Different Modes of Inhibition of Rat Gastric Mucosal 6-Keto-PGF_{1 α} Production by Indomethacin, Aspirin and Aminopyrine

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Abstract—The actions of aminopyrine on rat gastric mucosal cyclooxygenase activity *in vitro* were investigated and compared with those of the cyclooxygenase inhibitors indomethacin and aspirin. Aminopyrine is a phenyl-pyrazolone derivative with potent analgesic and antipyretic properties, but is weakly ulcerogenic, while indomethacin and aspirin are known to cause considerable ulcerogenesis. Aminopyrine was less potent in its ability to inhibit cyclooxygenase activity. The inhibition by all three drugs decreased with an increase in the substrate concentration. Pre-incubation with the enzymes greatly increased the inhibitory action of indomethacin and aspirin, but only slightly increased that of aminopyrine. The inhibitory action of aminopyrine was reversible, whereas those of indomethacin and aspirin were irreversible. These findings are discussed in relation to the low incidence of gastrointestinal irritation caused by aminopyrine.

Indomethacin, aspirin and many other nonsteroidal anti-inflammatory drugs (NSAIDs) have long been known to be ulcerogenic (1–3), particularly in conjunction with other factors such as stress (4), and have been known to be inhibitors of prostaglandin (PG) biosynthesis (5). Aminopyrine, a phenyl-pyrazolone derivative with analgesic and antipyretic properties, is as effective as aspirin and other NSAIDs in the therapy for acute rheumatic fever, and it is sometimes considered a superior anti-inflammatory agent because it causes less gastric irritation and ulcerogenesis, although its clinical use has declined due to its recently recognized bone marrow toxicity (6). PGs and PG synthetase may be present in the gastrointestinal tract in relation to their physiological functions (7–10). The inhibitory effect of many NSAIDs on PG biosynthesis causes ischemia of vascular beds, especially in areas already damaged by other factors (5, 11). Drugs which produce gastric mucosal ulceration or injury have been shown to reduce H⁺ secretion (12, 13). Spenny and Mize (14) reported that indomethacin, aspirin and other NSAIDs enhanced

the back-diffusion of H⁺ through the mucosa, increased the loss of H⁺ from the lumen, and consequently reduced H⁺ secretion.

The analgesic, antipyretic and anti-inflammatory properties and the PG synthesis-inhibiting activities of these drugs have been extensively studied, but little is known about the mechanisms underlying ulcerogenesis in the gastrointestinal tract. The present study compared the effects of aminopyrine, indomethacin and aspirin on rat gastric mucosal cyclooxygenase activity under various experimental conditions in order to try to establish the mechanisms of ulcerogenesis.

Materials and Methods

Preparation of enzyme: Male Wistar rats weighing 150–250 g were killed by exsanguination after a blow on the head. Following removal of their stomachs, mucosal sections were prepared by separating the overlying muscle by blistering (15). This procedure involved inserting the tip of a fine needle (26 gauge) between the muscle and mucosa and injecting 20 mM Tris-HCl buffer (pH 7.4). The raised muscle layer was then

carefully cut away. The parts of the mucosal membranes were gently homogenized (200 rpm/min) in a glass-Teflon homogenizer with ten volumes of 50 mM potassium phosphate buffer (pH 7.4). All procedures were performed at 0–3°C. The homogenates were used as the source of the enzyme (cyclooxygenase). The enzyme was incubated at 37°C for 6 min, after which the enzyme activity was stopped by addition of 50 μ l of ice-cold 2 M citric acid. The mixture was centrifuged at 20,000 g for 10 min at 0–3°C and then assessed for the production of 6-keto-PGF_{1 α} (the stable breakdown product of PGI₂) using radioimmunoassay kits (16). The results were expressed as ng (6-keto-PGF_{1 α})/mg mucosal protein and as the percentage of inhibition of the control value. Mucosal protein concentration was determined by the method of Lowry et al. (17) with bovine serum albumin as a standard.

Reaction medium: Unless otherwise stated, determination of cyclooxygenase activity was carried out at 37°C in a 1-ml reaction mixture of arachidonic acid (2×10^{-5} M), enzymes (0.7 mg gastric mucosal protein/ml), hydroquinone (4×10^{-4} M), glutathione (5×10^{-4} M), various concentrations of indomethacin, aspirin or aminopyrine, ethanol (1.0%, v/v) and potassium phosphate buffer (50 mM, pH 7.4). The pH value of the incubation medium was always confirmed to be pH 7.4 before the start of incubation or pre-incubation. Ethanol, used as a solvent for arachidonic acid and inhibitors, had no effect on the reaction at the final concentration (2.0%, v/v) employed.

Effects of indomethacin, aspirin and aminopyrine on cyclooxygenase activity under different experimental conditions: The inhibition of cyclooxygenase by indomethacin, aspirin and aminopyrine was studied under the following three incubation conditions:

a) Inhibitors (indomethacin, aspirin and aminopyrine) and arachidonic acid were simultaneously added to the medium, and the reaction was promptly started by adding the enzyme preparation at 37°C in the presence of the cofactors (hydroquinone and glutathione) without pre-incubation.

b) Inhibitors were pre-incubated with enzymes in phosphate buffer containing cofactors at 37°C for 2 min, and the reaction

was started by adding 1 to 5 volumes of phosphate buffer containing arachidonic acid and cofactors at 37°C; i.e., the concentration of inhibitors in the reaction medium was diluted to 1/2–1/6 of that in the pre-incubation medium.

c) Inhibitors were pre-incubated with enzymes in phosphate buffer containing cofactors at 37°C for 2 min, and the reaction was promptly started at 37°C with the cofactors.

Chemicals: The following drugs were used: 6-keto-PGF_{1 α} radioimmunoassay kits (NEN Research Product), arachidonic acid (Sigma, Grade 1; purity, approx. 99%), hydroquinone (Merck), reduced glutathione (Sigma), indomethacin (Sigma), aspirin (Sigma) and aminopyrine (Sigma).

Statistical analysis: Statistical differences were analyzed by the unpaired Student's *t*-test, and linear regression analysis was done in some experiments. Data are presented as the mean $\pm$ the standard error of the mean.

Results

Inhibitions of PG biosynthesis by indomethacin, aspirin and aminopyrine: The basal production of 6-keto-PGF_{1 α} was 2.8×10^{-10} moles/min/mg gastric mucosal protein.

Various amounts of indomethacin, aspirin and aminopyrine were added to the incubation test tube, and the percentage inhibition of cyclooxygenase activity was determined.

All drugs tested inhibited the formation of PGs as shown in Fig. 1. As expected, the inhibitory action of indomethacin was markedly higher than those of aspirin and aminopyrine. The inhibitory potency of aminopyrine was less than that of aspirin. The IC₅₀ values of indomethacin, aspirin and aminopyrine were calculated to be 8.1×10^{-7} , 1.1×10^{-3} and 3.8×10^{-3} M, respectively, from the best fit curves ($r^2=0.91$, $r^2=0.90$, $r^2=0.90$, respectively).

Effect of substrate concentration on inhibition of cyclooxygenase activity by drugs: Arachidonic acid at 5 to 80 μ M was added to the reaction mixture with the inhibitors, and the reaction was started by adding the enzyme preparation. Inhibitors were used at a fixed concentration of IC₅₀. The inhibitions of PG biosynthesis by indomethacin ($8.1 \times$

Fig. 1. Inhibition of rat gastric mucosal 6-keto-PGF_{1α} production (cyclooxygenase) by indomethacin, aspirin and aminopyrine. The reaction media contained arachidonic acid (8×10^{-5} M), hydroquinone (4×10^{-4} M), glutathione (5×10^{-4} M), various concentrations of inhibitors and 0.7 mg mucosal protein in 1 ml of 50 mM potassium phosphate buffer (pH 7.4). Incubation was carried out at 37°C for 6 min. For details, see "Materials and Methods." Points are means for 4 to 8 determinations of different samples.

Fig. 2. Effects of substrate concentration on inhibition of 6-keto-PGF_{1α} production by indomethacin, aspirin and aminopyrine. The reaction medium contained various concentrations of arachidonic acid (5–80 μ M), hydroquinone, glutathione, inhibitor (indomethacin, 8.1×10^{-7} M; aspirin, 1.1×10^{-3} M; aminopyrine, 3.8×10^{-3} M) and 0.7 mg mucosal protein in 1 ml of 50 mM phosphate buffer. Incubation was carried out at 37°C for 6 min. Columns represent mean values for 6 to 7 determinations with S.E.M. as a vertical line.

10^{-7} M), aspirin (1.1×10^{-3} M) and aminopyrine (3.8×10^{-3} M) increased with a decrease in the arachidonic acid concentrations. At the highest concentration of substrate used (80 μ M), the inhibition levels by indomethacin, aspirin and aminopyrine were 33.2,

32.3 and 21.5%, respectively, while at the lowest concentration of substrate (5 μ M), their inhibitions were 81.2, 80.2 and 78.8%, respectively (Fig. 2). By Lineweaver-Burk plot analysis, the K_m and V_{max} values were determined to be 5.0×10^{-5} M and 3.4×10^{-10}

mole/min/mg protein, respectively, in the absence of inhibitors. These three inhibitors appear to be competitive as our data on the Lineweaver-Burk plot yielded straight lines with an intercept at approximately $V_{\max}$.

Effect of dilution of drugs on cyclooxygenase activity: All inhibitors (two different concentrations of inhibitors were used: IC₅₀ and higher than IC₈₀) were pre-incubated with enzymes for 2 min, and the reaction was started by adding 1 to 5 volumes of phosphate buffer containing arachidonic acid and cofactors. Consequently, the concentration of inhibitors in the reaction medium was 1/2–1/6 of that in the pre-incubation medium. As shown in Fig. 3, when the reaction mixture was diluted, the inhibitory action by aminopyrine was reversed in proportion to the degree of dilution, but not those by indomethacin and aspirin. The effects of indomethacin (2×10^{-6} , 8.1×10^{-7} M) and aspirin (3.3×10^{-3} , 1.1×10^{-3} M) were not reversed until six-fold dilution, whereas the inhibition by aminopyrine (9×10^{-3} , 3.8×10^{-3} M) was almost fully reversed at five- to six-fold

dilutions (see Fig. 3).

Effects of pre-incubation of cyclooxygenase with drugs: The inhibition of cyclooxygenase activity by inhibitors with or without pre-incubation was studied. Indomethacin, aspirin and aminopyrine (two different concentrations of inhibitors were used: IC₅₀ and lower than IC₂₅) were added at the start of pre-incubation, and their inhibitory effects were compared with the effects without pre-incubation. As shown in Fig. 4, indomethacin and aspirin were markedly more inhibitory after pre-incubation with the enzymes for 2 min: without and with pre-incubation, 8.1×10^{-7} M indomethacin caused 50 and 90% inhibition, respectively, and 4×10^{-7} M indomethacin caused 23 and 73% inhibition, respectively; 1.1×10^{-3} M aspirin caused 50 and 94% inhibition, and 4×10^{-4} M aspirin, 22 and 74% inhibition, respectively. Aminopyrine inhibition was only slightly increased by pre-incubation: without and with pre-incubation, 3.8×10^{-3} M aminopyrine caused 50 and 60% inhibition, respectively, and 2.1×10^{-3} M aminopyrine, 24 and 34%, respectively.

Fig. 3. Effects of dilution of pre-incubation medium on inhibition of 6-keto-PGF_{1α} production by indomethacin, aspirin and aminopyrine. Inhibitors (indomethacin, 2×10^{-6} , 8.1×10^{-7} M; aspirin, 3.3×10^{-3} , 1.1×10^{-3} M; aminopyrine, 9×10^{-3} , 3.8×10^{-3} M) were pre-incubated with enzymes (0.7 mg protein/ml) in 50 mM phosphate buffer containing hydroquinone and glutathione at 37°C for 2 min, and the reaction was started by adding 1 to 5 volumes of 50 mM phosphate buffer containing arachidonic acid, hydroquinone and glutathione at 37°C for 6 min. Thus, the concentration of inhibitors in the reaction medium was 1/2–1/6 of that in the pre-incubated medium. Points represent means $\pm$ S.E.M. for 7 determinations. Significantly different from the response of the un-diluted sample, * $P < 0.05$, ** $P < 0.01$.

Fig. 4. Effects of pre-incubation of enzymes with indomethacin, aspirin and aminopyrine on 6-keto-PGF_{1α} production. □, no pre-incubation (substrate and inhibitors were simultaneously added to the incubation medium). ▨, pre-incubation (inhibitors were pre-incubated with enzymes at 37°C, and 2 min later, substrate was added). The reaction media contained arachidonic acid, hydroquinone, glutathione, inhibitor (indomethacin, 8.1×10^{-7} , 4×10^{-7} M; aspirin, 1.1×10^{-3} , 4×10^{-4} M; aminopyrine, 3.8×10^{-3} , 2.1×10^{-3} M) and 0.7 mg protein in 1 ml of 50 mM phosphate buffer. Incubation was carried out at 37°C for 6 min. Columns represent means $\pm$ S.E.M. for 5 to 7 determinations. Significantly different from response of the corresponding non-incubated sample, * $P < 0.01$, ** $P < 0.001$.

Discussion

The present results have shown that like indomethacin and aspirin, aminopyrine inhibited the rat gastric mucosal cyclooxygenase and the nature of the inhibition appears to be competitive. The order of inhibitory potency was indomethacin $\gg$ aspirin $>$ aminopyrine. Inhibitory effects of these drugs were similar to those reported by Flower (18) using a homogenate of sheep seminal vesicles, except that aspirin and aminopyrine were approximately equipotent in inhibiting cyclooxygenase activity. Several workers have already reported that indomethacin and aspirin or salicylate-like drugs are competitive inhibitors for PG synthetase (6, 18–21), although the mode of inhibition of aminopyrine has not yet been determined. The mechanism of the analgesic and antipyretic action of aminopyrine or its derivatives is still a matter of controversy (21–23).

The inhibitory activities of indomethacin and aspirin were greatly potentiated by pre-incubation with the enzymes and that of aminopyrine only slightly. Furthermore, the inhibitory actions of indomethacin and aspirin

were not changed by dilution of the pre-incubation mixture, whereas the inhibition by aminopyrine was suppressed largely by dilution (see Fig. 3). Ku and Wasvary (24) and Lands and LeTellier (25) found that indomethacin and aspirin had the dual nature of so-called "competitive-irreversible" inhibition. According to this concept, indomethacin and aspirin may interact with a binding site sufficiently close to the active site to reduce the catalytic activity of the enzyme in a time-dependent manner. On the other hand, aminopyrine inhibits it in a time-dependent but reversible manner. These results suggest that the *in vivo* effects of the reversible inhibitor aminopyrine, especially its inhibitory effect on the gastrointestinal tract, might be short-lasting, while that of indomethacin or aspirin might be long-lasting. In relation to this, Melarange and Rashbrook (10) recently compared the effects of nabumetone, 4-(6-methoxy-2-naphthyl)-butane-2-one, which has a potent anti-inflammatory action, with indomethacin on rat gastric mucosal cyclooxygenase activity (production of 6-keto-PGF_{1α}) and suggested that nabumetone did not cause gastric damage because it had a

minimal effect on gastric mucosal cyclo-oxygenase.

Natural and synthetic PGs have been shown to be potent inhibitors of gastric acid secretion and offer protection against peptic ulcers in animals and humans (26–31). PG-induced cytoprotective effects may involve increased blood flow (32, 33), bicarbonate secretion (34), mucus release (35), restoration of active sodium transport (36), decrease in back diffusion of acid or local stimulation of surface-active phospholipids (37), and maintenance of normal mucosal levels of DNA, RNA or protein (38). Furthermore, stress has been shown to decrease endogenous PG formation in the rat mucosa and to augment peptic ulceration (39).

From our results and these findings, we suppose that the low incidence of gastrointestinal irritation due to aminopyrine, in comparison with other analgesics, might be related to its reversible effect (short-lasting) as well as to its lower inhibitory action on prostaglandin biosynthesis.

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