Comparison of effects of aspirin and indomethacin on human platelet prostaglandin synthetase

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SUMMARY Human platelets were incubated *in vitro* with either aspirin or indomethacin and the prostaglandin synthetase activity of the resultant microsomal fraction from each incubation measured using a radiometric technique. Whereas aspirin produced a dose-related inhibition of the enzyme, indomethacin produced little or no inhibition over the same concentration range (10^{-6} mol/l– 10^{-3} mol/l). Furthermore, administration of aspirin (600 mg) to volunteers produced a highly significant, prolonged inhibition of platelet microsomal prostaglandin synthetase whereas no inhibition was found with indomethacin (50 mg). As indomethacin is considerably more potent than aspirin as an inhibitor of human platelet prostaglandin synthetase *in vitro*, the results suggest a fundamental difference in the nature of the inhibition produced by each drug, aspirin being an essentially irreversible inhibitor whereas the inhibition produced by indomethacin is reversible. Studies with [³H-acetyl] aspirin have confirmed previous findings (Roth and Majerus, 1975) that aspirin produces an irreversible acetylation of a particulate fraction protein from human platelets.

The hypothesis that aspirin-like drugs exert their various pharmacological actions via inhibition of prostaglandin (PG) biosynthesis (Vane, 1971) has received wide support from many experiments carried out on human and animal systems, both *in vitro* and *in vivo* (Flower, 1974). Little is known about the nature of this blockade of PG biosynthesis at the molecular level, however, though results obtained with the enzyme prepared from sheep seminal vesicular glands suggest that most of the common aspirin-like drugs, including indomethacin and aspirin itself, are 'competitive-irreversible' inhibitors (Smith and Lands, 1971; Ku and Wasvary, 1973; Raz *et al.*, 1973). Furthermore, the absence of intermediates from inhibited preparations suggests that these drugs block an early, possibly the initial, stage of PG synthesis (Tomlinson *et al.*, 1972).

During the course of studies of the effects of aspirin and aspirin-like drugs on PG synthetase prepared from human rheumatoid synovial tissue, we have observed (Crook *et al.*, 1976) that prolonged therapy with the commonly used aspirin-like drugs did not inhibit the *in vitro* activity of a microsomal fraction derived from this tissue; in contrast, aspirin

therapy completely destroyed subsequent *in vitro* enzyme activity. In order to investigate this unexpected difference further, we have studied the effect of aspirin and indomethacin, both *in vitro* and *in vivo*, on PG synthetase prepared from human platelets. Our results show a distinct difference between the nature of the inhibition produced by these two drugs.

Materials and methods

Platelet-rich plasma was obtained from healthy volunteers by centrifugation of heparinized blood at 50 *g* for 10 minutes, then at 100 *g* for 10 minutes. A platelet pellet was obtained by centrifugation of the combined plasmas at 2500 *g* for 5 minutes.

PREPARATION OF MICROSOMAL FRACTION
5 ml ice-cold Tris-acetate buffer (0.1 mol/l, pH 8.0) containing sucrose (0.25 mol/l), EDTA (1 mmol/l), hydroquinone (0.5 mmol/l), cysteine (1 mmol/l), and reduced glutathione (2 mmol/l) was added to each cell preparation. While maintained in ice the mixture was sonicated (Rapidis 300, Ultrasonics Ltd.) by two 30-second pulses of 200 watts. Cell debris and any unbroken cells were removed by centrifugation at 2500 *g* for 5 minutes, after which a microsomal fraction was obtained by centrifugation at 10⁵ *g* for 1 hour.

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PRODUCTION OF ^{14}C PG FROM ^{14}C ARACHIDONIC ACID

Each microsomal pellet was resuspended in fresh buffer mixture by a brief period of sonication and incubated with ^{14}C -arachidonic acid (100 nCi, final concentration $0.85\text{ }\mu\text{mol/l}$) at 37° for 1 hour, with shaking, in a volume of 2 ml. The reaction was terminated by the addition of 2 M citric acid (0.25 ml).

EXTRACTION, SEPARATION, AND QUANTITATION OF FORMED PGS

The formed ^{14}C PGs and unconverted substrate were extracted from the acidified incubates with diethyl ether ($2 \times 3.0\text{ ml}$). After evaporation of the combined extracts under nitrogen at 37° the residue was dissolved in $20\text{ }\mu\text{l}$ chloroform/methanol, 2:1, and $5\text{ }\mu\text{l}$ applied to a thin layer plate (silica gel G, 0.25 mm , containing 2.5% silver nitrate). Authentic arachidonic acid, PGE_2 and $\text{PGF}_{2\alpha}$ were applied in a marker lane and the plate developed in benzene-dioxan-acetic acid, 20:20:1 to a height of 15 cm.

After locating the markers with phosphomolybdic acid and scanning the plate for radioactivity, the zones in the extract lanes which corresponded to the markers were scraped from the plate and their activity determined by liquid scintillation counting, quench corrections being achieved using a channels ratio method.

INCUBATION OF WHOLE PLATELETS WITH ASPIRIN AND INDOMETHACIN

Aliquots of platelet-rich plasma were incubated with either aspirin or indomethacin over the concentration range 10^{-6} mol/l – 10^{-3} mol/l , at 37° for 30 minutes, with shaking. The platelet pellet obtained by centrifugation at 2500 g for 5 minutes was resuspended in Tyrode's solution (5.0 ml), incubated at 37° for a further 10 minutes and the platelet pellet again obtained by centrifugation. The preparation of the microsomal fraction and measurement of PG synthetase activity were then carried out as described above.

INCUBATION OF PLATELET MICROSOMES WITH ASPIRIN AND INDOMETHACIN

Human platelet microsomes were resuspended in Tyrode's solution and aliquots incubated with either aspirin or indomethacin over the concentration range 10^{-6} mol/l – 10^{-3} mol/l , at 37° for 30 minutes with shaking. The suspension was then layered on to Tris-acetate buffer (0.1 mol/l , pH 7.4) containing sucrose (0.25 mol/l) and a microsomal pellet obtained by centrifugation at 10^5 g for 1 hour. The

PG synthetase activity of each microsomal pellet was then measured as described above.

EFFECT OF ASPIRIN AND INDOMETHACIN IN VIVO

Three healthy male volunteers who had received no medication of any kind for at least 2 weeks beforehand were given indomethacin (50 mg) orally, and blood samples obtained over the following 96 hours, including a pre-drug, basal sample. 3 weeks later the procedure was repeated with the subjects taking aspirin (600 mg). Platelet-rich plasma was prepared from each sample, a platelet count performed, and the PG synthetase activity of the microsomal fraction measured as described above, using a constant number of platelets from each subject. Plasma levels of indomethacin and salicylate were measured by standard techniques.

PROTEIN ESTIMATION

Microsomal protein concentrations were measured by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

PREPARATION OF [^3H -ACETYL] ASPIRIN

Salicylic acid (2.5 mg) was dissolved in pyridine (2.0 ml , redistilled) and heated with ^3H -acetic anhydride (25 mCi , specific activity 3500 mCi/mmol , Amersham) at 37° for 3 hours. The solvent was evaporated under nitrogen and the product purified by thin layer chromatography (silica gel G, 0.25 mm , methanol washed; solvent system cyclohexane-chloroform-acetic acid, 80:20:10). The radioactive aspirin band was transferred to a small sinter eluted with redistilled ethanol (10 ml), and stored at -20° . Chromatographic properties and absorption spectra of the product showed it to be identical to authentic acetylsalicylic acid.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF ACETYLATED PG SYNTHETASE

This was carried out essentially as described by Roth and Majerus (1975). Platelet microsomes were incubated with [^3H -acetyl] aspirin ($60\text{ }\mu\text{mol/l}$) in 0.1 mol/l Tris-acetate buffer, pH 7.4, at 37° for 30 minutes. The suspension was then layered on to Tris-acetate buffer (0.1 mol/l , pH 7.4) containing sucrose (0.25 mol/l) and a pellet obtained by centrifugation at 10^5 g for 1 hour. This was solubilized using sodium dodecyl sulphate (SDS)/mercaptoethanol solution and aliquots run on 5% polyacrylamide gels in 0.1 mol/l phosphate buffer, pH 7.1, containing 0.2% SDS. Gels were sliced, digested with hydrogen peroxide, and counted for radioactivity. Quench corrections were made using an external standard ratio method.

Fig. 1 Inhibition of human platelet microsomal prostaglandin (PG) synthetase by aspirin and indomethacin. Human platelet microsomes were incubated with ^{14}C -arachidonic acid as described in the text. Production of PGE_2 ($\blacktriangle$, $\bullet$) and $\text{PGF}_{2\alpha}$ ($\triangle$, $\circ$) was inhibited in a dose-dependent manner by the addition of aspirin or indomethacin. Points represent the means of duplicate estimations.

Fig. 2 Inhibition of human platelet microsomal PG synthetase after preincubation of whole platelets with aspirin or indomethacin. Whole human platelets were preincubated with either aspirin or indomethacin at 37° for 30 minutes over the concentration range 10^{-6} mol/l– 10^{-3} mol/l and the PG synthetase activity of the subsequent microsomal fraction from each incubation measured as described in the text. Whereas aspirin produced a dose-dependent inhibition, indomethacin had little or no effect. Means $\pm$ standard error of the means for 4 estimations are shown.

Results

When added to active microsomal preparations of human platelet PG synthetase, both aspirin and indomethacin produced the expected dose-related inhibition (Fig. 1). Calculated from the dose of each drug required to produce 50% inhibition of PG biosynthesis, and expressed on a molar basis, indomethacin was approximately 150 times more potent than aspirin. The production of PGE_2 and $\text{PGF}_{2\alpha}$ was equally affected, and no evidence was found for 'selective' inhibition by either aspirin or indomethacin.

Preincubation of whole platelets with either aspirin or indomethacin showed a marked difference between the two drugs (Fig. 2). Thus while exposure to aspirin at 10^{-4} mol/l was capable of producing 80–90% inhibition of PG synthetase, indomethacin had little or no effect, even 10^{-3} mol/l producing less than 10% inhibition of subsequent microsomal PG synthetase activity. The apparent stimulation of PG synthesis by aspirin at 10^{-6} mol/l is not readily explained, though similar phenomena have been reported in other systems (Brocklehurst and Dawson, 1974; Stone *et al.*, 1975).

Preincubation of human platelet microsomes with

Fig. 3 Inhibition of human platelet microsomal PG synthetase after preincubation of platelet microsomes with aspirin or indomethacin. Human platelet microsomes were preincubated with either aspirin or indomethacin at 37° for 30 minutes over the concentration range 10^{-6} mol/l– 10^{-3} mol/l and the PG synthetase activity of the washed microsomes measured as described above. Means $\pm$ standard error of the means for 4 estimations are shown.

either aspirin or indomethacin confirmed the findings described above with whole platelets. Thus while aspirin produced a dose-related inhibition of PG synthetase activity of similar potency to that found when present in an incubation mixture containing platelet microsomes and ^{14}C -arachidonic acid, indomethacin was considerably less potent than aspirin in producing inhibition of PG synthetase by pre-incubation (Fig. 3). As indomethacin is approximately 150 times more potent than aspirin as a PG synthetase inhibitor when present in an incubation mixture containing platelet microsomes and ^{14}C -arachidonic acid (Fig. 1), these findings strongly suggest that indomethacin is a reversible inhibitor of human platelet PG synthetase.

Administration of aspirin (600 mg) to 3 volunteers produced a prompt fall in platelet microsomal PG synthetase activity to less than 15% of basal levels, and this blockade lasted for several days (Fig. 4).

Fig. 4 Effect of orally ingested aspirin and indomethacin on subsequent microsomal PG synthetase activity from human platelets. 3 volunteers each took indomethacin (50 mg) orally. Blood samples were taken at intervals over the following 96 hours, platelets were prepared from each sample, and the PG synthetase activity of the microsomal fraction from each one measured as described in the text. 3 weeks later the procedure was repeated with the volunteers taking aspirin (600 mg). Means $\pm$ standard error are shown.

The time course of the inhibition of platelet PG synthetase by aspirin is thus effectively a measure of platelet turnover. By contrast, ingestion of indomethacin (50 mg) produced no reduction of microsomal PG synthetase activity in 2 individuals, and in the third subject brought about a stimulation of activity which at 24 hours after the drug had been taken was more than twice the basal level.

Studies using [^3H -acetyl] aspirin of high specific activity support the results obtained with aspirin from both the *in vitro* and *in vivo* experiments described here, and confirm previous findings (Roth and Majerus, 1975). Fig. 5 shows a typical scan of the radioactivity incorporated into platelet particulate fraction protein and separated by polyacrylamide gel electrophoresis. Reference to a series of protein markers of known molecular weight showed that the labelled peak had a molecular weight of 81 000, and calculation of the amount of label incorporated was in agreement with the previously reported figure of 2000–3000 acetyl groups per platelet.

Fig. 5 Polyacrylamide/SDS gel electrophoresis of human platelet microsomes incubated with [^3H -acetyl] aspirin. Human platelet microsomes were incubated with [^3H -acetyl] aspirin (60 $\mu\text{mol/l}$) in 0.1 M tris-acetate buffer, pH 7.4, at 37° for 30 min. The microsomes were washed by centrifugation through tris-acetate buffer containing sucrose (0.25 mol/l) and the pellet obtained was solubilized using SDS/mercaptoethanol. An aliquot of this solution containing 170 μg protein was electrophoresed on 5% polyacrylamide, the gel cut into 3 mm slices, digested with hydrogen peroxide, and the radioactivity in each slice measured by liquid scintillation counting. The molecular weight of the labelled peak was calculated to be 81 000.

Discussion

The ability of the aspirin-like drugs to inhibit platelet aggregation has been known for many years, and much compelling evidence now exists to suggest that this action is brought about by the blockade of the biosynthesis of PGs, their cyclic endoperoxide precursors, and the recently discovered thromboxanes (Smith and Willis, 1971; Willis, 1973; Hamberg *et al.*, 1975). However, while the effect of orally ingested aspirin in inhibiting both platelet PG production and platelet aggregation lasts for several days, the affect of indomethacin is short-lived (Kocsis *et al.*, 1973). As aspirin has a short half-life *in vivo* and sodium salicylate does not produce these effects, a fundamental difference in the modes of action of aspirin and indomethacin is indicated.

Such a finding has great practical importance in view of the interest that has centred around the clinical potential of the aspirin-like drugs as anti-thrombotic agents. Platelets contain little RNA or DNA and are capable of very limited *de novo* enzyme synthesis (Steiner, 1970). Hence a drug which causes irreversible inhibition of platelet PG synthetase would appear to be superior in this respect to one which can exert its effect only for as long as an adequate plasma concentration is maintained. The equivocal results obtained so far using aspirin, therefore, might not augur well for the possible efficacy of other aspirin-like drugs.

Furthermore, as platelets are a potential source of PG-like material at inflammatory foci (Glatt *et al.*, 1974), the time courses of the inhibition of PG production by aspirin or indomethacin at such sites might also be expected to differ. However, it is difficult to draw any further conclusions from the findings presented here without a detailed knowledge of the turnover rates of PG synthetase from the different cells involved.

The finding that aspirin produces a rapid, saturable, irreversible acetylation of a platelet particulate fraction protein at low (30 $\mu\text{mol/l}$) concentrations provides convincing evidence that this may be its mode of action in blocking PG synthesis at the molecular level. Consideration of the structures of other aspirin-like drugs, however, suggests that this is unlikely to be a general property of this group of compounds, as most possess no labile functional group. Our results suggest that indomethacin probably does not function as a benzoylating agent in an analogous manner to aspirin, though it would clearly be of interest to investigate this possibility

using suitably labelled indomethacin of high specific activity.

References

- Brocklehurst, W. E., and Dawson, W. (1974). *Future Trends in Inflammation*. p. 37. Ed. by G. P. Velo, D. A. Willoughby, and J. P. Giroud. Piccin Medical Books, Padua.
- Crook, D., Collins, A. J., Bacon, P. A., and Chan, R. (1976). Prostaglandin synthetase activity from human rheumatoid synovial microsomes—the effect of 'aspirin-like' drug therapy. *Annals of the Rheumatic Diseases*, **35**, 327–332.
- Flower, R. J. (1974). Drugs which inhibit prostaglandin biosynthesis. *Pharmacological Reviews*, **26**, 33–67.
- Glatt, M., Peskar, B., and Brune, K. (1974). Leucocytes and prostaglandins in acute inflammation. *Experientia*, **30**, 1257–1259.
- Hamberg, M., Svensson, J., and Samuelsson, B. (1975). Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proceedings of the National Academy of Sciences of the USA*, **72**, 2994–2998.
- Kocsis, J. J., Hernandezovich, J., Silver, M. J., Smith, J. B., and Ingelman, C. (1973). Duration of inhibition of platelet prostaglandin formation and aggregation by ingested aspirin or indomethacin. *Prostaglandins*, **3**, 141–144.
- Ku, E. C., and Wasvary, J. S. (1973). Inhibition of prostaglandin synthetase by Su-21524. *Federation Proceedings*, **32**, Abst. 3302.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265–275.
- Raz, A., Stern, H., and Kenig-Wakshal, R. (1973). Indomethacin and aspirin inhibition of prostaglandin E_2 synthesis by sheep seminal vesicles microsome powder and seminal vesicle slices. *Prostaglandins*, **3**, 337–352.
- Roth, G. J., and Majerus, P. W. (1975). The mechanism of the effect of aspirin on human platelets. 1. Acetylation of a particulate fraction protein. *Journal of Clinical Investigation*, **56**, 624–632.
- Smith, J. B., and Willis, A. L. (1971). Aspirin selectively inhibits prostaglandin production in human platelets. *Nature (New Biology)*, **231**, 235–237.
- Smith, W. L., and Lands, W. E. M. (1971). Stimulation and blockade of prostaglandin biosynthesis. *Journal of Biological Chemistry*, **21**, 6700–6702.
- Steiner, M. (1970). Platelet protein synthesis studied in a cell-free system. *Experientia*, **26**, 786–789.
- Stone, K. J., Mather, S. J., and Gibson, P. P. (1975). Selective inhibition of prostaglandin biosynthesis by gold salts and phenylbutazone. *Prostaglandins*, **10**, 241–251.
- Tomlinson, R. V., Ringold, H. J., Qureshi, M. C., and Forchielli, E. (1972). Relationship between inhibition of prostaglandin synthesis and drug efficacy: support for the current theory of the mode of action of aspirin-like drugs. *Biochemical and Biophysical Research Communications*, **46**, 552–559.
- Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nature (New Biology)*, **231**, 232–235.
- Willis, A. L. (1973). Platelet synthesis of pro-aggregating material from arachidonate and its blockade by aspirin. *Circulation*, **48**, Suppl. 4, 55.