Epithelial COX-2 Expression Is Not Regulated By Nitric Oxide in Rodent Renal Cortex

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Abstract—In the adult rodent kidney cortex, cyclooxygenase-2 (COX-2), NO synthase (NOS1), and renin synthesis change in parallel on alterations in distal tubular NaCl concentration, and their products in part may mutually determine synthesis and activity of these enzymes. Epithelial NO synthesis has been postulated to exert a stimulatory role on COX-2 expression. Changes in COX-2 and NOS1 may be assessed histochemically by determining changes in the number of positive cells. In rat, macula densa and adjacent cells may co-express COX-2 and NOS1, whereas cell groups of the upstream thick ascending limb (cTAL) express COX-2 alone. We have tested whether the stimulation of COX-2 expression by short- and long-term unilateral renal artery stenosis, low salt, and furosemide treatment depends on co-expression of NOS1. These conditions produced significant respective increases (40% to 351%, P<0.05) in the number of COX-2 immunoreactive cells, regardless of whether NOS1 was present or not, suggesting that co-expression of NOS1 is not necessary to produce these changes. Under high-salt conditions, analogous though inverse changes were recorded (−62% to −73%, P<0.05). In mice with genetic deletion of NOS1, low- and high-salt diets caused similar changes of COX-2 immunoreactivity (106% and −52%, P<0.05) than those seen in wild-type mice (43% and −78%, P<0.05). We conclude that alterations of distal tubular NaCl concentration and presumably NaCl transport induce changes in epithelial COX-2 expression that does not depend on presence of co-expressed NOS1. It therefore seems unlikely that NO is part of a signal transduction chain between tubular chloride sensing and the modulating effects of prostaglandins in tubulo-vascular information transfer. (Hypertension. 2002;39:848-853.)

Key Words: nitric oxide synthase • macula densa • renin • juxtaglomerular apparatus • prostaglandins

The mammalian kidney is an important site for the synthesis and action of products derived from cyclooxygenase (COX) isoforms (prostaglandin synthase, G2/H2), which catalyze the metabolism of arachidonic acid into biologically active prostanoids.1 Functional roles for eicosanoid products of COX include the orthograde formation of the nephron during ontogenesis2 and the modulation of renal salt and water excretion.3 Recent evidence has highlighted the roles of COX-2 in the tubulo-vascular signaling mechanism of the juxtaglomerular apparatus.4–8 The macula densa mechanism for control of renin secretion depends on COX-2–mediated prostaglandin synthesis, because the stimulation of renin activity, secretion, and mRNA expression in response to a decrease in luminal NaCl concentration could be reduced by the administration of COX-2 inhibitors or nonsteroidal anti-inflammatory drugs.9–10 Additional evidence came from COX-2–deficient mice that failed to increase renal renin content under low-salt conditions.11 In the rodent renal cortex, the principal site of COX-2 expression is the cortical thick ascending limb (cTAL), including the macula densa region.12,13 COX-2 expression is increased in high-renin states whether induced by blockade of the renin angiotensin system,14,15 salt restriction,9,12,16 unilateral renal artery stenosis,10,17 or Na,K,2Cl-cotransport inhibition.18

Neuronal NO synthase (NOS1) is partly co-localized with COX-2–expressing cells of the macula densa and neighboring cTAL cells,13 and conditions that alter COX-2 expression have earlier been shown to induce analogous changes of NOS1.13,19–21 It has therefore been proposed that NO may also be involved in the modulation of the tubulo-vascular responses.3 Because NO can activate COX22 and because inhibition of NOS can decrease juxtaglomerular COX-2 levels,5,14 it was suggested that NO may be a positive regulator of COX-2. To further investigate whether local NO synthesis is required for a stimulation of COX-2 expression, we studied the co-expression of COX-2 and NOS1 at the level of the single cell in rat models in which global upregulation of these 2 enzymes has previously been established. NOS1-deficient mice were used to corroborate our results.
Methods

Animals
Adult male Sprague Dawley rats, male NOS1-null mutant mice (NOS1<sup>−/−</sup>), and wild-type control mice (NOS1<sup>+/+</sup>) were from the local animal facilities.

Treatments
All treatments and the settings of the control groups were as detailed earlier<sup>19,31</sup>: (1) 2-kidney, 1-clip Goldblatt model of renovascular hypertension during a 3-day early phase and a 28-day maintenance phase (total, n = 16) and (2) continuous infusion of furosemide by subcutaneously implanted minipumps for 5 days (total, n = 8). The treated rats received 0.3% NaCl and 0.1% KCl as drinking fluid to compensate for the loss of electrolytes.<sup>18</sup> (3) Low- (LSD), normal- (NSD), and high-salt (HSD) diets for 8 days in rats (total, n = 12), and (4) LSD and HSD for 11 days in mice (total, n = 8). Body weight and urine osmolality (24-hour values from metabolic cages) were controlled throughout all experiments. Kidneys of all animals were perfusion-fixed at the end of the experiments.

Histochemistry
For histochemical demonstration of NOS tissue activity, the enzymatic reduction of nitroblue tetrazolium in the presence of NADPH (NADPH diaphorase reaction) was used on cryostat sections as described.<sup>13,30</sup> For immunohistochemistry, the following primary antibodies were used: (1) rabbit monoclonal antibody against an exon 2-encoded NOS1 domain (amino acids 1 to 181 from rat brain; Sigma), (2) rabbit polyclonal antibody against an exon 12 to 21-encoded NOS1 domain (Calbiochem), (3) goat polyclonal antibody against a C-terminal COX-2 peptide (Santa Cruz Biotechnolgy), and (4) rabbit polyclonal antibody against rat Tamm Horsfall protein (THP; against the whole protein; gift from J. Hoyer, Philadelphia). For detection, Cy3-coupled goat anti-rabbit or donkey anti-goat, and Cy2-coupled mouse anti-rabbit antisera were used (Dianova). Cell nuclei were stained with 4′,6-diamino-2-phenylindole (Sigma). Standard incubation procedures were used as described.<sup>13,30</sup> In double-labeling experiments, suitable secondary antibodies coupled to different fluorochromes were applied.

In Situ Hybridization
For visualization of mRNA expression, in situ hybridization was performed using digoxigenin-UTP labeled riboprobes made from 1300-bp COX-2 cDNA fragments and using an alkaline-phosphatase–generated signal detection as given in a previously established protocol.<sup>13</sup>

Histochernical Quantification of COX-2 and NOS1 Signals
For histochemical quantification of COX-2– and NOS1-positive cells, an earlier described methodology was adapted.<sup>13,30</sup> Renal cortical immunoreactive signals were determined by counting COX-2– and NOS1-positive cells in 3 distinct microanatomical locations: (1) within the macula densa proper, as defined by the absence of THP Tamm Horsfall protein<sup>21</sup>; (2) in the macula densa region identified by double staining for both antigens; and (3) in the cTAL, counting only those cells that were NOS1-negative in COX-2/NOS1 double-stained sections. In mice, COX-2 staining was restricted to the macula densa, so only these cells were counted.

To verify changes in NOS1 activity, NADPH diaphorase staining was evaluated as described.<sup>19</sup> Results indicated the same tendency and extent of changes in all experimental groups, as reported in the previous paper, and are not shown.

COX-2 mRNA expression was quantified in sections treated with in situ hybridization. All positively labeled sites were counted throughout the renal cortex. Whenever >1 signal was located in the same tubular profiles, the signals were counted as a single signal.

Results from numerical evaluation were expressed as the number of labeled cells or sites per 100 glomeruli. For all quantifications, which were blinded to the evaluator, a total volume of 400 to 600 glomeruli was considered. For statistical analysis, the Lord test (1-way test), which is used for small sample numbers,<sup>24</sup> or ANOVA were applied where appropriate. Differences of a level of P < 0.05 were considered significant.

Results

Microanatomical Distribution of COX-2
COX-2 immunoreactive cells were encountered in 3 typical locations: in the macula densa proper, as defined by the THP-negative portions of the cTAL; in the macula densa region of the cTAL adjacent or opposite to the macula densa; and in upstream portions of the cTAL, located at varying distances to the glomerular attachment site (Figure 1). In macula densa cells, COX-2 was detected as a faint nuclear membrane–associated signal compared with that in cTAL cells, where the intracellular signal was generally more widespread and stronger. Double staining of COX-2 and NOS1 revealed that in the NOS1 immunopositive macula densa proper, no more than 1 or 2 COX-2–expressing cells were encountered per positive site, whereas cells co-expressing NOS1 and COX-2 were more frequent in the macula region (Figure 1a through 1m). In sites of the cTAL distant to the glomerulus, single cells or groups of cells showing COX-2 immunoreactivity alone were found (Figure 1n through 1r), whereas NOS1 co-expressing cells were principally absent from these portions, independent of the physiological status of the animal. In situ hybridization for COX-2 in rats showed a lower number of labeled cells compared with that obtained with the corresponding antibody (Figure 2i and 2k). With both techniques, however, signals were located in analogous positions of the distal nephron. In contrast to rats, COX-2 immunoreactivity in control mice was located mostly in macula densa cells proper, whereas cTAL cells were rarely stained (Figure 2g and 2h); as in rats, the COX-2 signal was confined to a perinuclear zone. COX-2 mRNA expression was restricted to single scattered cells in the juxtaglomerular region (data not shown). NOS1<sup>−/−</sup> mice were used to test whether COX-2 expression is regulated in the absence of epithelial NOS1 activity. Because residual NOS1 activity in these mice may potentially be derived from splice variants lacking exon 2 (NOS1<sub>β</sub> variant), we evaluated NOS1 activity by the NADPH diaphorase reaction, and NOS1 expression by in situ hybridization with a NOS1<sub>3′</sub>–specific riboprobe and by immunocytochemistry with an antibody against an N-terminal domain of the protein. These approaches did not identify NOS1 in macula densa or any other tubular epithelial sites in the NOS1<sup>−/−</sup> mice (Figure 2a through 2f).

Regulation of COX-2 Expression in Rats
The number of cells expressing COX-2 and/or NOS1 was determined in rats with short-term or long-term unilateral renal artery stenosis, and in rats treated with LSD, NSD, HSD, or furosemide, and compared with the mean of the respective control group.

The number of COX-2 immunoreactive cells in the macula densa proper, as defined by the lack of concomitant THP immunoreactivity and the obligatory expression of NOS1, was increased by 81% after short constriction and 90% after
long constriction \( (P<0.01) \) in the clipped kidney, by 40% in the LSD group \( (P<0.05) \), and by 195% in the furosemide group \( (P<0.01) \). By contrast, COX-2–positive cells were decreased by 53% in the nonclipped kidney after short constriction, by 70% after long constriction \( (P<0.01) \), and by 60% in the HSD group \( (P<0.01) \; \) Figures 1 and 3a).

The number of COX-2 immunoreactive cells of the macula densa region, where cells express both NOS1 and COX-2, increased by 71% after short constriction and 90% after long constriction \( (P<0.01) \) in the clipped kidney, by 45% in the LSD group \( (P<0.05) \), and by 70% in the furosemide group \( (P<0.01) \). The number of these cells had decreased by 41% in the nonclipped kidney after short constriction, by 31% after long constriction \( (P<0.05) \), and by 68% in the HSD group \( (P<0.01) \; \) Figures 1 and 3b).

Finally, the number of COX-2 immunoreactive cells of the upstream cTAL portions, which throughout all experiments did not express NOS1, had increased by 73% after short constriction and 75% after long constriction \( (P<0.01) \) in the clipped kidney, by 59% in the LSD group \( (P<0.05) \), and by 351% in the furosemide group \( (P<0.01) \). By contrast, the cell number had decreased by 14% after short constriction and 13% after long constriction \( (P=\text{NS}) \) in the nonclipped kidney and by 73% in the HSD group \( (P<0.01) \; \) Figures 1 and 3c).

The number of NOS1 immunoreactive cells of the macula densa and macula densa region together had increased by 35% \( (P<0.05) \) in the short-term and 50% \( (P<0.01) \) in the long-term clipped kidney, by 41% \( (P<0.01) \) in the LSD group, and by 44% \( (P<0.01) \) in the furosemide group and decreased by 14% in the short-term clipped kidney \( (P<0.05) \), by 16% \( (P<0.01) \) in the long-term clipped kidney, and by 30% in the HSD group \( (P<0.01) \; \) Figures 1 and 3d).

The number of cortical COX-2 mRNA-expressing sites was increased by 66% in the clipped kidneys and decreased by 36% in the nonclipped kidneys in the maintenance phase of renal artery stenosis \( (P<0.05) \). In the furosemide group, a 491% increase was measured \( (P<0.01) \; \) Figures 2i, 2k, and 3e). Results in rats are summarized in Figure 4.

**Figure 1.** Representative views of differences in specific immunoreactivity of the macula densa region (a through m) and TAL (n through r) from rat kidneys subjected to various experimental conditions as indicated on the left. a through m, Single fluorescent signals of COX-2 (red) and NOS1 (green), and overlay images of signals indicated by merge are documented. n through r, Only overlay images of COX-2 (red) and THP (green) are presented; NOS1 was absent from these segments. In the left block, signals for COX-2 and NOS1 are high in the clipped Goldblatt kidneys (a through c), low in the nonclipped kidneys (d through f), and high after LSD (g through i) and furosemide treatment (k through m). In the right block, analogous quantitative changes are shown for scattered COX-2 immunoreactive cells. Nuclei are 4',6-diamino-2-phenylindole–stained for better identification. G indicates glomerulus.

**Regulation of COX-2 Expression in Mice**

In these experiments, the number of COX-2–expressing cells was compared between wild-type control mice and NOS1 \(-/-\) mice. Because COX-2 immunoreactivity is restricted to the macula densa in mice, only macula densa cells were evaluated. The numbers of COX-2–positive cells were not different between NOS1 \(-/-\) and wild-type under control conditions. After LSD treatment, COX-2 immunoreactive cells were increased by 43% in weight and by 106% in NOS1 \(-/-\) compared with the respective genotype on a control diet \( (P<0.05) \), and decreased after HSD by 78% in wild-type and 52% in NOS1 \(-/-\) \( (P<0.05) \); Figure 2g and 2h. Absolute values are given in Figure 3f).
Discussion

Partial co-expression and the co-regulation of COX-2 and NOS1 have been the basis of the hypothesis that local NO synthesis may be a determinant of COX-2 activity or COX-2 expression. In the present studies, we have used a quantitative histochemical approach to examine whether known COX-2–inducing stimuli can be differentiated in their stimulatory potency depending on the exact location of the COX-2 expressing tubular cells. In particular, we aimed at disclosing whether co-expression of NOS1 is required for COX-2 regulation. Thus, we have distinguished levels of COX-2 expression between cells characterized by presence (macula densa region) or absence (upstream cTAL) of co-expression with NOS1. In view of the well-established similarities in the expression of transport-related gene products between these sites, a comparison between these cells is a useful test of a role of NOS1 on the regulation of COX-2 expression.

The experimental conditions used in rats in this study resulted in changes of COX-2 and NOS1 expression that were comparable to those reported earlier under similar circum-

Figure 2. Representative views of NOS1 activity/mRNA expression/immunoreactivity in control and NOS1−/− mice (a through f), of the effects of LSD and HSD on COX-2 immunostaining in NOS1−/− mice (g and h), and of COX-2 mRNA expression in furosemide-treated rats (i and k). NADPH diaphorase staining (a and b), NOS1 in situ hybridization using NOS1 3′-specific riboprobes (c and d), and NOS1 immunostaining using an antibody directed against an exon 12 to 21-encoded NOS1 domain of the protein (e and f) show clear signals in macula densa of the wild-type mice, but no signal in the knockout mice. COX-2 signal is markedly stronger after LSD and stains more macula densa cells (g) than after HSD (h). COX-2 mRNA expression is much stronger in furosemide-treated rats (k) than in untreated controls (i); macula densa between arrowheads. The mRNA signals in panels i and k are mostly in cTAL.

Figure 3. Calculated changes in numbers of COX-2– and NOS1-positive cells in kidney cortex. Experimental conditions in a through e (1, short-term clip; 2, short-term nonclip; 3, long-term clip; 4, long-term nonclip; 5, LSD; 6, HSD; and 7, furosemide treatment) are compared with the respective control levels within the distinct epithelial locations. f, Values show absolute cell numbers in wild-type (left) and NOS1−/− mice (right) under LSD, NSD, and HSD, respectively; statistical analysis compares LSD and HSD groups to their respective NSD controls. * indicates individual values; △, mean values. *P<0.05, #P<0.01.
It is conceivable that NO contributes to the regulation of COX-2 in cells that express NOS1, but that COX-2 regulation uses other pathways in cells without concomitant NOS1 expression. In fact, in the work of Cheng et al, it appears that suppression of COX-2 by 7-nitroindazole is most pronounced in the macula densa and less dramatic in the surrounding cTAL cells. A direct, cGMP-dependent activation of COX-2 in cultured rabbit cTAL cells has also been reported. We had previously found that at least in rat, however, neither cTAL nor macula densa cells contained detectable amounts of soluble guanylyl cyclase, the cGMP-catalyzing enzyme. Thus, it is unclear whether an NO-dependent cGMP pathway can affect prostaglandin synthesis within the same cell in situ.

Recently an alternative, cGMP-independent intracellular mechanism of COX-2 induction initiated by phosphorylation of extracellular signal-regulated kinase (ERK)1/2 as well as p38 kinase has been described that may be active in the present conditions; ERK and p38 activation may in fact stimulate COX-2 gene expression by transcriptional and posttranscriptional regulation.

Our complementary studies in mice show that macula densa COX-2 immunoreactivity was not different between wild-type controls and NOS1 knockout mice, suggesting that presence of local NOS1 is not a prerequisite for macula densa-specific expression of COX-2. Furthermore, stimulation of COX-2 expression in macula densa cells by a LSD was comparable in NOS1+/− and wild-type mice. Because we documented total absence of tubular NOS1 expression in the NOS1−/− mice, these results confirm our conclusions from the rat studies that an intracellular co-expression of COX-2 and NOS1 and the direct availability of NO is not required.

In summary, our results indicate that an activation of epithelial COX-2 expression by a reduction in NaCl intake in rats is comparable in macula densa cells and in cTAL cells near or distant from the macula densa, and that coexpression of NOS1 does not modify the degree of COX-2 stimulation. Furthermore, NOS1-deficient mice express COX-2 at similar levels as those of wild-type mice, and a low salt intake stimulates COX-2 expression to a similar degree in both strains of mice. We therefore conclude that intracellular or ambient NO levels are not required for the activation of COX-2.

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References


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