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DECREASE OF URINARY PROSTAGLANDIN E_2 AND PROSTAGLANDIN $F_{2\alpha}$ EXCRETION BY NONSTEROIDAL ANTI-INFLAMMATORY DRUGS IN RATS

RELATIONSHIP TO ANTI-INFLAMMATORY ACTIVITY

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Abstract—An analytical method for measuring *in vivo* inhibition of prostaglandin (PG) synthesis by nonsteroidal anti-inflammatory drugs was developed for estimation of urinary prostaglandin levels in rats. Drugs were administered orally to rats (Wistar, male, 200–250 g), and water (2.5 ml/100 g body weight) was given 1 hr after drug administration to yield a constant volume of urine. Urine was collected for 4 hr after drug administration, and urinary PGE_2 and $PGF_{2\alpha}$ were determined by radioimmunoassay. The urine volume in the 4-hr period was 5.0 ± 0.30 ml per rat, and prostaglandin contents in the 4-hr urine were 4.56 ± 0.56 ng PGE_2 and 1.31 ± 0.24 ng $PGF_{2\alpha}$ per rat in the no-drug control group. Administration of nonsteroidal anti-inflammatory drugs decreased the urinary PGE_2 and $PGF_{2\alpha}$ dose dependently. The activities of ten typical nonsteroidal anti-inflammatory drugs in reducing urinary PGE_2 excretion were compared with their anti-inflammatory activities in rats. A close correlation ($r = 0.98$, $P < 0.001$) between the dose required for 50% reduction of urinary PGE_2 excretion and the dose required for 50% inhibition of carrageenin edema was found for each drug. These drugs were also tested for their inhibitory effects on PGE_2 biosynthesis in a cultured system of mouse 3T6 fibroblast cells and on prostaglandin synthesizing system in bovine seminal vesicle microsomes. No close correlation was observed between anti-inflammatory activities and inhibition of prostaglandin biosynthesis *in vitro*.

Nonsteroidal anti-inflammatory drugs inhibit prostaglandin (PG) synthetase from various tissues and organs [1, 2], but the inhibitory activities do not always coincide with the anti-inflammatory potencies of the drugs [3]. In order to study the relationship between the inhibition of prostaglandin biosynthesis and the anti-inflammatory potencies of the drugs, it is necessary to measure the inhibition of prostaglandin synthesis in the *in vivo* system. However, the limiting problem has been the lack of a suitable system for quantitative assay of *in vivo* inhibition of prostaglandin synthesis, because prostaglandins are rapidly synthesized and metabolized in tissues and circulating blood.

Measurement of PGE_2 and $PGF_{2\alpha}$ in urine of humans and dogs has been reported by Frölich *et al.* [4], Ciabattoni *et al.* [5], and McGiff *et al.* [6]. Since urinary PGE_2 and $PGF_{2\alpha}$ are a good reflection of renal prostaglandin synthesis, and urinary levels of the primary prostaglandins are much higher than plasma levels [4–6], estimation of the prostaglandins in urine is easier and more reliable than in plasma where platelets generate prostaglandins rapidly. Scherer *et al.* [7] reported decreased urinary prostaglandins following administration of indomethacin. However, a difficulty in measuring urinary prostaglandins after administration of nonsteroidal anti-inflammatory drugs to animals is that the urine volume is reduced markedly by administration of the drugs. The quantitative analysis of inhibition of urinary prostaglandin excretion by nonsteroidal anti-inflammatory drugs in animals, therefore, has not yet been reported.

The present report describes a quantitative assay of the inhibition of PGE_2 and $PGF_{2\alpha}$ urinary excretion that follows nonsteroidal anti-inflammatory drugs administration to rats. In addition, inhibition by these drugs of prostaglandin synthesis in mouse 3T6 fibroblast cells and in bovine seminal vesicle microsomes was also measured. The inhibitory effects on prostaglandin synthesis in these systems were compared with the anti-inflammatory potencies of the drugs, as estimated from carrageenin edema in rats.

MATERIALS AND METHODS

Arachidonic acid, PGE_2 and $PGF_{2\alpha}$ were obtained from the Sigma Chemical Co., St. Louis, MO. [^{14}C]Arachidonic acid, [^{3}H]- or [^{14}C]PGE_2 and $PGF_{2\alpha}$ were from the New England Nuclear Corp., Boston, MA. Radioimmunoassay kits for PGE and $PGF_{2\alpha}$ (double antibody) were purchased from Clinical Assay, Cambridge, MA. Silicic acid (100 mesh) was from Mallinckrodt Chemicals, St. Louis, MO. NCS tissue solubilizer was purchased from Amersham, Arlington Heights, IL.

3T6 Fibroblasts, a cultured strain from Swiss albino mouse embryo, were obtained from the tissue culture center of the Dainippon Pharmaceutical Co., Tokyo. Dulbecco modified Eagle's minimum essen-

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tial medium (Dulbecco–Eagle MEM) [8] was from the Nissui Pharmaceutical Co., Tokyo.

Acetylsalicylic acid and salicylic acid were from Wako Chemicals, Osaka. Flurbiprofen was from Boots, Nottingham, U.K.; indomethacin from Merck, Rahway, NJ; and ketoprofen from Rhone-Poulenc, Vinty-sur-Seine, France. Meclofenamic acid and mefenamic acid were from Warner Lambert, Detroit, MI. Naproxen was from Tanabe-Syntex, Tokyo. Diclofenac sodium and phenylbutazone were from Fujisawa-Ciba-Geigy, Osaka. Hydrocortisone and prednisolone were from Sigma, and dexamethasone was from Organon-Sankyo, Tokyo.

Extraction of prostaglandins from rat urine. Male Wistar rats (200–250 g) were fasted from 16 hr before use. Drugs were suspended in 0.5% tragacanth and administered orally. Water (2.5 ml/100 g body weight) was given orally 1 hr after drug administration. Urine from four rats was collected into one ice-cold container using a urine collection cage during the 4 hr after drug administration. The urine was adjusted to pH 3.0 with formic acid, and prostaglandins were extracted twice with equal volumes of chloroform as reported by Scherer *et al.* [7]. Under the conditions, extraction recoveries of PGE_2 and $PGF_{2\alpha}$ were 96–97%. The chloroform layer was dried under a flow of nitrogen gas. Then the residue was dissolved in 0.3 ml of benzene–ethylacetate–methanol (60:40:10) followed by 1.2 ml of benzene–ethylacetate (60:40) and applied to a silicic acid column (0.5 g, 1 × 18 cm). The chromatography was done with increasing concentrations of methanol in benzene–ethylacetate (60:40) according to the method of Jaffe *et al.* [9]. PGE_2 was eluted from the column with 12 ml of benzene–ethylacetate–methanol (60:40:2), and $PGF_{2\alpha}$ was eluted with 3 ml of benzene–ethylacetate–methanol (60:40:20). Column recoveries for PGE_2 and $PGF_{2\alpha}$ were 74 and 88% respectively.

Radioimmunossay for PGE_2 and $PGF_{2\alpha}$. The PGE_2 fraction was evaporated to dryness in nitrogen gas and dissolved in 1.0 ml of 0.01 M Tris–HCl, pH 7.4, containing 0.1% gelatin and 0.14 M NaCl. To the PGE_2 solution was added 0.1 ml of 1 N NaOH, and the solution was heated at 80° for 10 min, after which the solution was neutralized by the addition of 0.1 ml of 1 N acetic acid. By this alkaline treatment, PGE_2 was completely converted to PGB_2. Radioimmunoassay was carried out in duplicate using anti-PGB rabbit serum [10]. A suitable dilution of the sample in a total volume of 600 μl was mixed with 50 μl of [^{3}H]PGB_2 (0.01 μCi) and 10 μl of anti-PGB rabbit serum. After incubation at 37° for 1 hr, 100 μl of rabbit normal serum and 100 μl of goat anti-rabbit serum were added, and incubation was continued at 4° overnight. The precipitate was collected by centrifugation at 2500 *g* for 20 min at 4° and solubilized in 300 μl NCS at 60° for 15 min. The radioactivity was determined in 10 ml of toluene scintillation fluid using a Packard Tri-Carb liquid scintillation spectrometer. In a similar way, the $PGF_{2\alpha}$ fraction was assayed with anti-$PGF_{2\alpha}$ rabbit serum. The detection limits for PGE_2 and $PGF_{2\alpha}$ were 50 and 10 pg respectively. The precision of the radioimmunoassay for PGE_2 and $PGF_{2\alpha}$ was determined by the coefficient of variation at levels of 100 and 300 pg PGE_2 and 30 and 100 pg $PGF_{2\alpha}$. The inter-assay variation was 9.8 and 11.9% (N = 6) for $PGE_{2\alpha}$ and 5.6 and 7.4% (N = 6) for $PGF_{2\alpha}$ respectively.

The anti-PGB serum does not identify the PGB compounds as either PGB_1 or PGB_2. However, similar results were obtained when these samples were reacted with anti-PGE_2 serum. Therefore, the PGB values obtained with the anti-PGB serum were considered to be mainly due to PGE_2.

Prostaglandin synthesis in 3T6 fibroblasts. 3T6 fibroblast cells (3×10^5 cells/well) were seeded and grown in 1 ml of Dulbecco–Eagle MEM supplemented with 10% calf serum in a multidish plate (Nunc Co., 24 wells, 14 mm in diameter) at 37° under 5% CO_2 in air. After 48 hr of incubation, the fibroblast cells (8×10^5 cells/well) were washed with 2 ml of the MEM medium, and 0.5 ml of the fresh MEM medium supplemented with calf serum containing drugs [solubilized in 0.01% dimethyl sulfoxide (DMSO)] was added. In separate experiments, 0.01% DMSO was shown not to affect prostaglandin formation by the cell. After the cell monolayers were incubated for an additional 2 hr, the medium was removed. The cells were washed a second time, and 0.25 ml of the MEM medium supplemented with calf serum containing drugs plus 5 μg/ml arachidonic acid was introduced [11]. One hour later, the medium was removed and saved for PGE_2 measurement by radioimmunoassay. Measurement of the PGE_2 content of the culture medium was carried out without solvent extraction. The medium (50–100 μl) was mixed with 50 μl of 1 N NaOH and heated at 80° for 10 min, and the mixture was neutralized by the addition of 50 μl of 1 N acetic acid. Radioimmunoassay was carried out using the anti-PGB rabbit serum as described above. Arachidonic acid and the anti-inflammatory drugs tested showed no interference with the radioimmunoassay.

Inhibition of prostaglandin synthetase. Microsomal fraction from bovine seminal vesicle was prepared according to the method of Takeguchi *et al.* [12], lyophilized, and stored below −60° before use. The reaction mixture consisted of 0.1 M Tris–HCl, pH 7.6, 1 mM epinephrine, 2 mM glutathione, 200 μg of the microsomal enzyme, and anti-inflammatory drug to be tested, in a total volume of 200 μl. The reaction was started by the addition of 10 μM [^{14}C]arachidonic acid (60 nCi), was performed at 30° for 10 min, and was stopped by the addition of 50 μl of 1 N HCl. Prostaglandins were extracted with 1.5 ml of ethylacetate, and the ethylacetate layer was dried with nitrogen gas, dissolved in 40 μl of methanol, and applied to a thin-layer plate (Merck, Kieselgel 60 F). The solvent of the chromatography was an organic phase of ethylacetate, acetic acid, iso-octane and water (11:2:5:10) [13]. Under the conditions, the R_f values for PGE_2 and arachidonic acid were 0.47 and 0.81 respectively. The PGE_2 fraction was detected with a radioactive scanner (Berthold Co.), scraped out, and dipped in 1 ml ethanol. Ten millilitres of toluene–ethanol (1:1) scintillation solution was added, and the decrease of the radioactivity by the drugs was estimated.

Carrageenin-induced rat paw edema. Anti-inflammatory potencies of drugs were estimated according to the method of Winter *et al.* [14]. Male Wistar rats

(120–130 g), fasted for 16 hr, were injected subcutaneously in the plantar region of the right hind paw with 0.05 ml of 1% carrageenin in saline. Drugs were administered orally in 0.5% tragacanth suspension. Foot volumes were measured with a plethysmograph before and 3 hr after the carrageenin injection. Edema intensity was calculated by the following equation:

$$\text{Edema intensity} = \frac{\text{paw volume 3 hr after carrageenin injection}}{\text{paw volume before carrageenin injection}} - 1$$

RESULTS

Decrease in urinary PGE_2 and $PGF_{2\alpha}$ following administration of anti-inflammatory drugs. Administration of nonsteroidal anti-inflammatory drugs decreased urine excretion in rats. For example, no detectable urine was produced for 4 hr after oral administration of indomethacin at doses of over 3 mg/kg body weight. However, essentially constant volumes of urine were excreted in the drug-treated animals by oral administration of water 1 hr after drug administration. Saline could not replace water. Following administration of water (2.5 ml/100 g body weight), about 5 ml of urine per rat was excreted over 4 hr from the rats that had received the various doses of nonsteroidal anti-inflammatory drugs (Table 1).

Table 1. Urine volume in rats after administration of nonsteroidal anti-inflammatory drugs*

	Dose (mg/kg)	4-hr urine (ml/rat)
No-drug control		5.0 ± 0.30†
Indomethacin	1	4.2
	3	4.6
	10	3.8
Flurbiprofen	0.1	5.0
	0.3	4.3
	1	4.0
Ketoprofen	0.3	5.0
	1	5.3
	3	3.8
Naproxene	0.3	5.3
	1	4.8
	3	4.0
Mefenamic acid	10	4.5
	30	3.8
	100	4.2
Phenylbutazone	10	5.9
	30	5.5
	100	4.5
Acetylsalicylic acid	30	4.8
	100	6.0
	300	5.5

* Drugs were administered orally. Water (2.5 ml/100 g body weight) was given 1 hr after drug administration. Urine from four rats was collected into one container for 4 hr after drug administration. Urine volume (ml) is expressed per one rat.

† Average of ten experiments (N = 40).

Excretion of PGE_2 and $PGF_{2\alpha}$ in the 4-hr urine of the no-drug control group was 4.56 ± 0.56 ng and 1.31 ± 0.24 ng per rat respectively. Administration of ten typical nonsteroidal anti-inflammatory drugs decreased the urinary PGE_2 excretion in a dose-dependent manner (Fig. 1). Flurbiprofen, ketoprofen and naproxen were most effective, having ID_{50} values (the drug dose required for 50% reduction of urinary PGE_2 excretion) below 0.5 mg/kg. Indomethacin and diclofenac sodium had ID_{50} values of 3–6 mg/kg. Meclofenamic acid, mefenamic acid and phenylbutazone displayed moderate inhibition (ID_{50} values of 30–60 mg/kg). Acetylsalicylic acid and

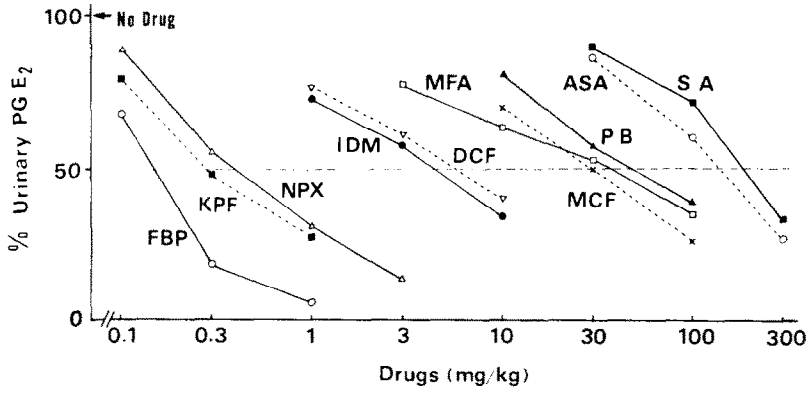

Fig. 1. Decrease of PGE_2 in 4-hr urine of rats administered nonsteroidal anti-inflammatory drugs. The drugs were administered orally, and water (2.5 ml/100 g body weight) was given 1 hr after drug administration. Urine from four rats was collected into one container during the 4 hr after the drug administration, and urinary PGE_2 contents were determined by radioimmunoassay as described in Materials and Methods. The PGE_2 contents in the urine are expressed as percentages of the no-drug control. The PGE_2 contents in the 4-hr urine of the no-drug control group were 4.56 ± 0.56 ng per rat (mean ± S.E.M. of six experiments, N = 24). Abbreviations: ASA, acetylsalicylic acid; DCF, diclofenac sodium; FBP, flurbiprofen; IDM, indomethacin; KPF; ketoprofen; MCF, meclofenamic acid; MFA, mefenamic acid; NPX, naproxen; PB, phenylbutazone; and SA, salicylic acid.

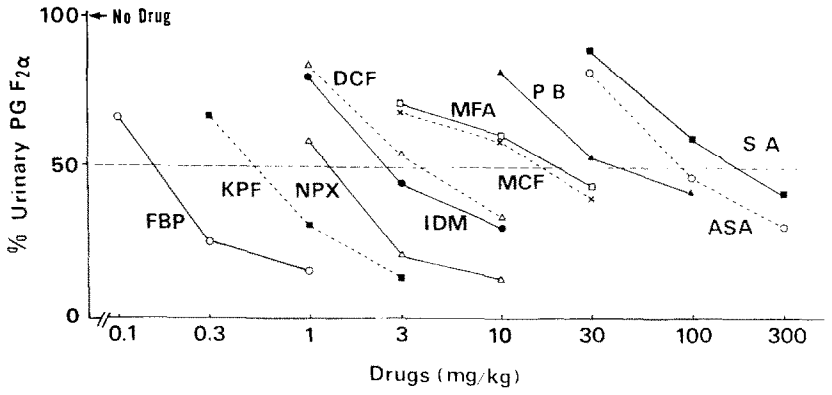

Fig. 2. Decrease of $PGF_{2\alpha}$ in 4-hr urine of rats administered nonsteroidal anti-inflammatory drugs. Urinary $PGF_{2\alpha}$ contents are expressed as percentages of the no-drug control. The $PGF_{2\alpha}$ contents in the 4-hr urine of the no-drug control group were 1.31 ± 0.24 ng/rat (mean ± S.E.M. of six experiments, N = 24). Procedures are the same as described for Fig. 1.

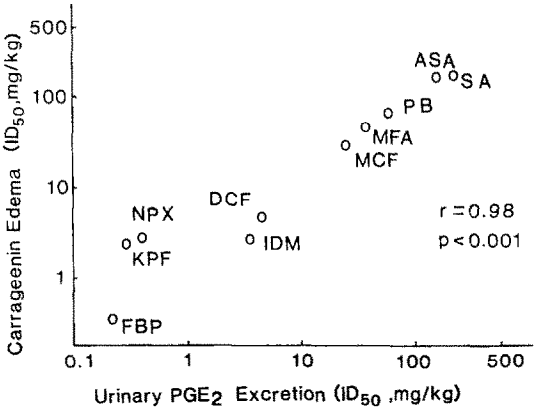

Fig. 3. Correlation between anti-carrageenin edema activity of nonsteroidal anti-inflammatory drugs and the suppression of urinary PGE_2 excretion in rats. Rats were injected subcutaneously with carrageenin, and nonsteroidal anti-inflammatory drugs were administered orally as described in Materials and Methods. The ID_{50} values of urinary PGE_2 excretion were calculated from Fig. 1.

salicylic acid had higher ID_{50} values (150–170 mg/kg). These nonsteroidal anti-inflammatory drugs decreased the urinary $PGF_{2\alpha}$ excretion with about the same potency as the urinary PGE_2 excretion (Fig. 2).

The activities of the drugs in reducing urinary PGE_2 excretion were compared with their anti-inflammatory activities. A close correlation was found ($r = 0.98$, $P < 0.001$) between the ID_{50} for reduction of urinary PGE_2 excretion and the ID_{50} values determined from the anti-carrageenin edema activity (Fig. 3).

This urinary prostaglandin excretion assay could not be applied to the evaluation of the anti-inflammatory activities of steroidal drugs, because hydrocortisone, dexamethasone and prednisolone (1–30 mg/kg, orally) increased the volume of urine under the conditions of the assay system, and their inhibitions of PGE_2 and $PGF_{2\alpha}$ excretion were not dose-dependent (Table 2).

Inhibition of PGE_2 synthesis in fibroblast cells and in seminal vesicle microsomes. The effects of the

Table 2. Effect of oral anti-inflammatory steroids on 4-hr urine volume and urinary PGE_2 and $PGF_{2\alpha}$ in rats*

	Dose (mg/kg)	4-hr urine (ml/rat)	PGE_2 (ng/rat)	$PGF_{2\alpha}$ (ng/rat)
No-drug control		5.0 ± 0.30	4.56 ± 0.56	1.31 ± 0.24
Hydrocortisone	1	5.8	7.2	2.4
	3	6.0	7.6	1.4
	10	7.0	4.6	1.5
	30	9.2	7.0	1.7
Dexamethasone	1	8.3	6.4	1.6
	3	9.0	7.2	1.0
	10	10.8	5.8	1.4
Prednisolone	1	8.5	6.0	1.4
	3	9.3	4.9	1.1
	10	10.0	7.8	1.7
	30	11.5	10.1	1.5

* Procedures were the same as in Table 1 and Figs. 1 and 2.

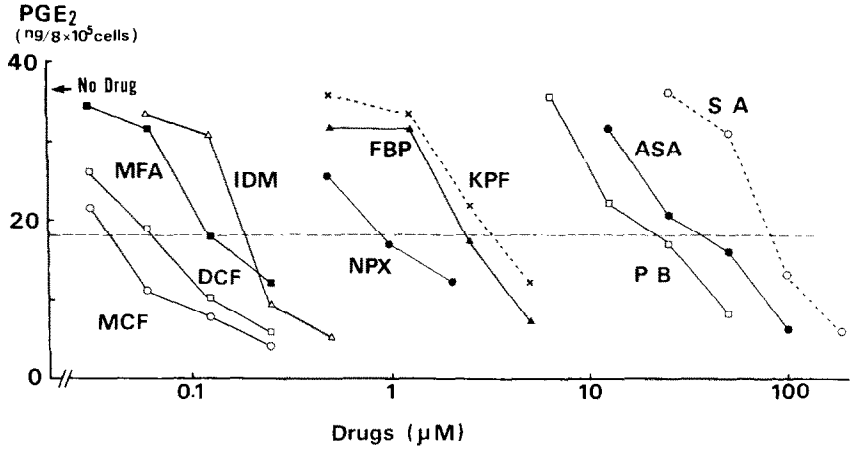

Fig. 4. Inhibition by nonsteroidal anti-inflammatory drugs of PGE_2 synthesis in 3T6 fibroblast cells. 3T6 cells were incubated with the drugs for 2 hr, and, after washing, the cells were further incubated with the drugs plus 5 μg/ml arachidonic acid for 1 hr. Formation of PGE_2 during the 1-hr incubation period was determined by radioimmunoassay as described in Materials and Methods. PGE_2 synthesis in the absence of inhibitor was 37 ± 1.7 ng per 8×10^5 cells per 1 hr.

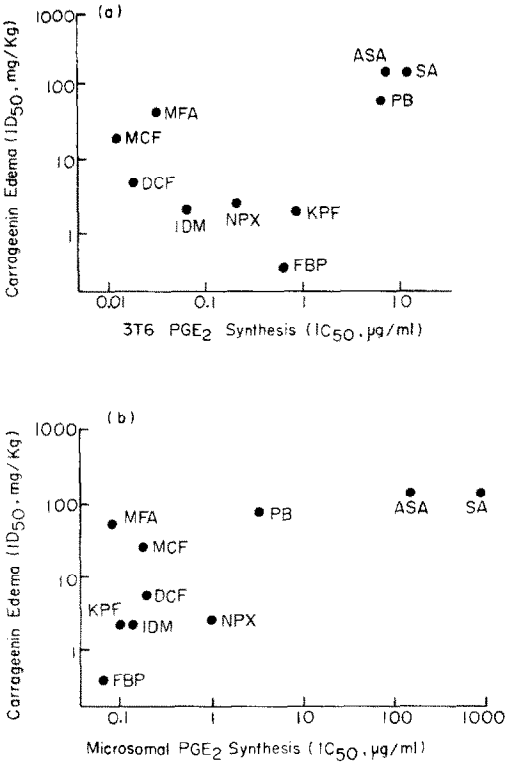

Fig. 5. Correlation between the anti-carrageenin edema activities of nonsteroidal anti-inflammatory drugs and their inhibitory activities toward PGE_2 synthesis in 3T6 fibroblast cells (a), and correlation between anti-carrageenin edema activity and inhibitory activity toward prostaglandin synthetase in bovine seminal vesicle microsomes (b). The IC_{50} values of PGE_2 synthesis in 3T6 fibroblast cells were calculated from Fig. 4. Prostaglandin synthetase activity in bovine seminal vesicle microsomes was determined by estimation of $[^{14}C]PGE_2$ synthesis from 10 μM $[^{14}C]$arachidonic acid as described in Materials and Methods.

nonsteroidal anti-inflammatory drugs on prostaglandin synthesis were examined using cultured mouse 3T6 fibroblast cells. The cells were pretreated with an anti-inflammatory drug for 2 hr and then incubated with fresh medium containing the same drug plus arachidonic acid for another hour. The cells released 37 ± 1.7 ng PGE_2 and 2.3 ± 0.6 ng $PGF_{2\alpha}$, per 8×10^5 cells, into the medium in 1 hr in the absence of inhibitor. Incubation of the 3T6 cells with nonsteroidal anti-inflammatory drugs resulted in a concentration-dependent inhibition of PGE_2 formation (Fig. 4). The IC_{50} values of meclofenamic acid, mefenamic acid, diclofenac sodium and indomethacin were under 0.2 μM. Flurbiprofen, ketoprofen and naproxen had IC_{50} values of about 2 μM, and phenylbutazone and acetylsalicylic acid had an IC_{50} value of 20–30 μM. The IC_{50} of salicylic acid was about 80 μM.

For comparison, these nonsteroidal anti-inflammatory drugs were examined for their inhibitory effects on PGE_2 synthesis by bovine seminal vesicle microsomes. When the seminal vesicle microsomes were incubated with 10 μM arachidonic acid, 1 mM epinephrine and 2 mM glutathione at 30° for 10 min, 20–30% of the arachidonic acid was converted to PGE_2 in the absence of inhibitor. No other prostaglandins were produced. Addition of the nonsteroidal anti-inflammatory drugs the reaction mixture inhibited the PGE_2 formation. The IC_{50} values obtained from these studies were compared with the ID_{50} values determined from the anti-carrageenin edema activity in rats (Fig. 5a and 5b). No good correlation was found between the inhibition of PGE_2 synthesis in the *in vitro* systems and the anti-inflammatory activity.

DISCUSSION

The present study describes an analytical method for measuring the inhibition of prostaglandin syn-

thesis by nonsteroidal anti-inflammatory drugs in rats by estimation of urinary prostaglandins. Estimation of prostaglandins in urine is more convenient and reliable than the determination of prostaglandins in tissues or blood, but constancy in urine excretion volume is indispensable because the stimulation or suppression of urine excretion results in changes in the amount of urinary prostaglandins [15]. In particular, the decrease in urine volume of rats that have been administered nonsteroidal anti-inflammatory drugs makes them inappropriate for quantitative analyses of drug effects on urinary prostaglandin excretion. As described in this paper, an essentially constant volume of urine (about 5 ml/4 hr) was obtained in rats administered nonsteroidal anti-inflammatory drugs, and then the excretion of PGE_2 and $PGF_{2\alpha}$ in the 4-hr urine was decreased dose dependently. This method, however, was not valid for anti-inflammatory steroids.

The ID_{50} values of ten typical nonsteroidal anti-inflammatory drugs for reduction of urinary excretion of PGE_2 correlated closely with the anti-inflammatory potencies of the drugs estimated by carrageenin edema formation in rats ($r = 0.98$, $P < 0.001$). Therefore, the ID_{50} for reduction of urinary PGE_2 is considered to be a good indicator of the inhibitory activities of nonsteroidal anti-inflammatory drugs toward *in vivo* prostaglandin synthesis. McGiff *et al.* [6] and Frölich *et al.* [4] reported that urinary PGE_2 and $PGF_{2\alpha}$ are a real reflection of renal prostaglandin synthesis. The close correlation between the reduction in the urinary prostaglandins and the inhibition of carrageenin edema suggests that renal prostaglandin synthesis was inhibited to the same extent as the prostaglandin synthesis in the edema tissue.

There were some discrepancies between the *in vitro* inhibition of prostaglandin synthesis by the drugs and the anti-inflammatory activities. For example, meclofenamic acid and mefenamic acid were potent inhibitors of the *in vitro* prostaglandin synthetase systems, but the anti-carrageenin edema activities in rats were not as potent. Salicylic acid, which had very weak inhibitory activity in the cell-free prostaglandin synthetase system, had essentially the same anti-carrageenin edema activity as acetylsalicylic acid [16]. In contrast, the inhibition of *in vivo* prostaglandin synthesis estimated by the urinary PGE_2 excretion was closely correlated with the anti-inflammatory activity. The difference between the inhibitory potencies of the drugs in the *in vitro* and *in vivo* prostaglandin synthesis systems may be ascribed to the metabolic activation, inactivation, or difference in the uptake of the drugs. This analytical method for measuring reduction of urinary PGE_2 excretion may be useful for evaluation of pro-drug type anti-inflammatory drugs which produce active metabolite *in vivo*.

3T6 fibroblasts, a cultured strain from Swiss albino mouse embryo, were found to release high concentrations of PGE_2 into the medium, and this release of PGE_2 was inhibited by typical nonsteroidal anti-inflammatory drugs, dose-dependently. Hong and Levine [17] reported that MC 5-5 cells, methylcholanthrene-transformed mouse fibroblast cells, secreted high concentrations of PGE_2 into the medium and that this PGE_2 secretion was inhibited by anti-inflammatory steroids. Carty *et al.* [11] reported the inhibition of PGE_2 production in the MC 5-5 cells by piroxicam, indomethacin, and some other nonsteroidal anti-inflammatory agents. Relative inhibitory activities of the drugs on PGE_2 synthesis almost coincided between the 3T6 and MC 5-5 cell systems.

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