PROSTAGLANDIN SYNTHETASE SYSTEMS IN RAT AND RABBIT RENAL MEDULLA AND INHIBITION BY NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

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Abstract—The properties of prostaglandin synthetase systems (PSSs) in the renal medulla of the rat and rabbit, and inhibition by ketoprofen, indomethacin, ibuprofen, phenylbutazone and aspirin were investigated in relation to their anti-inflammatory, analgesic, antipyretic and ulcerogenic activities. Rat and rabbit PSSs produced prostaglandin (PG) E1 and PGF from arachidonic and dihomo-γ-linolenic acids and had an optimal pH of 8.5 and 7.5 for PG E formation, respectively. Only a slight loss of activity occurred with lyophilization. In the rat PSS, all drugs tested were inhibitory in the order of ketoprofen, ibuprofen, indomethacin and aspirin, respectively. In the rabbit PSS, the same potency relationship was also found. Drug sensitivity of the rat PSS was remarkably lower than that of the rabbit PSS. Significant correlations were noted between the inhibitory potencies of the drugs against both PSSs and other in vivo pharmacological activities within the same species.

Vane has proposed that inhibition of the prostaglandin synthetase system (PSS) by non-steroidal anti-inflammatory drugs is involved in the mechanism of pharmacological actions (1). Since then, many investigators reported the inhibitory effects of drugs on PSSs obtained from the bovine and sheep seminal vesicles (2-5), guinea-pig lung (1), rabbit kidney (6) and dog spleen (7) which have a high ability of prostaglandin (PG) formation (8). However, these results indicated that the relative inhibitory potencies of the drugs against PSSs vary remarkably depending on the tissues employed as enzyme source, as well as the assay conditions. In addition, the inhibitory effects of non-steroidal anti-inflammatory drugs on PSSs do not necessarily correlate with their pharmacological ones, since comparison of the both effects has been done between data obtained from different animal species (7, 9-13). For a valid test of Vane’s hypothesis, correlations in the same species should be determined.

We carried out experiments to observe the correlations between inhibition of PSSs from the renal medulla of rats and rabbits and other pharmacological effects such as antiedema, analgesia and ulceration in the rat and antipyresis in the rabbit in non-steroidal anti-inflammatory drugs.

MATERIALS AND METHODS

Chemicals

Chemicals used were 3H-arachidonic acid, 14C-dihomo-γ-linolenic acid, 3H-PGs (E1,
E2, F10, F2a) (New England Nuclear), PG standards (Ono), arachidonic acid, l-epinephrine bitartrate (Sigma) and reduced l-glutathione (BDH). Drugs used were aspirin (Rhone-Poulenc), phenylbutazone (Fujisawa), ibuprofen (Kaken), indomethacin (Merck) and ketoprofen (synthesized in Daiichi Sefyaku Research Institute). All other chemicals were of analytical grade.

**Animals**

Male Donryu strain rats (130 to 200 g, Nihon Rat) and male albino rabbits (2.2 to 3 kg, Ichikawa) were used.

**Preparation of microsomes**

The animals were sacrificed and the kidneys were rapidly removed and chilled on ice. The renal medulla was isolated from the renal cortex and homogenized in 2 volumes of 0.1 M phosphate buffer (pH 8.5 and 7.5 for the rat and rabbit renal medulla, respectively) using a Polytron homogenizer (Kinematica) for 1 min and a Potter-Elvehjem homogenizer for 3 min at 4°C. After centrifugation at 12,000 x g for 20 min, the supernatant fluids were recentrifuged at 105,000 x g for 1 hr. The pellet was suspended in the respective phosphate buffer with a Potter-Elvehjem homogenizer and the suspension was again centrifuged at 105,000 x g for 1 hr. The pellet was finally resuspended in the respective phosphate buffer, lyophilized and stored for 7 days in a freezer at ---20°C. These lyophilized microsomes were used as PSS.

**Enzyme assay**

Unless otherwise stated, the enzymatic assays were conducted in a shaker incubator (Yamato) at 37°C for 30 min. Each component was dissolved in 0.2 M Tris-HCl buffer (rat PSS: pH 8.5, rabbit PSS: pH 7.5) and each incubation tube usually contained lyophilized microsomes (rat PSS: 10 mg, rabbit PSS: 7.5 mg), 30 nM 3H-arachidonic acid, 1 nM 14C-dihomo-1-linolenic acid, 1.7 mM reduced l-glutathione, 0.5 mM l-epinephrine, 0.07 mM EDTA-2Na, various concentrations of inhibitors as indicated and 0.2 M Tris-HCl buffer in a final volume of 1.5 ml. Following incubation, the mixtures were diluted with 1.5 ml distilled water, acidified with 0.5 ml of 1.0 N HCl and immediately extracted twice with 5 ml ethyl ether. Recovery of 3H-arachidonic acid, 14C-dihomo-1-linolenic acid and 3H-PGs (E2, E9, F10, F2a) from the mixtures was over 90% through extraction with ethyl ether. The resultant extracts were concentrated under a stream of nitrogen gas. The concentrates were chromatographed on thin-layer silica gel plates (Merck, Kieselgel 60 F254) in benzene-dioxane-acetic acid (20 : 10 : 1). The developed plates were scanned in a radiochromatogram scanner (Aloka, model JTC-203) to locate the radioactive spots. PG standards were visualized with iodine vapor. After spontaneous loss of the iodine, the spots on the chromatogram corresponding to PGE, PGF and the other fractions were scraped into scintillation vials, suspended in 15 ml of dioxane scintillation phosphor (PPO: 7 g, dimethyl-POPOP: 0.3 g, naphthalene: 100 g in 900 ml of dioxane) and assayed for radioactivity (3H, 14C) in a liquid scintillation spectrometer (Aloka, model LSC-652). In this enzyme assay, PGE and PGF were formed from the substrates and PGE2 formation rates were approx. 5 and
40% for the rat and rabbit PSSs, respectively. The % inhibition was obtained by comparison of % of radioactivity converted from the labeled substrates into PGs in the presence and absence of the drugs. The experimental values for the drugs were obtained by running triplicate assays and control assays were carried out as a standard practice in all experiments to insure comparable enzyme activity. IC50 values represent the molar concentration of the drugs resulting in a 50% inhibition of PG biosynthesis and calculated from the formula of the best fit straight lines for log concentration vs. % inhibition plots which were determined by the method of least squares.

Pharmacological assays

Anti-inflammatory activity: The experiment was carried out according to a modification of the method of Winter et al. (14). Seven rats were used for each dose level. A 1% carrageenan suspension in saline was given s.c. into the left hind paw in a volume of 0.1 ml. Test drugs were administered p.o. in a suspension of 0.5% carboxymethyl cellulose solution 1 hr before the carrageenan injection and foot volume was measured at 1 hr intervals for 4 hr. Difference in the foot volume measured before and after the carrageenan injection was converted into %. The determination of % inhibition of swelling was made by comparing the % inhibition between drug-treated and control groups. ID50 value, the dose of the test drug which inhibits the foot swelling by 50%, was estimated on the basis of the mean of the % inhibition determined 2, 3 and 4 hr after the carrageenan injection.

Analgesic activity: The analgesic activity was measured according to the method described by Randall and Selitto (15) and seven rats were used for each dose level. A volume of 0.1 ml of a 10% Brewer’s yeast suspension in saline was injected s.c. into the left hind paw. Test drugs were given p.o. in a suspension of 0.5% carboxymethyl cellulose solution 2 hr after the Brewer’s yeast injection and the pain threshold was measured on the inflamed and non-inflamed paws before the Brewer’s yeast injection and at 1 hr intervals for 4 hr after the drug administration. AID2.0 value, the dose of the test drug required to make the analgesic index (Formula 1) 2 as measured in the inflamed paw 2 hr after the drug administration, was determined.

Analgesic index = \( \frac{(TA/TA)}{(TA/TA)} \frac{(CA/CA)}{\text{Formula 1}} \)

In the Formula 1, TA and CA represent the pain threshold after the drug administration in the drug-treated group and in the control group, respectively. TB and CB represent the pain threshold before the Brewer’s yeast injection in the drug-treated and control groups, respectively.

Antipyretic activity: Five rabbits were used for each dose level. The hyperthermia was induced by i.v. injection of 10 µg/kg T.T.G., No. 2 (lipopolysaccharide prepared from pseudomonas fluorescens, Fujisawa). Test drugs were administered p.o. in a suspension of 0.5% carboxymethyl cellulose solution 90 min after injection of the pyrogen and rectal temperature was measured at 30 min intervals for 3.5 hr. ID50 value, viz., the dose of the test drug required to cause a 50% reduction in the area under the rectal temperature-time...
Ulcerogenic activity: The experiment was carried out according to the method described by Jahn and Adrian (16). Rats fasted for 24 hr before the experiment were used for each dose level. Test drugs were administered p.o. in a suspension of 0.5% carboxymethyl cellulose solution and 3.5 hr later the animals were sacrificed. The stomach of each rat was removed and examined macroscopically for gastric irritation which was defined as ulceration and/or hemorrhagia of the gastric mucosa. The presence of a single ulcer or hemorrhagic spot was taken as the positive and UD50 value was determined according to the method of Litchfield-Wilcoxon (17).

All ID50 and AID2.0 values described above were obtained from the regression line fitted by the least square method and 95% fiducial limits were calculated according to Filler's equation (18).

RESULTS

Determination of assay conditions

PGE and PGF were formed from arachidonic acid and dihomo-γ-linolenic acid by both rat and rabbit PSSs. The formation rates of PGE2 and PGF2α from arachidonic acid were approx. twice as high as those of PGE1 and PGF1α from dihomo-γ-linolenic acid in both

![Fig. 1. Effect of pH on PG biosynthesis by the rat and rabbit PSSs. Incubation and assay conditions are as described in Methods, excepting various pH buffers were used (0.1 M phosphate, : 0.2 M Tris-HCl, : 0.1 M bicarbonate, •).](image1)

![Fig. 2. Effect of incubation time on PGE biosynthesis by the rat and rabbit PSSs. Incubation and assay conditions are as described in Methods.](image2)
PSSs. As shown in Fig. 1, the PG formation rates depended on the pH. The optimal pH for PGE and PGF formation by the rat PSS was approx. 8.5. Contrary to the rat PSS, the optimal pH for PGE formation was different from that of PGF formation by the rabbit PSS, viz., the optimal pH for PGE and PGF formation was approx. pH 7.5 and 8.5, respectively. As can be seen in Fig. 2, at each optimal pH with 30 μM arachidonic acid and 1 μM dihomo-γ-linolenic acid, PG formation by both PSSs was increased linearly up to 30 min, thus incubation was carried out for 30 min.

Microsomes were lyophilized and stability of the enzyme preparation was investigated after storage at -20°C for 7 days. As shown in Table 1, the PG formation activity of the rabbit lyophilized microsomes was found to be approx. 90% of the activity of freshly prepared microsomes.

Effects of arachidonic acid concentrations on the PGE₂ formation rate are shown in Fig. 3. The apparent Km values for arachidonic acid were estimated by Lineweaver-Burk

### Table 1. Effect of lyophilization on activity of rabbit PSS

| PGs | % conversion of substrate into PGs | Lyophilized/Lyophilized | Lyophilized/Lyophilized |
|-----|----------------------------------|-------------------------|-------------------------|
| F₁  | 12.8 ± 0.47                      | 11.0 ± 1.01             | 0.86                    |
| F₂  | 19.4 ± 3.38                      | 17.0 ± 2.10             | 0.88                    |
| F₁  | 22.9 ± 0.07                      | 21.1 ± 1.50             | 0.92                    |
| F₂  | 40.0 ± 3.78                      | 34.9 ± 3.89             | 0.87                    |

a) Fresh microsomes. b) Microsomes stored at -20°C for 7 days after lyophilization. c) Values represent the means ± standard error for three experiments. Incubation and assay conditions are as described in Methods, except that incubation was carried out at pH 8.5.

![Fig. 3. Effect of arachidonic acid concentrations on PGE₂ biosynthesis by the rat and rabbit PSSs. Incubation and assay conditions are as described in Methods, except that the concentration of arachidonic acid was varied as indicated. ---: rat PSS, ---: rabbit PSS.](image)
plots to be $4.2 \times 10^{-5}$ and $5.9 \times 10^{-5}$ M in the rat and rabbit PSSs, respectively.

From these results, the basal reaction conditions described in Methods were derived for the determination of PG synthetase activity of rat and rabbit PSSs, and PG biosynthesis \textit{in vitro} with both PSSs was investigated in the presence and absence of the drugs.

\textit{Inhibition of PG biosynthesis in vitro by non-steroidal anti-inflammatory drugs}

As shown in Fig. 4, the drugs tested exerted a dose-dependent inhibition of the conversion of the substrates into PGs by the rat and rabbit PSSs and the dose-response curve for each drug was parallel within the same PSS. Therefore, inhibitory potencies of the drugs against both PSSs were compared by IC50 values. As shown in Table 2, in the rabbit PSS, all drugs tested were inhibitory in the order of ketoprofen, indomethacin, ibuprofen, phenylbutazone and aspirin. Ketoprofen and indomethacin were 3393 and 1587 times as potent as aspirin, respectively. In addition, the inhibitory potencies of the drugs against PGE synthetase were similar to those against PGF synthetase in the rabbit. Order of the inhibitory potencies of the drugs against the rat PSS was identical with that against the rabbit PSS, except for ibuprofen. Thus, all drugs tested were inhibitory in the order of ketoprofen, ibuprofen, indomethacin and aspirin in the rat PSS. Ketoprofen and indomethacin were 488 and 102 times as potent as aspirin, respectively.

\textit{Correlation among the inhibition of PG synthetase and the other pharmacological properties in non-steroidal anti-inflammatory drugs}

Table 2 also compares IC50 values of the drugs against the rat and rabbit PSSs with ID50, AID2.0, UD50 and UD50 values of the drugs against carrageenan-induced edema, Brewer's yeast-induced pain, T.T.G.-induced fever and gastric ulcer. There are significant correlations among the inhibitory potencies of the drugs against the rat PSS and anti-inflammatory and analgesic activities in the rat. Similarly, antipyretic activity significantly correlated with rabbit PSS inhibition. No correlation was found between the inhibition of the rat PSS and ulcerogenic activity in the rat.

\textbf{DISCUSSION}

According to reports by Iizuka et al. (19) and Pong and Levine (20), the kind of PGs formed from arachidonic acid depends on the assay method of PGs. PGE$_2$ was mainly deter-
TABLE 2. Inhibition of PG biosynthesis in rat and rabbit PSSs by non-steroidal anti-inflammatory drugs and related pharmacological activities

| Drugs      | Rat PSS | Rabbit PSS | Pharmacological activities<sup>a</sup> | Anti-inflammatory ID<sub>50</sub> | Analgesic AID<sub>2.0</sub> | Antipyretic ID<sub>50</sub> | Ulcerogenic UD<sub>50</sub> |
|------------|---------|------------|---------------------------------------|-------------------------------|---------------------------|---------------------------|---------------------------|
|            | IC<sub>50</sub> (pM) |            |                                       |                               |                           |                           |                           |
| Aspirin    | 4400    | 984        | 977                                   | 721                           | 687                       | 175.1                     | 157.6                     | 296.6                     | 17.4                     |
| Phenylbutazone | NT<sup>b</sup> | 22.0       | 14.6                                  | 13.8                          | 7.4                       | 77.7                      | 76.0                      | 92.9                      | 96.0                     |
| Ibuprofen  | 34.9    | 10.4       | 29.1                                  | 9.6                           | 17.4                      | 20.8                      | 29.0                      | 69.0                      | 148.0                    |
| Indomethacin | 43.3    | 0.62       | 0.44                                  | 0.46                          | 0.33                      | 8.4                       | 2.9                       | 12.5                      | 6.6                      |
| Ketoprofen | 9.0     | 0.29       | 0.58                                  | 0.26                          | 0.37                      | 12.2                      | 2.4                       | 4.2                       | 6.8                      |
| Correlation coefficient |          |            |                                       | 0.998<sup>c</sup> | 0.986<sup>c</sup> | 0.956<sup>d</sup> | 0.263<sup>e</sup> |
| Significance (P at t-test) |          |            |                                       | &lt;0.001                     | &lt;0.01                    | &lt;0.001                   | N.S.<sup>f</sup> |

<sup>a</sup> Described in Methods.  
<sup>b</sup> Not tested.  
<sup>c</sup> Correlation coefficient of the inhibition by the drugs of PGE<sub>2</sub> formation by rat PSS to the anti-inflammatory, analgesic and ulcerogenic activities in rats.  
<sup>d</sup> Correlation coefficient of the inhibition by the drugs of PGE<sub>2</sub> formation by rabbit PSS to the antipyretic activity in rabbits.  
<sup>e</sup> Not significant.  
<sup>f</sup> N.S.
mined by bioassay but only PGF$_{2\alpha}$ was detected by radioimmunoassay after incubation with rabbit PSS. Our present results clarified that rat and rabbit PSSs produced PGE$_2$ and PGF$_{2\alpha}$ from arachidonic acid simultaneously and that in rabbit PSS, the optimal pH for PGE$_2$ formation was different from that for PGF$_{2\alpha}$ formation but the optimal pH for PGE$_2$ and PGF$_{2\alpha}$ formation was the same in the rat PSS. Furthermore, it has been reported that PGE type PGs are recovered from various inflamed tissues such as carrageenan-induced edema and air pouch injected with phlogistic agents (21, 22), and have more powerful actions on the inflammatory process than PGF type PGs (23–25). Therefore, the optimal pH for PGE formation was employed in both PSSs.

The drugs tested in this study exhibited a dose-dependent inhibition of both PSSs and drug sensitivity of rabbit PSS was higher than that in the rat. In addition, the order of their inhibitory potencies against both PSSs was similar except for ibuprofen. Ferreira and Vane have reported that inhibitory potencies of ibuprofen against various preparations of PSS vary remarkably (26). Of the drugs tested, ketoprofen was the most potent against both PSSs and was followed by indomethacin and aspirin. IC$_{50}$ values of ketoprofen and indomethacin for both PSSs were considerably less than peak plasma levels attained by the administration of effective doses of both drugs to rats (27, 28). This result suggests that both drugs may induce inhibition in the in vivo PG biosynthesis in the rat and that this inhibition may be involved in the mechanism of the other pharmacological properties. Moreover, the present findings that the inhibitory potencies of all the drugs tested against PGE formation are similar to those against PGF formation in the rabbit PSS are compatible with the data that these same compounds inhibit the formation of cyclic endoperoxides by dioxygenases from unsaturated fatty acids (29).

According to other investigators (7, 9–13), the inhibitory actions against PSSs from the bovine and sheep seminal vesicles by non-steroidal anti-inflammatory drugs do not necessarily correlate with other pharmacological actions of the drugs in the mouse, rat and rabbit. However, when we compared our data obtained from the same species, significant correlations were seen to exist between IC$_{50}$ values for PGE$_2$ formation of both PSSs and ID$_{50}$, AID$_{2.0}$ and ID$_{50}$ values for anti-inflammatory, analgesic and antipyretic activities, respectively. Such observations support Vane’s proposal that inhibition of PG synthetase by non-steroidal anti-inflammatory drugs is the mechanism of action involved in reducing inflammation, pain and fever (1). Although Ferreira and Vane reported that inhibition of PG biosynthesis may also lead to untoward effects in organs that depend upon PG for normal physiological function (26), no clear-cut correlation was found between the drug inhibition of the rat PSS and drug-induced gastric ulceration in the rat.

Investigation of inhibition of PG biosynthesis in vitro was found to be useful for elucidation of the mechanism of pharmacological actions of non-steroidal anti-inflammatory drugs.

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