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

Masaru Miyataka, Kathryn A. Rich, Marylou Ingram, Tadahiko Yamamoto, Richard J. Bing

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. PG12 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

N onsteroidal anti-inflammatory drugs (NSAIDs) are frequently used as analgesics and in the treatment of osteoarthritis and other chronic inflammatory diseases.1 NSAIDs act by inhibition of cyclooxygenases.2 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.3 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.3 To overcome this side effect, selective COX-2 inhibitors, such as rofecoxib (VIOXX), celecoxib (Celebrex), and NS-398 have been developed.2,4 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.4 The decline in renal function is especially pronounced in the elderly and in patients with preexisting renal disease.5 The renal effects are related to depletion of prostacyclin.6 On the other hand, Wang et al7 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.7

Prostanoids result from the activity of cyclooxygenases on arachidonic acid.2 Prostaglandins modulate renal microvascular hemodynamics, renin release, and tubular salt and water reabsorption.8 Prostaglandins diminish vascular resistance in the renal vascular bed, increase perfusion, and mediate natriuretic processes; their presence maintains glomerular filtration rate.9 Diminution of renal prostacyclin results in papillary necrosis or in interstitial nephritis.9 Urinary sodium excretion is also reduced after application of celecoxib.9 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.8

Recently, we described the synthesis of a new compound, B-NOD, which in vitro and in situ releases NO.10 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.10

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 (PG12) 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.

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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.\(^1\) Administration was by stomach tube, given in 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.\(^{1,2}\) Fifty to 100 mg of tissue were prepared from the frozen specimens.\(^{1,2}\) After homogenization of the tissue in 900 \(\mu\)L of cold Tris-HCl buffer (0.05 mol/L, pH 7.4) containing \(DL^-\)dithiothreitol (1.0 mmol/L), leupeptin (10.0 \(\mu\)mol/L), phosphoramidon (25.0 \(\mu\)mol/L), and aprotinin (100.0 \(\mu\)mol/L) (all from Sigma Chemical Co) and after sonication for 15 seconds, the homogenate was centrifuged (1200 \(g\), 5 minutes), and EDTA (0.5 mmol/L) and NADPH (1 mmol/L) (Sigma) were added to 65 to 75 \(\mu\)L of supernatant. \(L^-\)Citrulline (200 000 cpm, Amersham Life Science) was added, and the samples were incubated for 30 minutes in a 37\(^\circ\)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 (\(\sim\)3.5 mL) was mixed in 10 mL of scintillation fluid and counted in triplicate in a Beckman LS 100SC 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 PGI\(_2\) and TXA\(_2\)

PGI\(_2\) and TXA\(_2\) were measured as their stable metabolites, 6-ketoprostaglandin \(F_1\alpha\) (PGF\(_{1\alpha}\)) and thromboxane \(B_2\) (TXB\(_2\)), by using enzyme immunoassay kits (Cayman Chemical Co) as previously described.\(^{11,12}\) 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\(^\circ\)C for 5 minutes and then centrifuged (1000 \(g\), 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 (Sep-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). PGF\(_{1\alpha}\) and TXB\(_2\) 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 Brunette\(^{13}\) and of Guesdon\(^{14}\) 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 (Invitrogen) and run at 120 V. The proteins were transferred to a nitrocellulose membrane at 30 mA overnight. The membrane was incubated in a blocking buffer (3% nonfat dry milk in 20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween 20 (TBST)) for 2 hours at room temperature and then washed with TBST (20 mmol/L Tris pH 7.5, 150 mmol/L NaCl, and 0.1% Tween 20). The membrane was then incubated with primary antibody goat polyclonal anti-COX-2 (C-20, Santa Cruz Biotechnology; 1:150) overnight at 4\(^\circ\)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 (Vector) 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 band intensities were quantified with Image ProPlus software (Media Cybernetics).

Statistical Analysis

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

Results

Both selective and nonsselective COX-2 inhibitors (aspirin, celecoxib, and NS-398) significantly depressed PGI\(_2\) and TXB\(_2\) concentrations in the kidneys (\(P<0.01\)) (Figures 1 and 2, Table). Control values for the stable form of prostaglandin \(F_1\alpha\) (PGF\(_{1\alpha}\)) and the stable form of thromboxane \(B_2\) (TXB\(_2\)) concentrations were 592 and 16.73 pg/mg, respectively (Table). Aspirin was the most effective compound, lowering the concentration of PGI\(_2\) to 75.50 pg/mg and of TXB\(_2\) 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, Table). 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 offsets the effect of aspirin on renal medullar prostacyclin \( \text{F}_1 \) and \( \text{TXB}_2 \) (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 \( \text{PGF}_{1 \alpha} \) and \( \text{TXB}_2 \) (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., 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. The authors believed that COX-2 inhibition decreases production of prosclerotic eicosanoids and that modulation of inflammatory stimuli or of glomerular hemodynamics is responsible. 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. B-NOD increases cyclic guanosine monophosphate (cGMP) and prevents platelet aggrega-
viative COX-2 inhibitors seem to have no distinct advantage which increases prostaglandin formation. B-NOD has expression in medullary interstitial cells in the tip of the medulla exceeds that in the cortex.

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.

References


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