PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 159, 165–170 (1978)

Inhibition of Renal Prostaglandin Synthesis and Metabolism by Indomethacin in Rats (40306)

RICHARD J. ROMAN,[1] MICHAEL L. KAUKER, NORBERTO A. TERRAGNO, AND PATRICK Y-K WONG[2]

Department of Pharmacology, University of Tennessee, Center for the Health Sciences, Memphis, Tennessee 38163

Indomethacin, because of its potency as an inhibitor of prostaglandin biosynthesis *in vitro* (1), is widely employed as a pharmacologic agent to investigate the renal actions of endogenous prostaglandins. Evidence for inhibition of renal prostaglandin production *in vivo* has usually been established by demonstrating a reduction of prostaglandin release, i.e., a lowering of prostaglandin (PG) concentration in renal venous blood or a decreased urinary excretion of prostaglandins. However, there are few studies in which the ability of indomethacin to inhibit renal prostaglandin synthesis has been systematically evaluated, and none of the studies has been done in rats surgically prepared for acute experimentation. Furthermore, release of prostaglandins by the kidney probably reflects the net activity of enzymes that synthesize and degrade prostaglandins. Assessment of prostaglandin release may not be adequate to determine the extent of inhibition of prostaglandin synthesis by indomethacin *in vivo* since indomethacin has been reported to inhibit *in vitro* the primary prostaglandin catabolizing enzymes: 15-hydroxyprostaglandin dehydrogenase (PGDH) and prostaglandin E_2 9-ketoreductase (9-KRD). Indeed, Terragno *et al.* (2) have recently shown that indomethacin does not inhibit renal release of prostaglandin E_2 in conscious dogs. In the present study, the effect of indomethacin (2 mg/kg) on prostaglandin release, synthesis and catabolism was investigated in anesthetized, nondiuretic rats.

Materials and methods. Male Wistar rats weighing between 200–400 g were anesthetized with ip Inactin, 100 mg/kg of body wt. After tracheostomy, cannulas were placed in the right external jugular vein for infusions and the right carotid artery for recording of blood pressure. The left kidney was exposed and a polyethylene cannula (PE-50) was placed in the left ureter to allow for urine collections (3). The following drugs were used in the present study: indomethacin (Merck, Sharp and Dohme), meclofenamate (Parke Davis & Co.), phenylbutazone (Geigy Co.), RO 20-5720 (Hoffman La Roche, Inc.). The following three types of studies were carried out.

(a) *Prostaglandin bioassay.* In each experiment, two rats were surgically prepared as described above and, after one hour equilibration, both members of the pair received either indomethacin (2 mg/kg), meclofenamate (2 mg/kg), RO 20-5702 (2 mg/kg), phenylbutazone (50 mg/kg) or 3 m*M* sodium carbonate vehicle alone. Drugs were infused iv at a rate of 40 μl/min in an approximate total volume of 0.2 ml/100 g body wt. After 30 min, a 5 ml blood sample was collected from the left renal vein over a 1 to 2 min period. Blood samples from the two rats were pooled and injected into ice-cold ethanol. Samples were bioassayed for prostaglandin E_2-like activity after an acidic lipid extraction as described previously (4). Since the extracts of blood samples were not chromatographed to separate the various prostaglandins, the reported values represent total prostaglandins and are expressed as the concentration of PGE_2-like substance in the original samples without correction for losses (10–15%) that occur during the extraction procedure (4).

(b) *Prostaglandin synthesis.* In each of these experiments, two rats were prepared as above. After a 1 hr equilibration, urine flow and blood pressure were recorded during two clearance periods of 10 min each. The rats were then infused with either indomethacin

[1] Present Address: Biotechnology Resource in Electron Probe Analysis LHRRB, Harvard Medical School, Boston, Massachusetts 02115.

[2] Send Reprint Requests to: Department of Pharmacology, University of Tennessee, Center for the Health Sciences, 800 Madison Avenue, Box CR-301, Memphis, Tennessee 38163.

0037-9727/78/1592-0165$01.00/0

(2 mg/kg, 4 experiments) or vehicle (3 experiments) as described above. Urine flow and blood pressure were again measured during two experimental clearance periods after a 30-min equilibration. The kidneys from the two rats were removed and the renal papillae were quickly excised and homogenized in ice-cold 0.05 *M* KH_2PO_4 buffer, pH 7.4, with a Polytron homogenizer. Aliquots of papillary homogenates equivalent to 50 mg of wet tissue were incubated at 37° for 30 min in 2 ml of 0.05 *M* KH_2PO_4 buffer containing 0.4 μCi of 1-[^{14}C]arachidonic acid and 2 m*M* reduced glutathione (5). The reaction was stopped by acidification with 1 *M* citric acid (final pH 3.0). The reaction mixture was extracted 3 times with 6 ml of ethylacetate. The combined extract was evaporated under nitrogen. The resulting residue was dissolved in 100 μl of chloroform: methanol (1:1, v/v), quantitatively spotted on thin-layer chromatographic plates, and separated by chloroform:methanol:acetic acid:water (90:9:1:0.65, v/v) as the solvent system. Assays were run in duplicate. Prostaglandin production in boiled tissue controls was subtracted to correct for nonenzymatic formation (5).

(c) *Prostaglandin metabolism.* Eight additional rats were prepared and infused with indomethacin or vehicle as in the prostaglandin synthesis studies. In each experiment, the kidneys were removed after the experimental clearance periods, the renal cortex and outer medulla were excised and homogenized as described. The soluble enzyme fractions containing the PG metabolic enzymes were obtained by high speed centrifugation (105,000*g*). The fractions thus acquired were used to determine the effect of indomethacin on the activity of 9-KRD and PGDH (both NAD^+ and $NADP^+$ dependent) using procedures described previously (6, 7). In brief, PGDH activity was assayed by incubating aliquots of the high speed supernatant at 37° for 10 min with NAD^+ or $NADP^+$ (4 m*M*), 3H-PGE_2 (0.56 μ*M*; 300,000 dpm, NEN, Boston, MA) and 0.05 *M* KH_2PO_4 buffer, pH 7.4, in a final volume of 1 ml. The reaction was stopped by acidification with 1 *M* citric acid to pH 3.0. Authentic PGE_2 and 15-keto PGE_2 standards were added to the assay mixture and extracted 3 times with 2 ml of ethylacetate. The extract was dried under a stream of nitrogen. The residue was redissolved in 100 μl of chloroform:methanol (1:1, v/v). An aliquot of 50 μl of the extract was applied to a thin-layer chromatographic plate (TLC plate, 0.25 mm thick, 20 × 10 cm, silica gel precoated plastic sheets, Brinkman, NY) and separated in iso-octane: ethyl acetate: acetic acid:water (25:55:10:50, v/v). PGE_2 and its 15-keto metabolite were located by exposing the TLC plate to iodine vapor, followed by spraying the plate with 10% phosphomolybdic acid in ethanol. Areas corresponding to authentic PGE_2 and 15-keto PGE_2 standards were cut out and suspended in 10 ml of 0.4% Omnifluor toluene liquid scintillation fluid and counted in a Nuclear Chicago Mark II liquid scintillation spectrometer. The observed cpm were converted to dpm using a quench correction curve and external standard channel ratios. The results are expressed as p moles of 15-keto PG formed per min per mg of protein.

9-KRD activity was determined in the presence of an NADPH generating system (7) containing: NADPH, 0.15 m*M*,, glucose-6-phosphate, 3.5 m*M;* 2 units of glucose-6-phosphate dehydrogenase; ^{3}H-PGE_2 and 0.05 *M* KH_2PO_4 buffer (pH 7.4), and the soluble enzyme fraction in a final volume of 1 ml. After 10 min incubation at 37°, the reaction was stopped by acidification with 1 *M* citric acid to pH 3.0. Samples were extracted and separated by thin-layer chromatography as described above. Areas corresponding to authentic PGE_2 and $PGF_{2\alpha}$ standards were cut out and the radioactivity was determined as before. Protein concentration was determined by the method of Lowry *et al.* (8) using bovine serum albumin as standard. All assays were carried out in triplicate and controls were run simultaneously using boiled supernatant. Results are presented as the mean ± SE and significance was determined by Student's *t* test. $P < 0.05$ was considered significant.

In order to establish the relationship of different *in vitro* doses of indomethacin to the inhibition of renal cortical enzyme activity, the effect of increasing concentrations of indomethacin (0–50 μg/ml) on three major cortical metabolic enzymes were investigated. Assay procedures were similar to those described above, different concentrations of indomethacin were added to the incubation as

indicated (Fig. 1).

Results. Urine flow and blood pressure were measured in these studies to obtain an indication of the physiologic state of the rats under the experimental conditions. Control urine flows were similar in both vehicle and indomethacin treated rats, averaging 1.70 ± 0.26 and 2.07 ± 0.51 μl/min/100 g b wt respectively. After indomethacin urine flow decreased 41% ($P < 0.05$), whereas after infusion of an equal volume of vehicle alone it increased 61% ($P < 0.05$). Mean systemic blood pressure was unchanged after administration of indomethacin (from 116 ± 4 to 113 ± 4 mm Hg, $P > 0.1$) or vehicle (from 124 ± 4 to 123 ± 3 mm Hg, $P > 0.2$).

The concentration of prostaglandin E_2-like substance in renal venous blood of vehicle pretreated rats (Table I) was approximately 17-fold greater than levels measured in arterial blood of two additional pairs of animals (66 ± 6 pg/ml, $P < .01$), indicating that prostaglandin found in the venous blood of these rat kidneys was of renal origin. Mean renal venous blood prostaglandin levels were significantly lowered, by 69% and 90%, respectively, in rats infused with indomethacin or meclofenanate. Similarly, in single experiments 2 other nonsteroidal anti-inflammatory drugs (NSAID), phenylbutazone and RO 20-5702 appeared to reduce renal prostaglandin release (Table I).

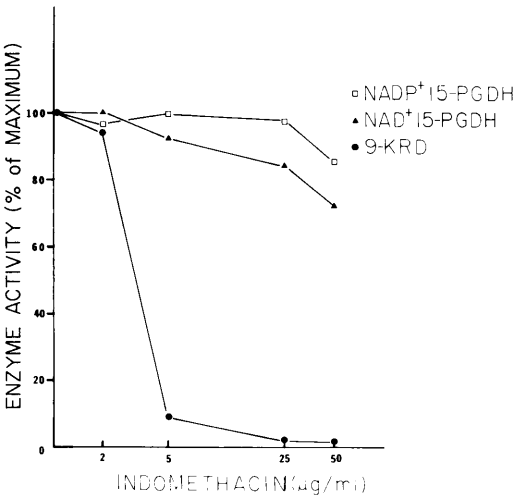

FIG. 1. Dose-response relationship of indomethacin on renal cortical prostaglandin metabolic enzyme activities *in vitro*. The effect of indomethacin was expressed as per cent of enzyme activity after correction for the control. Each point represents the mean of duplicate determinations; controls were run without indomethacin.

The effect of indomethacin pretreatment on prostaglandin synthetase activity of renal papillary homogenates was also studied *in vitro*. Pretreatment with indomethacin, 2 mg/kg, significantly reduced the synthesis of prostaglandins E_2, D_2 and $F_{2\alpha}$ from their precursor arachidonic acid (Table II). Renal papillary PGE_2 production was inhibited 97% by *in vivo* indomethacin pretreatment. Addition of indomethacin, 5 μg/ml, to incubations of renal papillary homogenates obtained from vehicle pretreated rats also diminished prostaglandin production. The degree of prostaglandin synthetase inhibition produced by addition of indomethacin *in vitro* (5 μg/ml) and pretreatment with indomethacin *in vivo* (2 mg/kg) was similar.

Indomethacin pretreatment also interfered with renal prostaglandin metabolism in the present studies. The effect of indomethacin on the key prostaglandin metabolic enzymes is shown in Table III. Treatment with indomethacin inhibited renal cortical-medullary 9-KRD activity by 61% ($P < 0.05$). NAD^+-dependent PGDH activity was also diminished by 46%, however this decrease was not statistically significant. The enzyme $NADP^+$-dependent PGDH was not affected by indomethacin.

The effect of indomethacin pretreatment

TABLE I. EFFECT OF INDOMETHACIN AND OTHER NSAID[a] ON THE CONCENTRATION OF PROSTAGLANDIN E-LIKE SUBSTANCE IN RENAL VENOUS BLOOD OF RATS.

	Prostaglandin concentration[b] pg/ml PGE_2-like equivalents
Vehicle (7)	1102 ± 167
Indomethacin, 2 mg/kg (6)[e]	343 ± 87[c]
Meclofenamate, 2 mg/kg (3)	108 ± 17[d]
Phenylbutazone, 50 mg/kg (1)	129
RO 20-5702, 2 mg/kg (1)	496

Mean values ± SE are presented.

[a] NSAID = nonsteroidal anti-inflammatory drugs.

[b] PGE_2-like material was assayed on a cascade of rat stomach strip, rat colon and chick rectum.

[c] $P < 0.005$, statistically different from vehicle treated animals.

[d] $P < 0.001$, statistically different from vehicle treated animals.

[e] Numbers in parentheses = number of samples assayed. Each sample contained two 5 ml samples of renal venous blood obtained from two rats.

TABLE II. PROSTAGLANDIN BIOSYNTHESIS BY HOMOGENATES OF RENAL PAPILLAE FROM RATS PRETREATED WITH VEHICLE OR INDOMETHACIN.

	Rate of prostaglandin biosynthesis[a]			
	PGE_2	$PGF_{2\alpha}$	PGD_2	Total PG
Vehicle pretreated (3)[d]	2.65 ± 0.48	0.68 ± 0.20	0.17 ± 0.02	3.49 ± 0.48
Indomethacin pretreated (2 mg/kg) (4)	0.08 ± 0.04[c]	0.06 ± 0.01[b]	0.02 ± 0.01[c]	0.16 ± 0.01[c]
Indomethacin added *in vitro* (5 μg/ml) (4)	0.18 ± 0.08[c]	0.15 ± 0.12	0.05 ± 0.03[b]	0.38 ± 0.18[c]

Mean data ± SE are presented.
[a] Values expressed as picomoles of prostaglandin formed/min per mg wet wt of tissue.
[b] $P < 0.05$, compared to vehicle pretreated.
[c] $P < 0.005$, compared to vehicle pretreated.
[d] Numbers in parentheses = number of experiments.

on the PG metabolic enzymes were also confirmed by the *in vitro* experiments. Indomethacin at a dose of 5 μg/ml *in vitro* produced marked inhibition of PG 9-KRD but was less effective on NAD^+-dependent PGDH. At a dose of 25 μg/ml 9-KRD was inhibited 95% while NAD^+-dependent PGDH activity was lowered only 15%. However, at this dose range indomethacin produced little or no effect on $NADP^+$-dependent PGDH (Fig. 1).

Discussion. In the present investigation, inhibition of renal prostaglandin synthetase after administration of 2 mg/kg indomethacin to anesthetized nondiuretic rats was assessed by two methods. These experiments demonstrated the following: (a) the concentration of a PGE-like substance in the renal venous blood was reduced 69% by indomethacin; (b) indomethacin pretreatment decreased, by greater than 90%, the conversion of radiolabeled arachidonic acid to various prostaglandins (PGE_2, $F_{2\alpha}$ and D_2) by renal papillary homogenates; (c) NSAID other than indomethacin were also effective in lowering renal venous prostaglandin levels. Indomethacin *in vivo* reduced, but did not completely abolish, net renal prostaglandin output in anesthetized rats prepared for acute experimentation. Associated with an inhibition of prostaglandin production was a significant decline in urine flow, which is consistent with the proposal that prostaglandins affect tubular handling of water by attenuating the antidiuretic action of ADH (9).

The extent of renal prostaglandin synthetase inhibition by indomethacin, as determined by the decline in renal venous prostaglandin levels (69%), was lower than that estimated by *in vitro* prostaglandin production by papillary homogenates (97%). The dissimilar degree of inhibition indicated by the two methods may reflect inherent differences in the experimental procedures, Homogenization of the renal papallae in the tissue incubation studies, for example, may have allowed indomethacin greater access to the enzyme cyclo-oxygenase thus producing a more complete blockade of prostaglandin synthesis than that which occurred *in vivo*. On the other hand, the present studies provide evidence suggesting an alternative explanation; i.e., the degree of prostaglandin syn-

TABLE III. METABOLISM OF PROSTAGLANDIN E_2 BY THE SOLUBLE ENZYME FRACTION OF RENAL CORTEX AND OUTER MEDULLA FROM RATS PRETREATED WITH VEHICLE OR INDOMETHACIN.

	15-PGDH[a]		PG-9-KRD[b]
	NAD^+ dependent	$NADP^+$ dependent	NADPH dependent
Vehicle pretreated (4)[e]	2.12 ± 0.66	0.98 ± 0.12	0.88 ± 0.18
Indomethacin pretreated, 2 mg/kg (4)	1.14 ± 0.31[c]	1.04 ± 0.12[c]	0.34 ± 0.12[d]

Mean data ± SE are presented.
Values are expressed as picomoles of $PGF_{2\alpha}$ or 15-keto PGE_2 formed/min per mg protein.
[a] 15-PGDH = 15-hydroxyprostaglandin dehydrogenase activity.
[b] PG-9-KRD = Prostaglandin E_2 9-ketoreductase activity.
[c] Not significant $P > 0.05$.
[d] $P < 0.05$ compared to vehicle pretreated.
[e] Numbers in parentheses = number of experiments.

thesis inhibition after indomethacin may not have been accurately reflected by the decline in renal prostaglandin release because the drug impaired prostaglandin metabolism as well as synthesis. Such a conclusion is supported by our finding that 9-KRD activity of renal cortico-medullary homogenates was reduced by 61% after indomethacin pretreatment. Additionally, although a significant difference was not detected in the present prostaglandin metabolism study, the decline of 46% in mean NAD^+-dependent PGDH activity after indomethacin is consistent with the view that indomethacin affects both prostaglandin synthesis and metabolism. The finding that indomethacin inhibited the soluble enzyme, 9-KRD, after systemic administration implies that this compound gained access to sites located in the intracellular compartment.

Inhibition of renal cyclo-oxygenase, 9-KRD and PGDH by indomethacin and other NSAID *in vitro* has been reported previously (10, 11). The concentrations used for half-maximal inhibition of PG synthesis were of the same order of magnitude as the concentration shown to produce half-maximal inhibition of prostaglandin metabolic enzymes. The present observations, however, provide the first evidence that a standard *in vivo* dose of indomethacin, 2 mg/kg, producing an estimated unbound plasma concentration of 5 μg/ml, interferes with prostaglandin metabolism. The effect on the PG metabolic enzymes was confirmed by the *in vitro* experiments which indicated that indomethacin indeed affected the major metabolic route of PGs in the kidney. The additional observation, both *in vitro* and *in vivo,* that 9-KRD activity was markedly reduced by NSAID especially by indomethacin whereas the enzyme $NADP^+$-dependent PGDH was unaffected, suggests that these enzymes may have different active site(s) even though they have been reported to be identical (12).

In conclusion, indomethacin, meclofenamate and other NSAID markedly reduced net renal prostaglandin production in rats surgically prepared for acute experimentation. It appears from the data reported here that indomethacin, after *in vivo* administration, may have a complex action to impair both synthesis and metabolism of renal prostaglandins. Differential inhibition of the enzymes involved in net prostaglandin production and alterations in the types of prostaglandins formed in various parts of the kidney complicate the interpretation of data obtained during indomethacin treatment. The usefulness of this agent to evaluate the role of prostaglandins in the regulation of renal function may thus be limited. However, due to species differences which exist with respect to prostaglandin degradation, the conclusion of this study may not be extrapolated to other species.

Summary. The effect of indomethacin and other NSAID on renal prostaglandin synthesis and metabolism was studied in nondiuretic rats prepared for acute experimentation. Thirty minutes after the administration of a 2 mg/kg iv dose of indomethacin, the concentration of prostaglandin in renal venous blood as determined by bioassay was reduced 69%. In addition, conversion of radiolabeled arachidonic acid to prostaglandin E_2 *in vitro* by the renal papillae of indomethacin pretreated rats was inhibited 97%.

Pretreatment with indomethacin also inhibited renal cortical–medullary prostaglandin E_2 9-ketoreductase activity by 61%. NAD^+-dependent 15-hydroxy-prostaglandin dehydrogenase activity was diminished 46%; however, this inhibition was not statistically significant. $NADP^+$-dependent 15-hydroxy-prostaglandin dehydrogenase activity was unaffected by pretreatment. It is concluded that indomethacin alters net renal prostaglandin production by inhibiting both prostaglandin synthesis and its metabolism.

This work was supported in part by research grants from the USPHS: HL-22075, AM-17711 and HL-1922801 HED; and the American, Tennessee and Memphis Heart Associations. Prostaglandins and their metabolites were gifts of Dr. Udo Axen of the Upjohn Company and R0 20-5702 was kindly supplied by Dr. J. R. Paulsrud of Hoffman and La Roche, Inc. We also would like to thank Misses Judy Early and Pat Goldstein, and Mr. Grant Barr for their assistance in these experiments.

1. Flower, R. J., Pharmacol. Rev. **26,** 33 (1974).
2. Terragno, N. A., Terragno, D. A., and McGiff, J. C., Circ. Res. **40,** 590 (1977).
3. Roman, R. J., and Kauker, M. L., Circ. Res. **38,** 185 (1976).
4. McGiff, J. C., Crowshaw, K., Terragno, N. A., and Lonigro, A. J., Circ. Res. **27** (Suppl. 1), 121 (1970).

5. Wong, P. Y-K, Majeska, K. J., and Wuthier, R. D., Prostaglandins **14,** 839 (1977).
6. Wong, P. Y-K, and McGiff, J. C., Biochim. Biophys. Acta **500,** 436, 1977.
7. Wong, P. Y-K, Terragno, A., Terragno, N. A., and McGiff, J. C., Prostaglandins **13,** 1103 (1977).
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951).
9. Anderson, R. J., Berl, T., McDonald, K. M. and Schrier, R. W., J. Clin. Invest. **56,** 420 (1975).
10. Stone, K. J., and Hart, M., Prostaglandins **12,** 197 (1976).
11. Wright, J. T., Jr., Corder, C. N., and Taylor, R., Biochem. Pharmacol. **25,** 1669 (1976).
12. Hassid, A., and Levine, L., Prostaglandins **13,** 503 (1977).

Received February 8, 1978. P.S.E.B.M. 1978, Vol. 159.