Nitric Oxide, Anti-Inflammatory Drugs on Renal Prostaglandins and Cyclooxygenase-2

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Abstract—Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used as analgesics. They inhibit cyclooxygenases (COX), preventing the formation of prostaglandins, including prostacyclin and thromboxane. A serious side effect of COX-1 and COX-2 is renal damage. We report here that both a nonselective NSAID (aspirin, acetylsalicylic acid) and COX-2 selective NSAIDs (celecoxib and NS-398) diminished renal prostacyclin and thromboxane concentration in the renal medulla. NSAIDs failed to change COX-2 and iNOS (the inducible form of NO synthase) expression. A NO donor, B-NOD, preserved renal prostacyclin and thromboxane after administration of aspirin. PGI2 and COX-2 protein were mainly expressed in the renal medulla, whereas iNOS expression was greater in the cortex. B-NOD preserved renal prostacyclin levels after administration of NSAIDs. (Hypertension. 2002;39:785-789.)

Key Words: prostaglandins ■ kidney ■ aspirin ■ nitric oxide

Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used as analgesics and in the treatment of osteoarthritis and other chronic inflammatory diseases. NSAIDs act by inhibition of cyclooxygenases. Two isoforms of COX have been identified: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). They are homodimers, heme-containing glycosylated proteins with 2 catalytic sites. Aspirin, the classical nonselective NSAID, inhibits platelet thromboxane A2 formation through inhibition of COX-1; this forms the basis for the therapeutic and preventive effects in coronary artery disease.

Inhibition of COX can cause severe gastric disturbances because of diminished prostacyclin synthesis in the gastric mucosa. To overcome this side effect, selective COX-2 inhibitors, such as rofecoxib (VIOXX), celecoxib (Celebrex), and NS-398 have been developed. These selective COX-2 inhibitors protect the gastric mucosa but, like nonselective COX inhibitors, cause renal damage, which is expressed as a reduction in glomerular filtration rate, renal blood flow, and diminished sodium and potassium excretion.

The decline in renal function is especially pronounced in the elderly and in patients with preexisting renal disease. The renal effects are related to depletion of prostacyclin. On the other hand, Wang et al found that chronic administration of a selective COX-2 inhibitor decreased proteinuria and inhibited development of glomerular sclerosis in rats with reduced functional renal mass. The decrease in proteinuria was comparable to that seen with an ACE inhibitor.

Prostanoids result from the activity of cyclooxygenases on arachidonic acid. Prostaglandins modulate renal microvascular hemodynamics, renin release, and tubular salt and water reabsorption. Prostaglandins diminish vascular resistance in the renal vascular bed, increase perfusion, and mediate natriuretic processes; their presence maintains glomerular filtration rate. Diminution of renal prostacyclin results in papillary necrosis or in interstitial nephritis. Urinary sodium excretion is also reduced after application of celecoxib. The role of COX-2 in the mammalian kidney has been thoroughly investigated. It was found that it is constitutionally expressed and that COX-2 mRNA is present at detectable concentrations in normal adult rat kidneys, particularly in microsomes, cortex, and papilla. Immunoreactivity of COX-2 mRNA was also localized in cells of the macula densa and adjacent cortical thick ascending limb.

Recently, we described the synthesis of a new compound, B-NOD, which in vitro and in situ releases NO. B-NOD does not cause a fall in blood pressure and can be orally administered. We previously suggested that a combination of aspirin with different concentrations of B-NOD might be useful in diminishing the side effects of aspirin. This paper presents the effect of a nonselective COX-1 and COX-2 inhibitor, aspirin, and of the selective COX-2 inhibitors celecoxib (Celebrex) and NS-398 on the renal concentration of prostacyclin (PGI2) and thromboxane A2 (TXA2), as well as on the activation in situ of the inducible form of NO synthase (iNOS) in the renal cortex and medulla of the rabbit. The data clearly show that the administration of aspirin, celecoxib, and NS-398 causes depletion of renal prostacyclin and thromboxane in the kidney, and that the NO donor B-NOD partially offsets the decline caused by aspirin. COX-2, thromboxane, and prostacyclin are mainly concentrated in the medulla, whereas iNOS expression is greater in the cortex.
Methods

Healthy male New Zealand rabbits (3.2 to 3.5 kg; Irish Farms, Norco, Calif) were used. The protocol was approved by the Institutional Animal Care and Use Committee. The animals were divided into 5 groups: a control group (n=6), a group receiving acetylsalicylic acid (aspirin, n=5), a group (n=6) receiving aspirin together with the NO donor B-NOD, a group (n=5) exposed to NS-398, and a group receiving celecoxib (n=5). The total administered doses were as follows (in mg/kg): aspirin 105, aspirin with B-NOD 180, NS-398 15, and celecoxib 15. We used a relatively high dose of aspirin to compare the present data with those previously reported on the infarcted heart in situ. Administration was by stomach tube, giving 3 divided doses; on the first day, administration was 6 hours apart. On the second day, compounds were administered 5 hours before euthanasia. All compounds were dissolved in DMSO (2 mL) and carboxymethylcellulose (8 mL). Before euthanasia, the animals were anesthetized with ketamine (40 mg/kg IM), xylazine (5 mg/kg IM), and sodium pentobarbital (50 mg/kg IV). The kidneys were exposed and excised, and specimens from the cortex and medulla were collected and weighted. Samples of both kidneys were analyzed and averaged.

Biochemical Methods

Assay of iNOS Activity

The iNOS activity was determined as previously described using the conversion of L-[14C]arginine to L-[14C]citrulline.22 Fifty to 100 mg of tissue were prepared from the frozen specimens. After homogenization of the tissue in 900 μL of cold Tris-HCl buffer (0.05 mol/L, pH 7.4) containing D,L-dithiothreitol (1.0 mmol/L), leupeptin (10.0 μmol/L), phosphoramidon (25.0 μmol/L), and aprotonin (100.0 μmol/L) (all from Sigma Chemical Co) and after sonication for 15 seconds, the homogenate was centrifuged (1200g, 5 minutes), and EDTA (0.5 mmol/L) and NADPH (1 mmol/L) (Sigma) were added to 65 to 75 μL of supernatant. L-[14C]arginine (200 000 cpm, Amersham Life Science) was added, and the samples were incubated for 30 minutes in a 37°C water bath. The reaction was stopped by adding ice-cold Tris-HCl buffer (pH 5.5). Radiolabeled citrulline was separated from arginine by cation exchange chromatography (Dowex 50-WX8, 200 to 400 mesh, Na-form, BioRad Laboratories). The eluate (~3.5 mL) was mixed in 10 mL of scintillation fluid and counted in triplicate in a Beckman LS 100S scintillation counter. All values were corrected for protein content of the samples (determined by the modified Lowry Assay) and calculated in picomole per milligram of protein per minute.

Assay of PGI2 and TXA2

PGI2 and TXA2 were measured as their stable metabolites, 6-ketoprostaglandin F1α (PGF1α), and thromboxane B2 (TXB2), by using enzyme immunoassay kits (Cayman Chemical Co) as previously described.22 In brief, 5 to 10 mg of tissue were prepared from each of the 4 specimens. The tissue from each sample was weighed and homogenized in 2 mL of ethyl alcohol. The homogenate was stored at 4°C for 5 minutes and then centrifuged (1000g, 15 minutes) to remove precipitate. The supernatant was added to 8 mL of double distilled water, and pH was adjusted to 4.0 with dilute HCl. The sample was passed through the C-18 reverse-phase cartridge (Seph-Pak Cartridge, Waters Corp), and the cartridge was rinsed with 5 mL of double distilled water followed by 5 mL high-performance liquid chromatography grade hexane (Sigma). PGF1α and TXB2 were eluted with 5 mL ethyl acetate containing 1% methanol and evaporated under a stream of dry nitrogen. The dried samples were reconstituted by enzyme immunoassay buffer and used for enzyme immunoassay analysis.

Western Blotting

In principle, the procedures of Brunette22 and of Guesdon22 were followed. In brief, samples from rabbit kidney cortex and medulla were minced with scissors and then lysed with in RIPA-lysis buffer for 30 minutes in ice, followed by sonication and centrifugation. The protein concentration of the supernatant was determined by bicinchoninic acid protein assay. Fifty micrograms of each protein extract were loaded into a 10% Tris-glycine gel (C-20, Santa Cruz Biotechnology; 1:150) overnight at 4°C. After washing 3 times in TBST, the membrane was incubated in a biotinylated anti-goat IgG secondary antibody (Vector) for 1 hour at room temperature. The membrane was again washed 3 times in TBST before incubating with avidin and biotinylated horseradish peroxidase macromolecular complex from Vectastain ABC kit (ABC reagent) for 1 hour. Antibody binding was visualized by development with 3,3’-diaminobenzidine substrate kit for peroxidase (DAB) solution (Vector). Blots were scanned into Adobe PhotoShop, and then band intensities were quantified with Image ProPlus software (Media Cybernetics).

Statistical Analysis

Data are presented as a mean±SEM. All differences were compared by the unpaired Student’s t test. Values of P<0.05 were considered significant.

Results

Both selective and nonselective COX-2 inhibitors (aspirin, celecoxib, and NS-398) significantly depressed PGF1α and TXB2 concentrations in the kidney (P<0.01) (Figures 1 and 2). Control values for the stable form of prostaglandin F1α (PGF1α) and the stable form of thromboxane B2 (TXB2) concentrations were 592 and 16.73 pg/mg, respectively (Table). Aspirin was the most effective compound, lowering the stable form of PGF1α to 75.50 pg/mg and of TXB2 to 3.5 pg/mg (Table 1). The difference between the effect of aspirin and the specific COX-2 inhibitors was significant (P<0.05) (Figures 1 and 2). Apparently specific COX-2 inhibitors also depressed prostanoids in the renal medulla.

The effect of the NO donor B-NOD in countering the loss of renal prostaglandin concentration was marked. As shown
in Figures 1 and 2 and the Table, B-NOD partially offset the effect of aspirin on renal medullar prostacyclin F\textsubscript{1}\text{a}H\textsubscript{2}\text{a} (75.5 and 261.43 pg/mg for aspirin versus aspirin and B-NOD, respectively, \(P<0.01\)) (Table). Although NSAID compounds depressed formation of prostaglandin, they failed to alter the expression of the iNOS (Figure 3, Table). Equally, COX-2 protein levels were not influenced by administration of either selective or nonselective COX-2 inhibitors (Figure 4).

Concentrations of prostaglandins and the expression of COX-2 were predominant in the renal medulla, whereas the renal cortex was the main location of iNOS expression. (Figures 1 and 4).

**Discussion**

It was the purpose of this study to explore the effect of nonselective (aspirin) and selective NSAIDs (celecoxib and NS-398) on prostacyclin, thromboxane, iNOS, and COX-2 in the renal medulla and cortex. Additionally, the relationship of a NO donor, B-NOD, on these parameters was investigated.

**NSAIDs and B-NOD**

In the renal medulla, aspirin causes a significant decline in PGF\textsubscript{1}\text{a} and TXB\textsubscript{2} (Figures 1 and 2, Table). The 2 selective COX-2 inhibitors, celecoxib and NS-398, had similar effects (Figures 1 and 2, Table).

Our results differ with those of Wang et al,\textsuperscript{7} who used a specific COX-2 inhibitor SC58236 on rats with renal ablation. Using proteinuria and glomerular sclerosis as indicators, they found that the COX-2 inhibitor decreased proteinuria and glomerular injury.\textsuperscript{7} The authors believed that COX-2 inhibition decreases production of prosclerotic eicosanoids and that modulation of inflammatory stimuli or of glomerular hemodynamics is responsible.\textsuperscript{7} It is possible that the discrepancy between the data of Wang and our result is owing to different criteria for renal damage. Wang’s experiments were performed on rats with renal ablation, whereas our studies were made on normal kidneys of rabbits.

The NO donor, B-NOD, administered jointly with aspirin offsets this decline (Figures 1 and 2). It releases NO for several hours without causing a fall in blood pressure or an increase in heart rate.\textsuperscript{10} B-NOD increases cyclic guanosine monophosphate (cGMP) and prevents platelet aggrega-
tion. B-NOD accomplishes this through NO release, which increases prostaglandin formation. B-NOD has potential clinical relevance because the release of NO counters the diminution of prostacyclin by aspirin and thereby inhibits platelet aggregation (Figures 1 and 2, Table).

Deterioration of renal function by selective COX-2 inhibitors has been repeatedly demonstrated. Rofecoxib causes renal damage, particularly when administered as a single dose, and celecoxib and rofecoxib induce a transient decline in renal function, particularly in patients with hypertension, cardiovascular disease, and renal failure. Therefore, selective COX-2 inhibitors seem to have no distinct advantage over nonselective NSAIDs as far as renal toxicity is concerned. Graham described the history of a patient who developed acute renal failure after having been prescribed 200 mg/d for 10 months for osteoarthritis. Moreland and St Clair also reviewed the adverse renal side effects of COX-2-selective NSAIDs.

iNOS and COX-2
In contrast to prostaglandins, iNOS expression is significantly greater in the renal cortex compared with the medulla, possibly because of the presence of NO synthase in the macula densa. Both selective and nonselective COX-2 inhibitors fail to diminish iNOS expression (Figure 3).

COX-1 and COX-2 proteins are constitutively expressed in the kidney. Both iNOS and COX-2 are activated by cytokines. Harris found that COX-2-mRNA is expressed in normal adult rat kidneys and is present in microsomes from cortex and papilla. COX-2 is also present in the macula densa and adjacent cortical thick ascending limb, where it regulates renin release. The direct role of COX-2 in the macula densa in mediating renin production was shown by the inhibition of the increase in renin expression during low-salt diet by administration of NS-398. In the renal medulla, Harris found localized COX-2 expression in medullary interstitial cells in the tip of the papilla. COX-2 is also localized in medullary collecting duct cells and in intercalated cells in the cortex. No COX-2 was detected in arterioles, glomeruli, or cortical medullary collecting ducts. COX upregulation in the macula densa in response to ACE inhibitors and angiotensin receptor antagonist has been previously shown. In addition, COX-2 in the rat kidney is under control by adrenal steroids and is upregulated under stimuli that call for increased renin synthesis and release.

Figures 1 and 2 show that PGI₂, TXA₂, and COX-2 are significantly higher in the renal medulla. In the rat kidney, the highest expression of COX-1 and COX-2 is also present in the inner medulla. In the mammalian kidney, prostaglandins regulate renal hemodynamics and electrolyte homeostasis and influence the renin-angiotensin system through changes in the macula densa, where COX-2 immunoreactivity is present.

In the macula densa, COX-2 is also upregulated by dietary salt restriction and by partial renal ablation and disruption of the renin-angiotensin signaling.

The effect of aspirin and celecoxib was previously described in the infarcted heart in situ. Similar to the kidney, in the heart, aspirin did not change myocardial iNOS production and celecoxib lowered myocardial PGI₂ production but did not interfere with the induction of iNOS.

In summary, we have shown that both selective and nonselective COX-2 inhibitors cause a significant decline in prostacyclin and thromboxane in the renal medulla, whereas expression of COX-2 and iNOS remains unchanged. Concentrations of prostacyclin in the renal medulla exceed those in the cortex, while expression of iNOS is greater in the cortex. A NO donor (B-NOD) offsets the effect of aspirin on renal prostaglandin.

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References
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