments in the distal nephron. Fluorescence labeling was specific, because omitting or blocking the primary antibody with the mPGES protein (data not shown) resulted in no staining. Also, labeling was present throughout the cytoplasm and was confined to small granules, consistent with microsomal localization. Strong labeling for mPGES was found in the macula densa (Fig. 2A), surrounding cortical thick ascending limb cells, in the connecting segment (CNT), and in the cortical collecting duct (CCD; Fig. 2B). No staining was detected in the proximal or distal convoluted tubules, in the glomerulus, or in vascular structures. Careful analysis revealed that only a subpopulation of cells in the CNT and CCD (Fig. 2B) was immunoreactive. Double labeling with an AQP2 antibody that selectively binds to mainly the apical membrane of principal cells (PC) found that mPGES protein was localized only in PC of CNT and CCD (Fig. 2C) and labeling was below the limits of detection in intercalated (IC) cells.

Localization of mPGES in the Renal Medulla

The medulla was abundant in mPGES-immunoreactive structures. In the outer medulla, descending thin limbs (DTL; Fig. 3, A–E) and medullary collecting ducts (MCD; Fig. 3, C–E) displayed strong labeling. Similar to the distinct localization of mPGESs in the CDD, only a subpopulation of cells in the outer MCD (Fig. 3E) was immunoreactive. mPGES-positive cells, similar to CDD, also labeled with AQP-2, indicated a PC phenotype (not shown). No evidence for mPGES was found in descending vasa recta (DVR; Fig. 3, B and E). Vasa recta was identified by red blood cells in the lumen and also based on their expression of the AQP1 water channel. Double labeling with an AQP1 antibody that selectively binds to DTL segments and vasa recta re-
revealed that mPGES protein was localized only in DTLs and not in DVR or ascending thin limbs (Fig. 3E). In the inner medulla, heavy labeling of mPGES in MCD continued to the papilla. In addition to MCD, numerous thin-walled tubular structures were immunoreactive (Fig. 3D), resembling the terminal part of long DTLs. mPGES was also localized to renal medullary interstitial cells (RMIC; Fig. 3D).

**Regulation of MD mPGES Protein Expression by Salt Diet and Captopril**

Because mPGES is considered as an inducible enzyme similar to COX-2, we tested whether well-defined stimuli for COX-2, such as changes in salt intake and angiotensin I-converting enzyme (ACE) inhibition by captopril, would also regulate mPGES expression in the MD segment of renal cortex. Figure 4 demonstrates that mPGES and COX-2 were colocalized in the MD segment. A low-salt diet significantly increased the number of both COX-2- and mPGES-immunoreactive MD cells per MD plaque (≈5.5- and 3-fold, respectively, n = 6 MD plaques in each group, P < 0.05). High-salt intake caused small reductions in both COX-2 and mPGES expression (Fig. 4). ACE inhibition with captopril, similar to low-salt diet, greatly increased the expression of both COX-2 and mPGES in the MD (≈8- and 3.5-fold, respectively, P < 0.05). No specific change in mPGES localization, compared with control tissue, was observed in response to varying salt intake and captopril treatment including expression in PC of the collecting tubule.

**DISCUSSION**

The present study provides a detailed description of the localization of mPGES protein in the rabbit kidney (Fig. 5). In contrast to the renal cortex, a high level of mPGES expression was found in the medulla, the major site of PGE2 synthesis (1, 24). Strong labeling in the
collecting duct system and DTL is also consistent with earlier enzyme immunoassay measurements (1, 8), demonstrating that these nephron segments are the major sites of PGE$_2$ synthesis in the kidney. However, this enzyme immunoassay (8) used isolated nephron segments microdissected from rabbits, the same species we used for immunohistochemistry, so there was no information obtained from vascular structures and the interstitium. Also, the use of dissected tubules (8, 27) or cell cultures with mixed phenotype (7, 9) did not allow comparison between different cell types within the individual segments. Present immunolocalization data are similar to findings of a very recent report (6) that described the site-specific expression of key enzymes for renal prostaglandin synthesis in rodent kidney. The only exception seems to be the significant mPGES expression in the DTL in rabbit kidney that was apparently not present in rat and mouse.

This study is an extension of our recent work that detected PGE$_2$ release from MD cells and first described, in preliminary form, the localization of mPGES in MD cells (22, 23). Expression of mPGES in the MD is consistent with the presence of COX-2 in these cells (4, 13, 23, 25, 28) and also with PGE$_2$ production and release from MD cells (22, 23). However, a recent report failed to detect mPGES mRNA in MD cells from the rat kidney (27). These conflicting results may represent different sensitivities of methods used. Identification of PC in the collecting tubule was based on double labeling immunohistochemistry using an AQP2 antibody that selectively binds to mainly the apical membrane of PC in various species (17, 20). Consistent with this, we found strong AQP2 labeling of PC at the apical membrane (Fig. 2C).

Distinct localization of mPGES in PC but not in IC cells has not been reported previously. Each of the PGH$_2$-producing COX isoforms, but particularly COX-1, is expressed in the collecting duct system; however, the localization seems to be species, cell type, and physiological status dependent (4, 9, 13, 14, 26). A
high level of PGE2 synthesis was detected in both isolated CCD and MCD (8), and there are well-established inhibitory effects of PGE2 on PC Na+ and water transport (1). However, there are no known specific effects of PGE2 on IC cells, except that a recent report suggested, indirectly, the presence of a PGE receptor [most likely E prostanoid (EP) receptor EP3] in these cells (15). At present, the functional importance of this distinct mPGES localization in the CCD and OMCD is not known. It is possible that other as yet to be localized PGE synthase isoforms (18) are also expressed in the collecting tubule and participate in PGE2 biosynthesis. Understanding the role of PC-specific, mPGES-derived PGE2 synthesis on collecting tubule function will require knowledge of cell-specific expression of different EP receptors in this nephron segment. PGE2 interacts with four different G protein-coupled EP receptors designated EP1, EP2, EP3, and EP4 (2, 3), and all of these receptors appear to be expressed in the collecting tubule (3, 11). Thus PGE2 synthesis by COX and PGES isoforms may exert local autocrine or paracrine actions in the collecting tubule via one or more of these receptors, and this complex interaction needs to be further investigated.

Expression of mPGES in the DTL is consistent with earlier work (8) that detected significant PGE2 synthesis in individual DTLs microdissected from the rabbit kidney. It has been suggested that PGE2, synthesized in this segment, may regulate tubular transport (1, 8), but it may also influence medullary blood flow by acting on nearby pericytes of DVR (Fig. 2E). Identification of DVR was based on the presence of red blood cells in the lumen (Fig. 3B) and double labeling immunohistochemistry using an AQP1 antibody that selec-
tively binds to DTL and DVR (21). Consistent with this, we found strong AQP1 labeling in these structures (Fig. 3D). Absence of mPGES in DVR (Fig. 3, B and E) suggests that this vascular segment cannot synthesize PGE₂; however, it does not rule out that PGE₂ production by DTL, MCD, and medullary interstitial cells may cause DVR vasodilation and an increase in medullary blood flow. Indeed, mPGES labeling was intense in RMIC (Fig. 3D), a special cell type in the medulla that expresses COX-2 (10) and interconnects tubular structures with each other and with vasa recta. The absence of mPGES labeling in both cortical and medullary vascular structures, but its presence in adjacent tubular segments and cell types, suggests a paracrine effect for PGE₂ on renal hemodynamics.

The present studies also demonstrated that mPGES, an enzyme capable of converting COX-2-derived PGH₂ to PGE₂, was not only present in the rabbit MD (Fig. 2A) but that it colocalizes with COX-2 in these cells (Fig. 4). In addition, we examined if well-established stimuli for COX-2 (i.e., changes in salt intake and ACE inhibition) would also induce mPGES expression. We found that MD expression of both COX-2 and mPGES was greatly upregulated in response to low-salt intake and ACE inhibition by captopril (Fig. 4). Also, high-salt intake caused a small reduction in COX-2 and mPGES staining in the MD. These findings indicate that, consistent with other cell types (16, 18, 19), mPGES in the MD is an inducible enzyme, similar to COX-2. Low-salt intake upregulates cortical COX-2 expression in various species including rabbit (14, 28). Colocalization of COX-2 and mPGES in the MD and their upregulation by a low-salt diet support the notion that these enzymes share a common gene-regulatory mechanism (16, 18, 19) and may contribute to the increase in PGE₂ synthesis associated with stress, inflammatory, and pyretic responses. Consistent with these immunofluorescence findings, recent functional experiments using a biosensor technique (22, 23) demonstrated significantly increased PGE₂ release from MD cells on a low-salt diet, compared with normal-salt intake. Further work is needed to characterize the regulation of mPGES expression in response to various other stimuli and in the renal medulla.

In summary, the present studies localized a PGES in DTL, MD, and PC of the collecting duct system and medullary interstitial cells in the rabbit kidney. Regulation of the mPGES protein expression by various salt diets and ACE inhibition suggests that mPGES is an inducible enzyme, similar to COX-2. Understanding the importance and complex effects of PGE₂ synthesized by mPGES at these sites of the nephron requires further studies.

We thank J. Hosmer, University of Alabama at Birmingham (UAB) Animal Resources Program, Histology Division, for excellent technical assistance with tissue processing and sectioning. Also, the authors thank A. Tousson and S. Williams at UAB High Resolution Imaging Facility for help with fluorescence imaging.

DISCUSSIONS

This work was supported by grants from National Institute of Diabetes and Digestive and Kidney Diseases (153032) to P. D. Bell and American Heart Association SDG (0230074N) and ASN Carl W. Gottschalk Research Scholar Grant to J. Peti-Peterdi.

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