

Prostaglandin synthetase activity from human rheumatoid synovial microsomes

Effect of 'aspirin-like' drug therapy

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Crook, D., Collins, A. J., Bacon, P. A., and Chan, R. (1976). *Annals of the Rheumatic Diseases*, 35, 327-332. Prostaglandin synthetase activity from human rheumatoid synovial microsomes. Effect of 'aspirin-like' drug therapy. Using a radiometric technique, prostaglandin synthetase activity was measured *in vitro* in the microsomal fraction of 19 synovial tissues taken from 17 rheumatoid arthritis patients. The enzyme was inhibited *in vitro* by low concentrations of several 'aspirin-like' drugs, though paracetamol and salicylic acid were virtually inactive.

While the synthetase preparations from patients receiving indomethacin, ibuprofen, or naproxen therapy exhibited considerable activity *in vitro*, we were unable to show any activity in preparations from patients taking aspirin, even in low doses. These findings suggest that *in vivo* aspirin may be unique in being an irreversible inhibitor of the enzyme, compared with other 'aspirin-like' drugs.

The role of prostaglandins as mediators of the inflammatory response is still not entirely clear. Their ability to increase vascular permeability in man and animals (Crunkhorn and Willis, 1969), and their ability to cause leucocyte emigration (Kaley and Weiner, 1971), suggest that they may be important mediators of the acute phase of the inflammatory reaction. Significant levels of prostaglandin E₂ (PGE₂) and prostaglandin F_{2α} (PGF_{2α}) have been recovered from inflammatory exudates (Willis, 1969). Control of the inflammatory response, with special reference to the prostaglandins has been reviewed by Willoughby and others (1973). The observations of Vane and his colleagues that aspirin, and other 'aspirin-like' drugs, are potent inhibitors of prostaglandin synthesis may explain the anti-inflammatory action of these drugs *in vivo* (Vane, 1971; Flower, 1974).

Using a radioimmunoassay technique to measure PGB levels in human synovial fluids, Levine (1973) showed that in a group of patients with inflammatory effusions the mean PGB concentration was significantly higher than that in a group with non-inflammatory lesions. A group with inflammatory lesions who had received salicylate or indomethacin therapy, however, had the same mean PGB concentration as the non-inflammatory group.

Furthermore, when culture fluids of human synovial tissues were analysed for prostaglandins the addition of indomethacin (5 µg/ml) reduced prostaglandin levels to less than 2% of those found in both control and colchicine-stimulated cultures. A study by Higgs and others (1974), using a bioassay technique to measure prostaglandins in rheumatoid arthritis (RA) synovial fluid, showed the average concentration in fluids from untreated patients to be seven times higher than that in fluids from patients receiving aspirin-like drugs.

The present study was designed to measure the activity of prostaglandin synthetase in rheumatoid synovium, rather than to measure endogenous prostaglandins which would be altered by surgical manipulation (Piper and Vane, 1971). The microsomal fraction of the synovial tissue homogenate was used as the enzyme source, and its activity measured by its ability to convert arachidonic acid to prostaglandins.

Materials and methods

We report the findings from 19 synovial tissue samples taken from 17 patients who had undergone surgical synovectomy of the knee or elbow. All had classical RA with a positive latex test. Brief clinical details of these patients are shown in Table I.

Table I *Clinical and therapeutic details of 17 patients studied*

Case no.	Sex	Age (years)	Joint	Duration of RA (years)	Aspirin-like drugs
1	F	45	Knee	8	Indomethacin 100 mg/d
2	F	22	Knee	8	Indomethacin 75 mg/d
3	F	65	Knee	12	Indomethacin 100 mg/d
4	M	63	Knee	24	Indomethacin 75 mg/d
5a	F	51	Knee	10	Indomethacin 75 mg/d
6	F	37	Knee	7	Indomethacin 75 mg/d
7	F	67	Knee	17	Naproxen 500 mg/d
8	F	52	Knee	14	Naproxen 750 mg/d
9	F	63	Knee	4	Ibuprofen 1200 mg/d
10a	F	38	Knee	5	Ibuprofen 1600 mg/d
10b	F	38	Knee	5	Ibuprofen 1600 mg/d
11	F	57	Knee	18	None
12	F	54	Knee	3	Benorylate 8 g/d
13	F	60	Elbow	23	Aspirin 3.6 g/d
14	F	23	Knee	2	Aspirin 2.4 g/d
15	F	68	Knee	8	Aspirin 1.2 g/d
5b	F	51	Knee	10	Aspirin 600 mg/d
16	F	67	Knee	14	Aspirin 600 mg/d
17	F	60	Knee	11	Aspirin 600 mg/d

PREPARATION OF SYNOVIAL TISSUE

Synovial tissue taken at synovectomy was kept on ice and used within one hour. The tissue was minced, and a 20% (w/v) homogenate prepared in 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). The mixture was further homogenized for one minute in a Potter-Elvehjem homogenizer. A cell-free homogenate was prepared by centrifugation at 4000 g (2 × 10 min) from which a microsomal pellet was obtained by centrifugation at 10⁵ g, for one hour.

PRODUCTION OF PROSTAGLANDINS FROM ¹⁴C ARACHIDONIC ACID SUBSTRATE

The synovial microsomes were resuspended in fresh buffer mixture, and 1.9 ml aliquots were incubated with ¹⁴C arachidonic acid* (100 nCi/0.1 ml buffer) at 37°C, for one hour, with shaking. The reaction was terminated by addition of 0.25 ml of 2 mol/l citric acid.

EXTRACTION, SEPARATION, AND QUANTITATION OF ¹⁴C PROSTAGLANDINS

The formed ¹⁴C prostaglandins and unconverted ¹⁴C arachidonic acid were extracted from the incubate with diethyl ether (2 × 3.0 ml). After evaporation of the combined extracts under nitrogen at 37° the residue was dissolved in 20 µl chloroform methanol 2:1, and 5 µl applied to a thin layer plate (silica gel G, 0.25 mm, containing 2.5% silver nitrate). Authentic arachidonic acid, PGE₂, and PGF_{2α} were applied in a marker lane and the plate developed in benzene/dioxan/acetic acid 20:20:1. After locating the markers with phosphomolybdic acid, the zones in the extract lanes which corresponded to the markers were scraped from the plate, transferred to counting vials, and methanol (1.0 ml) added.

* 58 mCi/mmol, Radiochemical Centre, Amersham.

After shaking briefly, 8 ml of liquid scintillant was added (0.5% PPO and 0.03% dimethyl POPOP in toluene) and the samples counted in a Phillips liquid scintillation system. Quench corrections were made using a channel ratio method.

CHARACTERIZATION OF FORMED PROSTAGLANDINS

The formed ¹⁴C prostaglandins were characterized by running in various chromatographic systems with authentic prostaglandin standards, by chemical conversion to other prostaglandins followed by further chromatographic identification, and by bioassay of TLC eluates.

The chromatographic systems used were: (1) system AI, with and without silver nitrate; (2) system AII, with silver nitrate; (3) chloroform/methanol/acetic acid/water (90:6:1:0.75).

Prostaglandin E₂ was further identified by: (1) conversion to PGB₂ with sodium hydroxide followed by chromatographic characterization in system AI; (2) conversion with sodium borohydride into equal amounts of PGF_{2α} and PGF_{2β} followed by chromatographic characterization in system AI.

The possibility that the microsomal preparation might contain prostaglandin-metabolizing activity was investigated by adding ³H-PGE₂ and ³H-PGF_{2α} as substrate, in place of ¹⁴C arachidonic acid, to separate microsomal incubates. These were then taken through exactly the same incubation, extraction, and separation procedures as for the arachidonate incubates.

BIOASSAY OF TLC ELUATES

A portion of the chloroform/methanol extract was applied to a TLC plate (silica gel G, 0.25 mm) and developed for 15 cm in the AI solvent system. 1 cm bands were eluted with methanol (1.2 ml), an aliquot of which (0.2 ml) was used to measure the radioactivity present. The remainder was evaporated under nitrogen at 40°.

redissolved in Tyrode solution (1.0 ml) and aliquots (0.1 ml) used for bioassay.

A rat stomach strip was superfused with Tyrode's solution containing a mixture of antagonists (Gilmore, Vane, and Wyllie, 1968). Standard solutions of PGE₂ and PGF_{2α} were used for calibration.

PROTEIN ESTIMATION

Microsomal protein concentration was measured by the method of Lowry and others (1951), using bovine serum albumin as standard. Concentrations varied between experiments but were in the range of 0.4–2.0 mg/ml.

Results

The microsomal preparation from human rheumatoid synovial tissue was found to be capable of producing significant metabolism of added ¹⁴C arachidonic acid. The chromatographic separation of one such incubate is shown in Fig. 1. The radioactive products were tentatively identified as PGE₂, and a trace amount of PGF_{2α}, as might be anticipated under the incubation conditions described. No other radioactive products were separated, and 95% of the radioactivity present was recovered as ¹⁴C PGE₂, ¹⁴C PGF_{2α}, and unchanged ¹⁴C arachidonic acid. A similar chromatographic pattern, with no other radioactive peaks, was found when the other chromatographic systems described were used.

Treatment of formed ¹⁴C PGE₂ with sodium hydroxide followed by further chromatography showed that 83% of the original activity was now

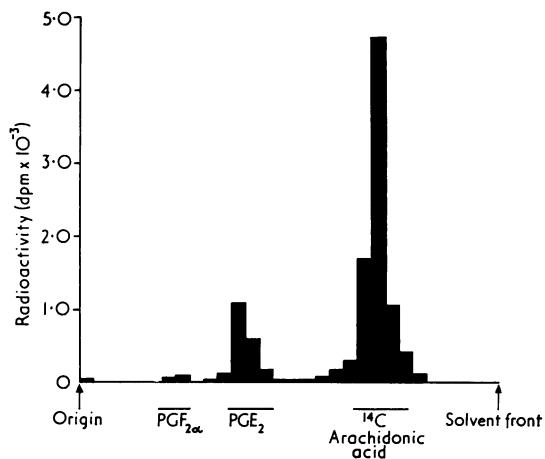


FIG. 1 Chromatographic separation of an incubation containing ¹⁴C arachidonic acid and microsomes from RA synovial tissue. The formed prostaglandins, PGE₂ and PGF_{2α}, were located by reference to the position of marker prostaglandins, shown below the scan. No other radioactive spots were detected on the plate. The combined ¹⁴C PGE₂, ¹⁴C PGF_{2α}, and unchanged ¹⁴C arachidonic acid made up 95% of the radioactivity present. The solvent front was run to 15 cm

coincident with a standard PGB₂ mark. Similarly, treatment with sodium borohydride gave two peaks which were coincident with standard PGF_{2α} and PGF_{2β}, containing 39% and 37% of the original activity respectively (figures not corrected for procedural losses). No other significant peaks of radioactivity were found in either extract.

When ³H PGE₂ and ³H PGF_{2α} were added to separate microsomal incubates instead of ¹⁴C arachidonic acid, chromatography showed that 94% and 97% of the radioactivity present corresponded to standard PGE₂ and PGF_{2α}, respectively and no other peaks of radioactivity were detected. This suggests that under the incubation conditions described the microsomal preparation from human rheumatoid synovial tissue contains no prostaglandin metabolizing activity.

When the fifteen 1 cm band eluates were each bioassayed for their prostaglandin activity as described, a single major peak of activity was found which corresponded to the radioactive peak designated PGE₂, and was coincident with standard PGE₂ run in parallel. A small amount of activity was seen in the PGF_{2α} region but no other significant peaks of activity were found. Combined chromatographic, chemical, and bioassay results thus strongly support the original tentative designations of the products of arachidonic acid metabolism as PGE₂ and PGF_{2α}.

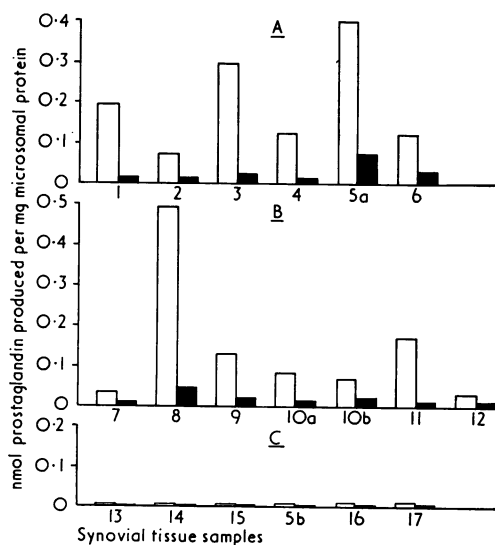


FIG. 2 The amounts of PGE₂ (open columns) and PGF_{2α} (solid columns) formed in incubations of ¹⁴C arachidonic acid with synovial microsomes taken from the patients shown in Table I. The three groups (A) patients taking indomethacin, (B) other 'aspirin like' drugs, and (C) aspirin, show inhibition of PG synthetase in the aspirin group. The results are expressed as radioactive products formed per mg synovial microsomal protein added to the incubation

Fig. 2 shows the amounts of PGE₂ and PGF_{2α} produced by the microsomal preparations from each of the 19 synovial tissues studied. Whereas the preparations from patients who were receiving indomethacin, ibuprofen, or naproxen therapy were all capable of prostaglandin synthesis *in vitro* (mean substrate conversion 16.0%, range 5.6–27.4%), no significant prostaglandin synthetase activity (<0.8% substrate conversion) could be detected in any patient being maintained on aspirin, even in low doses. The one patient we studied who was not taking any 'aspirin-like' drugs had about the same level of PG synthetase activity as the group being maintained on indomethacin. The preparation from a single patient taking benorylate produced a small but measurable amount (1.7%) of substrate conversion.

INHIBITION OF SYNOVIAL PG SYNTHETASE BY 'ASPIRIN-LIKE' DRUGS *IN VITRO*

Addition of low concentrations of several 'aspirin-like' drugs to active microsomal preparations *in vitro* (i.e. those derived from patients taking indomethacin, ibuprofen, or naproxen) produced a dose-related inhibition of prostaglandin synthesis. Fig. 3 shows a composite dose response curve to aspirin derived from four consecutive preparations. On each occasion when inhibition by other 'aspirin-like' drugs was studied *in vitro* aspirin was also assayed on the same preparation as a standard compound, and the concentration of drug necessary to produce 50% inhibition of PGE₂ synthesis (IC₅₀) calculated graphically from the dose response curve obtained. Drug potencies expressed on a molar basis relative to aspirin are shown in Table II.

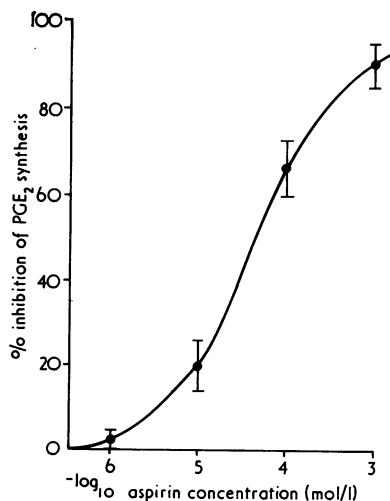


FIG. 3 Inhibition of synovial PG synthetase *in vitro*, by aspirin, showing the means of four consecutive synovial preparations. The vertical bars indicate the standard error of the means

Table II Relative molar potencies of some 'aspirin-like' drugs for 50% inhibition of PGE₂ synthesis, *in vitro*. Potency of aspirin was taken as unity

Drug	Relative molar potency for 50% inhibition of PGE ₂ synthesis
Paracetamol	<0.01
Salicylic acid	<0.02
Aspirin	1
Phenylbutazone	2.7
Ibuprofen	22
Naproxen	45
Indomethacin	257
Flurbiprofen*	5610

*Flurbiprofen (2-(2-fluoro-4-biphenyl) propionic acid), Boots Co., Nottingham.

Discussion

Prostaglandin synthetase activity from human rheumatoid synovial tissue was found, as in other tissues, in the microsomal fraction. Its biochemical characteristics have been shown to be similar to the synthetase systems prepared from other tissues. (Crook and Collins, 1975).

When several aspirin-like drugs were tested *in vitro* for their ability to inhibit prostaglandin synthesis, a wide range of potencies was found. Paracetamol, a drug with no anti-inflammatory activity, was virtually inactive in our assay, though reported to be more active than aspirin in some animal tissues (Flower and Vane, 1974). However, salicylic acid, which is equipotent with aspirin as an antirheumatic and anti-inflammatory agent *in vivo*, was also virtually inactive *in vitro* in our assay, in agreement with the findings in other assay systems (Vane, 1971; Willis and others, 1972). This apparent discrepancy has been discussed in detail by Smith, Ford-Hutchinson, and Elliott (1975); our findings provide evidence against the possibility that human inflammatory tissue contains an isoenzyme of prostaglandin synthetase which is susceptible to inhibition by salicylic acid. The possibility that human rheumatoid synovial tissue is sensitive to inhibition by a salicylic acid metabolite is currently under investigation.

It was of interest to find that microsomal preparations of synovial tissue from patients receiving indomethacin and other nonsteroidal anti-inflammatory drugs were capable of considerable prostaglandin synthesis *in vitro*. In contrast, there appeared to be a fundamental difference in this respect after aspirin therapy. We were unable to detect any significant PG synthetase activity in synovial microsomes taken from patients after aspirin therapy, lasting from 2 days to more than 6 months.

When indomethacin and the other drugs that apparently did not irreversibly inhibit the synthetase

in vivo were tested in an *in vitro* system, all these 'aspirin-like' drugs were more potent inhibitors of the enzyme system than aspirin itself (Table II).

These findings may be explained if the inhibition of the PG synthetase system by aspirin *in vivo* is considered to be of a noncompetitive (irreversible) type, while the *in vivo* inhibition caused by indomethacin and the other 'aspirin-like' drugs used here is considered to be competitive (reversible). In this latter case the manipulation of the synovial tissue through the preparative stages described may have removed these drugs from an enzyme(s) receptor site, allowing an *in vitro* activity of the enzyme(s). It may be that such a kinetic situation exists when the drugs are given therapeutically.

It is known that in some systems indomethacin *in vitro* irreversibly inhibits PG synthetase; for example the enzyme derived from sheep seminal vesicle microsomes (Smith and Lands, 1971; Raz, Stern, and Kenig-Wakshal, 1973). However, in a bovine brain microsomal preparation Ku and Wasvary (1974) found the inhibition by indomethacin to be reversible. The PG synthetase system in

human rheumatoid synovium appears to be similar to the synthetase system in human platelets, where the inhibitory effect of indomethacin *in vivo* on prostaglandin production and platelet aggregation is short lived compared with aspirin, which produces irreversible inhibition (Kocsis and others, 1973). Furthermore, the use of [³H-acetyl] aspirin (Roth and Majerus, 1975) has shown that aspirin produces an irreversible acetylation of a human platelet particulate fraction protein *in vivo* and *in vitro*. The time course of action of synovial PG synthetase inhibition by aspirin might well be studied by withdrawal of the drug at varying times before synovectomy.

The finding that PG synthetase was inactive in the tissue preparation from 3 patients taking only 600 mg aspirin per day supports the view of Smith and others (1975) that the anti-inflammatory efficacy of aspirin may have important components other than its well-documented inhibition of prostaglandin synthesis. Kinetic studies are now in progress to further elucidate the nature of the inhibition of synovial PG synthetase produced by aspirin and indomethacin.

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