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Salicylic acid fails to inhibit generation of thromboxane A_2 activity in platelets after *in vivo* administration to the rat

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Arachidonic acid-induced aggregation of rat platelets and the accompanying generation of thromboxane A_2 activity were inhibited by aspirin, whereas 20 times higher doses of salicylic, gentisic and salicyluric acids were inactive. Salicylic acid administered to the rats before aspirin prevented the inhibition of the cyclo-oxygenase-mediated effects of arachidonic acid. These results do not support the hypothesis that the anti-inflammatory activity of salicylic acid is due to inhibition of prostaglandin synthetase (cyclo-oxygenase) by an unknown metabolite and indicate that salicylic acid displays an anti-inflammatory activity independent inhibition of prostaglandin biosynthesis.

Since salicylic acid prevents experimental inflammation (Smith, Ford-Hutchinson & Elliot, 1975), but fails to inhibit prostaglandin (PG) biosynthesis (Smith & Willis, 1971; Vane, 1971), it has been suggested (Willis, Davison & others, 1972) that it is converted *in vivo* into an hypothetical metabolite endowed with PG synthetase inhibiting properties, responsible for the anti-inflammatory activity. Non-steroidal anti-inflammatory drugs suppress platelet aggregation due to the PG precursor arachidonic acid, by preventing the formation of PGG_2 and of PGH_2, which are converted into thromboxane A_2 (Vargaftig & Zirinis; 1973; Willis & Kuhn, 1973; Hamberg, Svensson & Samuelsson, 1975). Thromboxane A_2 is considered to be the mediator of platelet aggregation due to arachidonic acid.

I have now compared the ability of aspirin, salicylic acid, and their major metabolites given *in vivo* to inhibit the effects of arachidonic acid on rat platelets. It is hypothesized that if salicylic acid is indeed biotransformed into a metabolite with anti-inflammatory activity, the platelet reactions to arachidonic acid should be inhibited, as with aspirin.

MATERIALS AND METHODS

Groups of 3 male Wistar rats (300–350 g) were injected subcutaneously with aspirin, salicylic acid or their derivatives, and bled from the carotid artery under pentobarbitone (30 mg kg^{-1}, i.p.) directly into plastic tubes containing heparin (10 U ml^{-1}, final concentration). Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 200 *g* for 10 min, and adjusting to 300 000 platelets μl^{-1}. Aggregation was studied in a Bryston aggregometer at 37°, with stirring at 1100 rev min^{-1}, by adding to PRP either arachidonic acid or adenosine diphosphate (ADP). Samples from the aggregating platelet suspension were bioassayed for thromboxane and PG-like activities on strips of rabbit mesentery or coeliac artery, rabbit aorta and rat stomach, which were superfused with Krebs solution containing mepyramine, atropine, propranolol, phenoxybenzamine, methysergide and indomethacin, used to prevent the effects of other smooth muscle-contracting substances (Vane, 1964; Vargaftig & Dao, 1971; Vargaftig, Tranier & Chignard, 1974; Bunting, Moncada & Vane, 1976). The thrombocytopenic effect of arachidonic acid was studied in pentobarbitone-anaesthetized rats ventilated with a Starling miniature pump, blood samples being collected from the carotid artery and platelets counted at intervals (10 s, 1, 3 and 10 min) after 1 mg kg^{-1} of arachidonic acid intravenously, with a Coulter Counter.

Drugs used were: aspirin (lysine salt, Aspegic, Laboratories Egic, France), salicylic acid (Prolabo), arachidonic acid (Grade I), potato apyrase, gentisic and salicyluric acids (Sigma). Drugs were dissolved either in 0·9% (w/v) NaCl or in polyethylene-glycol 300 (Karl Roth), which by itself has no effect on platelets.

RESULTS

Arachidonic acid (0·05–0·2 mM) induced a slight change in shape of the platelets, followed by a return of light transmission to pre-drug concentrations, for

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0·5–3 min, when transmission decayed suddenly and large aggregates were formed, as indicated by visual inspection and by the sharp oscillation of the recording pen (Fig. 1). Amounts of arachidonic acid above

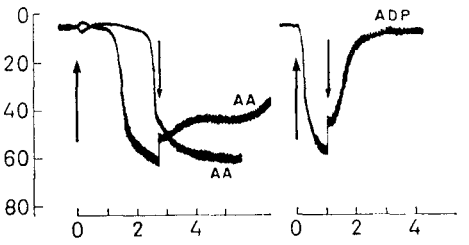

FIG. 1. Failure of apyrase to inhibit aggregation of rat platelets induced by arachidonic acid. Superimposed tracings of aggregation of rat platelets induced by arachidonic acid (0·2 mM, AA) in presence of 5 mg mg ^{1} of apyrase. Arrows placed at the end of the tracings indicate addition of apyrase, partially reversing aggregation by AA, and completely reversing aggregation by 10 μM of ADP. Ordinate percentage light transmission. Abscissa: Time (min).

0·5 mM failed to induce aggregation, although the shape change was present. When the ADP scavenger apyrase was added to PRP after aggregation was completed, reversion was obtained, although at a slower rate than with ADP-induced aggregation (Fig. 1). Addition of apyrase before arachidonic acid failed to prevent aggregation, but only increased the delay before it was triggered (Fig. 1). As seen in Fig. 2, thromboxane A_2 and PG-like activities were produced by platelets challenged with arachidonic acid. Those activities were present during 1–3 min before aggregation, and this could be seen particularly when the interval between addition of arachidonic acid and start of aggregation was prolonged.

Aggregation and formation of thromboxane A_2 activity were prevented when PRP was prepared from blood collected from animals treated with aspirin (Fig. 2 and Table 1). Other drugs, up to 200 mg kg^{-1}, were practically inactive. In some instances, treatment with salicylic acid appeared to increase the yield of rat stomach-contracting activity (PG-like). When salicylic acid was injected to the rats at 200 mg kg^{-1} 30 min before 10 mg kg^{-1} of aspirin, and PRP prepared from blood collected within 4 or 16 h, no inhibition of aggregation or of generation of thromboxane A_2 activity were observed (Table 2).

Intravenous injection of 1 mg kg^{-1} of arachidonic acid to 16 rats was followed by thrombocytopenia of 39 mean with 30% (s.d.) within 10 s, which faded by 1 min. Thrombocytopenia when present was reproducible for each animal but in some of the animals it was absent leading to a large standard deviation. Since thrombocytopenia peaked at 10 s and was over within 1 min, failure to detect it may be due to slightly different kinetics from one animal to another. ADP, injected at 0·1 mg kg^{-1} (i.v.) to three rats, induced thrombocytopenia of 74 ± 8% within 10 s, of 23 ± 16 at 1 min, and of 15 ± 5% within 10 min.

DISCUSSION

Aggregation of rat platelets induced by arachidonic acid was triphasic: an initial shape change was

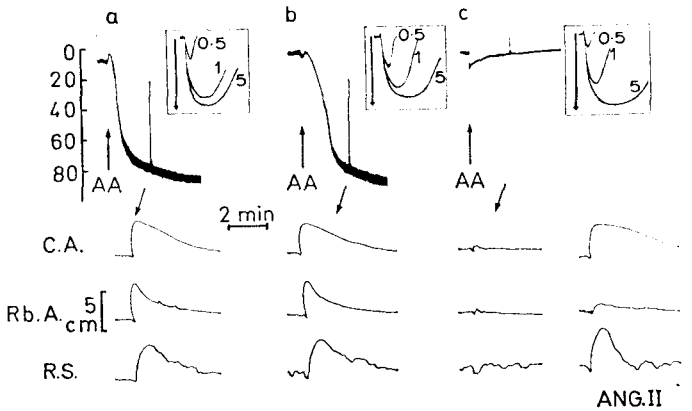

FIG. 2. Failure of salicylic acid to interfere with platelet aggregation by arachidonic acid, as compared with the effect of aspirin. Platelet-rich plasma was prepared from blood collected from rats pre-treated a—with solvent, b—with alicylic acid (200 mg kg^{-1}) or c—with aspirin (10 mg kg^{-1}), 16 h before. Aggregation was started with 0·1 mM of arachidonic acid (AA) and, after 2 min, the samples were bioassayed for thromboxane A_2 and prostaglandin-like activities on a coeliac artery (C.A.), a rabbit aorta (Rb. A.) and on a rat stomach (R.S.). Aliquots were collected when indicated by an artifact on the tracings. Scales as in Fig. 1. The inserts show that aggregation by ADP was unaffected by the various treatments. Last contraction due to 50 ng of angiotensin II. Figures indicate concn. of ADP (μM) used.

Table 1. *Comparison between the ability of aspirin and of three of its metabolites to inhibit generation of thromboxane A_2 and of prostaglandin-like activities from arachidonic acid by rat platelets.*

Treatment and dose (mg kg^{-1})	Time of blood coll. (h)	% Inhibition with s.d. of the contraction of strips of Rb. MS*	Rb. A*	R.S.*
Aspirin				
10	1	98	100	52
10	4	88 s.d. 21	95 s.d. 8	87 s.d. 22
10	16	84 s.d. 12	77 s.d. 25	60 s.d. 16
10	40	79 s.d. 8	90 s.d. 16	38 s.d. 6
10	64	0	8·5	0
30	16	100	87·5	25
30	40	88 s.d. 1·5	100	65 s.d. 12
30	64	54	38	33
100	16	100	92	/
100	40	88 s.d. 4	100	74 s.d. 4
100	64	60	49	42·5
100	160	28	41·5	32
Salicylic acid				
200	4	0	0	−17†
200	16	5·5 s.d. 7	0	−27†
Gentisic acid				
200	1	0	14	0
200	4	20	0	0
200	16	16	0	0
Salicyluric acid				
200	1	14	40	26
200	4	−9†	−40†	0
200	16	0	0	0

† Potentiation.
Rb. MS—Rabbit mesenteric artery. Rb. A—Rabbit aorta. Rat stomach—R.S.

followed by a shallow increase in light transmission, which was maintained for 1–3 min. After, a rapid increase in transmission took place with the appearance of large aggregates. Thromboxane A_2 activity appeared early, before aggregation started, which is compatible with its hypothesized role as an aggregation inducer. The steep aggregation curve might have been due to sudden release of ADP from the platelet into its plasma environment, during the release reaction, but failure of apyrase, at concentrations effective against ADP-induced aggregation, to prevent aggregation by arachidonic acid, rules out this hypothesis. Except for rabbit platelets, which do not aggregate when challenged with arachidonic acid or with ADP in the presence of apyrase, the latter fails to inhibit significantly aggregation of platelets from other species due to arachidonic acid. Since apyrase reversed rat platelet aggregation due to arachidonic acid after its completion, it appears that ADP might play a stabilizing role and reinforce the aggregate once it is formed. Aggregation by arachidonic acid did not reverse spontaneously, and this contrasts with the very short thrombocytopenia which can be induced *in vivo*. It appears that the *in vivo* situation is much more complex, involving clearance of aggregates by other substances, such as prostacyclin, and/or mechanical disruption of aggregates in microvessels. Similar *in vivo* results have been obtained for ADP (Kobayashi & Didisheim, 1973) and for collagen particles (Kobayashi, Mashimo & others, 1974).

Table 2. *Inhibition by aspirin and reversion by salicylic acid of rat platelet aggregation due to arachidonic acid and of the accompanying generation of thromboxane A_2 activity.*

Treatment (mg kg^{-1})		n	% Inhibition of the contraction of the n Rb. MS* with s.d.	Extent of aggregation (%) with s.d.
Saline	—	3	0	77 s.d. 11
Salicylic acid	200	3	0	73 s.d. 13
Aspirin	10	4	99 s.d. 2	0
Salicylic acid + aspirin	200 + 10	3	2·6 s.d. 4	76·8 s.d. 8

*Rb. MS.—Rabbit meserteric artery, bioassay performed 2 min after addition of 0·1 mM of arachidonic acid to platelet-rich plasma.

Aspirin was active against arachidonic acid-induced aggregation and release of thromboxane A_2 activity when used down to 10 mg kg^{-1}, and inhibition was persistent, particularly above 30 mg kg^{-1}. Those doses are markedly below the anti-inflammatory doses, which are 200–600 mg kg^{-1}, and when given 24 h beforehand they do not block experimental inflammation (Vargaftig, 1977). This indicates that the sites of action of aspirin in antagonizing inflammation and in antagonizing aggregation differ, and that the mechanism for either effect is also different, probably involving irreversible membrane acetylation (Rosenberg, Gimber-Phillips & others, 1971; Roth, Stanford & Majerus, 1975) with platelets, and interference at other sites for inflammation. The failure of salicylic acid to affect the platelet responses to arachidonic acid, under conditions where doses of 10 mg kg^{-1} of aspirin are inhibitory, indicates clearly that no metabolite of salicylic acid is produced in rats that might block biosynthesis of PG-related substances. The anti-inflammatory activity of salicylic acid is thus PG-synthetase independent. This does not rule out the possibility of a contributory role of aspirin by itself from the anti-inflammatory activity of salicylates in general, particularly when the membrane of invading cells (polymorphs) may be acetylated and thus lose the ability to generate PG.

Salicylic acid prevented inhibition by aspirin of the effects of arachidonic acid on platelets. Similar findings have been reported for aspirin-induced gastric lesions (Ezer, Palosi, Hajos & Szporny, 1976), and for inhibition of bronchoconstriction and hypotension due to arachidonic acid in guinea-pigs

and in rabbits (Vargaftig & Lefort, 1977; Lefort & Vargaftig, submitted for publication). Moreover, pre-incubation of rabbit platelets with salicylic acid, before the addition of aspirin, also prevents inhibition of aggregation and of generation of thromboxase A_2 activity (Vargaftig, 1977). It thus appears that in the three species tested, *in vivo* administration of salicylate counteracts the effects of aspirin, adding weight to the demonstration that no PG inhibitor is formed from salicylate at the height of its experimental anti-inflammatory activity. Binding of aspirin to its site of action is probably involved with the salicylate-aspirin antagonism (Vargaftig, in preparation).

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