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SALICYLATE REVERSES *IN VITRO* ASPIRIN INHIBITION OF RAT PLATELET AND VASCULAR PROSTAGLANDIN GENERATION

JAIME MERINO,* MANUELA LIVIO,† GRAZYNA RAJTAR‡ and GIOVANNI DE GAETANO§
Laboratory of Cardiovascular Clinical Pharmacology, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62, 20157 Milan, Italy

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Abstract—It is generally accepted that aspirin inhibits platelet function by irreversible acetylation of prostaglandin cyclo-oxygenase. The salicylate moiety seems not to be causally involved in the inhibitory effect of aspirin, a concept supported by the virtual inactivity of sodium salicylate. However, prostaglandin synthesis is also inhibited by numerous compounds which have no acetylating properties. Recent evidence indicates that salicylate may prevent the inhibitory effect of aspirin on rabbit platelet cyclo-oxygenase, suggesting that interaction of the salicylate moiety of aspirin with this enzyme is important. This study was aimed at evaluating whether the inhibitory effect of aspirin on platelet prostaglandin generation could be reversed by sodium salicylate. We therefore measured spectrophotometrically malondialdehyde (MDA) generated by arachidonate in rat platelet-rich plasma and evaluated the effect of short-term incubation with either aspirin or salicylate or both. In the experimental conditions used, salicylate not only prevented but also reversed aspirin-inhibition of MDA formation. This interaction was not peculiar for platelets, since salicylate also reversed the *in vitro* inhibitory effect of aspirin on vascular prostacyclin generation (measured by a bioassay). These findings suggest that irreversible acetylation of cyclo-oxygenase does not account for the early *in vitro* inhibitory effect of aspirin on prostaglandin synthesis.

Non-steroidal anti-inflammatory drugs inhibit platelet function by preventing the enzymatic transformation of arachidonic acid into prostaglandin cyclic endoperoxides [1]. Since aspirin is a potent inhibitor of platelet function and prostaglandin synthesis, whereas salicylic acid is almost inactive [2–5], acetylation of the enzyme cyclo-oxygenase by the former is considered essential for its effect [6]. This is in agreement with previous *in vitro* experiments in which platelet-bound radioactivity was only found when platelets were incubated with acetyl group- but not carboxyl group-labelled aspirin [6, 7]. However, both platelet function and prostaglandin synthesis are inhibited by numerous compounds, such as indomethacin, which have no acetylating properties [4, 5, 8, 9].

On the other hand, evidence has recently been presented that the aspirin-induced inhibition of rabbit platelet cyclo-oxygenase can be prevented by salicylate [10]. This pharmacological antagonism suggests that interaction of the salicylate moiety of aspirin with cyclo-oxygenase may be more important than is currently believed for the inhibitory effect of this drug.

The present study was aimed at evaluating whether aspirin's inhibitory effect on platelet prostaglandin synthesis and platelet aggregation could not only be prevented but also reversed by salicylate. Since aspirin is also a strong inhibitor of PGI_2 (prostacyclin) generation in the vessel wall of rats [11], we also investigated whether salicylate reversed this effect. We present here the results of *in vitro* experiments in the rat indicating that the inhibitory effect of aspirin on both platelet and vascular prostaglandin generation can be reversed by sodium salicylate.

MATERIALS AND METHODS

Animals. Male CD-COBS rats (250–300 g body wt) from Charles River, Calco, Italy were used.

Platelet malondialdehyde generation. Platelet malondialdehyde (MDA) generation on stimulation with sodium arachidonate (0.6–1 mM, Sigma, Mascia Brunelli, Milan, Italy) was measured by a modification [12] of the spectrophotometric method described by Smith *et al.* [13]. Platelet-rich plasma (0.5 ml) [12] was stirred in the cuvette of an aggregometer (Elvi 840, Elvi Logos, Milan, Italy) at 37° with microliter amounts, firstly of either sodium salicylate (1–2 mM, Carlo Erba, Milan, Italy) or aspirin (50–350 μM, lysine salt, Flectadol, Maggioni, Milan, Italy) or redistilled water for 1 min, then with redistilled water or the other drug for 1 additional min. Then 6–10 μl sodium arachidonate were added to the cuvette and platelet aggregation was followed for 2 min on a chart recorder connected to the aggregometer [11, 12]. The reaction was stopped by adding 0.5 ml 50% trichloroacetic acid and the sam-

* On leave of absence from the Department of Internal Medicine, University of Santander, Medical School, Santander, Spain.
† Research Fellow of the Italian National Research Council (C.N.R.).
‡ On leave of absence from the Department of Pharmacology, Lublin University Medical School, Lublin, Poland.
§ To whom reprint requests should be addressed.

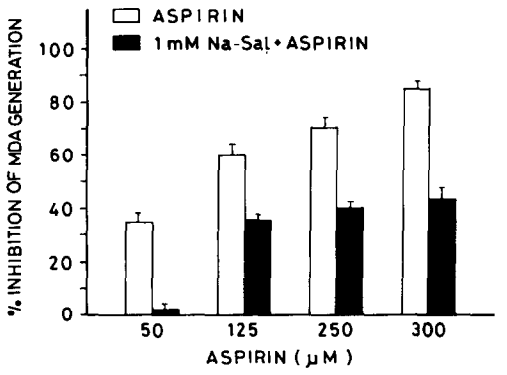

Fig. 1. Inhibition by aspirin (50–300 μM, final concentration [f.c.]) of sodium arachidonate (0.6 mM, f.c.)-induced platelet malondialdehyde (MDA) generation. Incubation of platelets with sodium salicylate (1 mM, f.c.) prior to aspirin partially prevents the inhibitory effect. Means ± S.E.M. of 3 experiments. For further details see text.

ple was processed for MDA assay as previously described [12].

Platelet aggregation inhibitory activity. The platelet aggregation inhibitory activity of prostacyclin (PGI_2) generated by rings of rat thoracic aorta was measured and characterized as described [11, 12]. This activity was completely inhibited by an antiserum (kindly provided by Drs. M. J. Silver and J. B. Smith) which antagonised the platelet antiaggregating activity of authentic PGI_2 (Upjohn, Kalamazoo, MI, U.S.A.) [14].

Aortic rings (2–6 mg wet wt) were incubated at room temperature in 50 μl 0.05 M Tris buffer, pH 7.4, with or without aspirin (600–1200 μM). After 2 min, 50 μl of buffer with or without sodium salicylate (1–4 mM) were added and incubation was continued for another 6 min. Microliter amounts of the supernatant buffer were incubated in the cuvette of the aggregometer with 250 μl human platelet-rich plasma, which was then challenged with 1–2 μM ADP (Sigma) [11, 12].

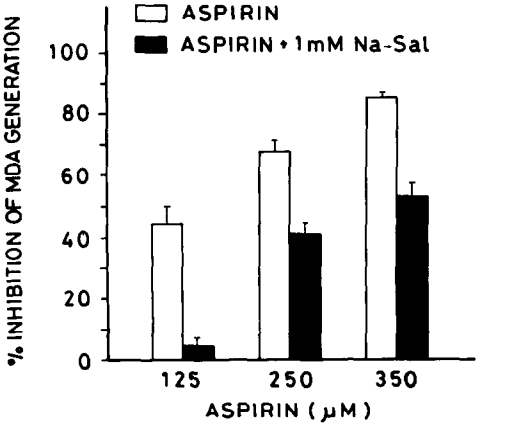

Fig. 2. Inhibition by aspirin (125–350 μM, f.c.) of sodium arachidonate (0.6 mM, f.c.)-induced platelet malondialdehyde (MDA) generation. Incubation of platelets with sodium salicylate (1 mM, f.c.) after aspirin partially reverses the inhibitory effect. Means ± S.E.M. of 3 experiments. For further details see text.

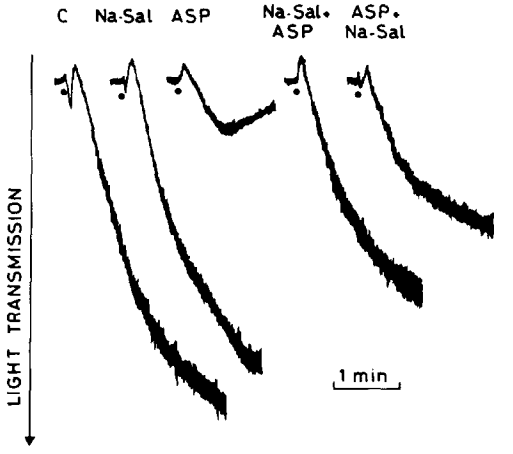

Fig. 3. Representative tracings of platelet aggregation induced by sodium arachidonate (0·6 mM, f.c.). Platelets were preincubated for 1 min with redistilled water (tracing C), 1 mM sodium salicylate (tracing Na-Sal) or 250 μM aspirin (tracing ASP) before addition of sodium arachidonate. The last two tracings show that the inhibitory effect of aspirin was prevented (tracing Na-Sal + ASP) or reversed (tracing ASP + Na-Sal) by sodium salicylate. For further details see text.

RESULTS

Aspirin (50–350 μM) strongly inhibited platelet MDA formation, whereas sodium salicylate was inactive even at 2 mM final concentration. However, salicylate (1 mM) prevented and reversed the inhibitory effect of aspirin (Figs. 1 and 2). Similarly, 0·75–2 mM sodium salicylate, though inactive itself, prevented or reversed aspirin (250–350 μM)-induced inhibition of platelet aggregation brought about by 0·6 mM sodium arachidonate (Fig. 3). Vascular PGI_2 generation was also inhibited by aspirin (600–1200 μM) but not by salicylate (1–4 mM). The latter, however, reversed the effect of aspirin (Fig. 4).

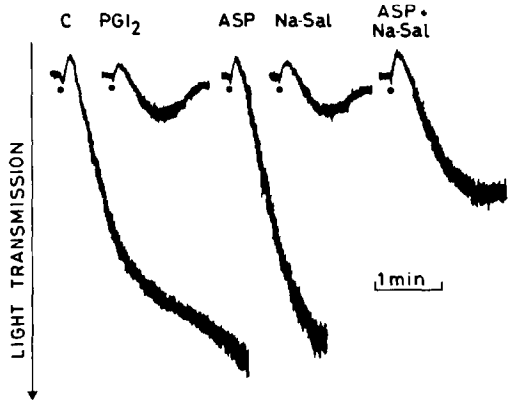

Fig. 4. Biological assay of prostacyclin activity generated by rat aortic rings. Representative tracings of human platelet aggregation induced by 2 μM ADP. The inhibitory effect of prostacyclin (tracing PGI_2 vs tracing C) was inhibited by incubating the vascular rings with 900 μM aspirin (tracing ASP) but was unaffected by incubation with 1 mM sodium salicylate (tracing Na-Sal). The last tracing (ASP + Na-Sal) shows that the inhibitory effect of aspirin was partially reversed by sodium salicylate. For further details, see text.

DISCUSSION

The most interesting finding in this study was that the inhibitory effect of aspirin (after short-term *in vitro* incubation) on both platelet and vascular prostaglandin generation could be *reversed* by sodium salicylate. This observation implies that in the test systems used, sodium salicylate, although virtually inactive, interfered with the prostaglandin synthesis pathway. If salicylate binding to prostaglandin cyclo-oxygenase is a generalized phenomenon throughout the body, its pharmacological effect on prostaglandin synthesis could also be evident in tissues other than platelets or vessel walls. This might be relevant to our understanding of the long known anti-inflammatory and antirheumatic effects of sodium salicylate [15].

The early inhibitory effect of aspirin on prostaglandin synthesis was reversible and may not necessarily be linked to irreversible acetylation of cyclo-oxygenase. Work in progress in our laboratory indicates that, in experimental conditions similar to those used here, the inhibitory effect of aspirin on rat platelet MDA formation (induced by sodium arachidonate or thrombin) is apparently competitive (with K_i of 120–160 μM, analysed by Dixon plots).

These observations are in agreement with previous findings [6, 16] that arachidonic acid is an apparently competitive inhibitor of cyclo-oxygenase acetylation by aspirin. Roth *et al.* [6] have suggested that the binding of arachidonic acid to the enzyme's active site might prevent aspirin binding to it (or to another, closely associated site). This mechanism could also explain why sodium salicylate prevents the effect of aspirin [10]. However, the observed reversion of aspirin's inhibitory effect by sodium salicylate suggests that reversible aspirin binding to the enzyme is sufficient to inhibit its activity, before irreversible acetylation takes place. It can therefore be postulated that aspirin inhibits cyclo-oxygenase activity through fast, reversible binding to its active site. This inhibitory effect is subsequently made persistent by irreversible, slower acetylation [6, 8, 17]. It is unlikely that acetylation accounts for the increasingly greater inhibition of platelet function observed as the incubation time of platelets with aspirin was increased [8], since the same time-dependent inhibition was seen with indomethacin and other compounds which lack acetyl groups (ref. 18 and unpublished observations of the authors). The presence of the acetyl residue on the salicylic acid moiety could make it more accessible to and/or more effective on cyclo-oxygenase.

The fact that the above drug interaction was observed not only in platelets (suspended in their own plasma) but also in the vessel wall (in the absence of plasma) suggests that salicylate–aspirin antagonism did not occur at the level of plasma protein binding [2].

Reversibility by salicylate of the aspirin inhibitory effect in platelet-rich plasma or in the vessel walls has apparently not been described so far.

Zucker and Peterson [8], in experiments designed to explore possible additive effects of several anti-inflammatory agents on the release of platelet-bound ^{14}C-serotonin by connective tissue, reported that human platelets preincubated for 15 min with aspirin (156 μM) and for another 15 min with sodium salicylate (9 mM), released slightly larger amounts of ^{14}C-serotonin than when they were preincubated with aspirin alone.

In the context of overall results obtained with different drug combinations, the authors concluded that no additive effect was observed. No specific comment was made on aspirin–salicylate interactions. In the light of our present data, Zucher and Peterson's findings could also indicate that salicylate partially reversed the aspirin effect.

These observations are potentially useful for a better understanding of the mechanism of action of this old and popular antiaggregating agent which is currently being investigated in many clinical trials for its potential antithrombotic effect. Approximately 50 per cent of orally administered doses of aspirin are hydrolysed to salicylic acid before they reach the blood stream. The percentage of hydrolysed drug may in some cases be larger than this, depending on the dosage form used [19]. If the aspirin–salicylate interaction described here also occurs *in vivo* in humans, the kinetic disposition of aspirin should be taken into account when evaluating its pharmacological and clinical effects. For example, 30 min after oral administration of 650 mg of aspirin to normal subjects, plasma levels of unchanged drug were about 4 times lower than those of the hydrolysed product (salicylic acid) [19]. Recent reports of a sex difference in the antithrombotic activity of aspirin [20–22] and of the paradoxical effects of high doses of aspirin on bleeding time [23] could also be re-assessed in the light of these data.

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REFERENCES

1. B. Samuelsson, M. Goldyne, E. Granström, M. Hamberg, S. Hammarström and C. Malmsten, *A. Rev. Biochem.* **47**, 997 (1978).
2. H. J. Weiss, L. M. Aledort and S. Kochwa, *J. clin. Invest.* **47**, 2169 (1968).
3. M. B. Zucker and J. Peterson, *Proc. Soc. exp. Biol. Med.* **127**, 547 (1968).
4. J. R. O'Brien, *Lancet* **I**, 894 (1968).
5. J. B. Smith and A. L. Willis, *Nature New Biol.* **231**, 235 (1971).
6. G. J. Roth and P. W. Majerus, *J. clin. Invest.* **56**, 624 (1975).
7. H. Al-Mondhiry, A. J. Marcus and T. H. Spaet, *Proc. Soc. exp. Biol. Med.* **133**, 632 (1970).
8. M. B. Zucker and J. Peterson, *J. Lab. clin. Med.* **76**, 66 (1970).
9. N. Stanford, G. J. Roth, T. Y. Shen and P. W. Majerus, *Prostaglandins* **13**, 669 (1977).
10. B. B. Vargaftig, *Eur. J. Pharmac.* **50**, 231 (1978).
11. S. Villa and G. de Gaetano, *Prostaglandins* **14**, 1117 (1977).

12. S. Villa, M. Livio and G. de Gaetano, *Br. J. Haemat.* **42**, 425 (1979).
13. J. B. Smith, C. M. Ingerman and M. J. Silver, *J. Lab. clin. Med.* **88**, 167 (1976).
14. J. B. Smith, M. L. Ogletree, A. M. Lefer and K. C. Nicolaou, *Nature, Lond.* **274**, 64 (1978).
15. L. S. Goodman and A. Gilman (Eds.), in *The Pharmacological Basis of Therapeutics*, 4th. Edn, p. 314. MacMillan, New York (1970).
16. L. H. Rome and W. E. M. Lands, *Prostaglandins* **10**, 813 (1975).
17. J. R. O'Brien, W. Finch and E. Clark, *J. clin. Path.* **23**, 522 (1970).
18. L. H. Rome and W. E. M. Lands, *Proc. natn. Acad. Sci. U.S.A.* **72**, 4863 (1975).
19. S. Riegelman, in *Aspirin, Platelets and Stroke. Background for a Clinical Trial* (Eds. W. S. Fields and W. K. Hass), p. 105. Warren H. Green, St. Louis (1971).
20. Canadian Cooperative Study Group, *New Engl. J. Med.* **299**, 53 (1978).
21. W. H. Harris, E. W. Salzman, C. A. Athanasoulis, A. C. Waltman and R. W. De Sanctis, *New Engl. J. Med.* **297**, 1246 (1977).
22. J. G. Kelton, J. Hirsh, C. J. Carter and M. R. Buchanan, *Blood* **52**, 1073 (1978).
23. J. O'Grady and S. Moncada, *Lancet* **II**, 780 (1978).