Dose-dependent inhibition of phosphoinositide metabolism in human platelets by aspirin *in vitro* and *in vivo*

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1 The effects of aspirin on the metabolism of phosphoinositides in human platelets were studied *in vitro* and *in vivo*.

2 Eight volunteers received, at two-weekly intervals, a single dose of 10, 30, 100 or 600 mg aspirin. Before the first dose platelets were taken and incubated *in vitro* with a range of concentrations (10 nM–100 μ M) of aspirin.

3 Formation of inositol phosphates (IP) was measured in [3 H]-inositol labelled platelets after incubation with collagen and thrombin for 30 min, a time at which a maximal increase in [3 H]-IP was observed.

4 The *in vitro* IC₅₀ for inhibition of the response to collagen by aspirin was approximately 1 μ M; the *in vivo* ID₅₀ was 40–50 mg. Aspirin did not fully inhibit the collagen stimulated IP formation either *in vitro* or *in vivo*, and the response to thrombin was unaffected.

5 The ID₅₀ and IC₅₀ of aspirin is thus in accord with the doses of aspirin associated with inhibition of platelet aggregation and thromboxane production in other studies. The possible relevance of these data to the clinical uses of aspirin is discussed.

Keywords aspirin platelets phosphoinositides collagen

Introduction

Aspirin is used extensively in the prophylaxis of vascular thrombotic disease, and is known to have a variety of antiplatelet effects; these include inhibition of aggregation and decreased release of platelet contents. These effects result from the irreversible inhibition of the enzyme cyclo-oxygenase, which is responsible for the conversion of arachidonic acid into prostaglandins and thromboxanes (Smith & Willis, 1971).

In the platelet arachidonic acid is thought to be produced following breakdown of inositol phospholipids by phospholipases C and A₂ (Lapetina, 1983) when stimulated with agents such as thrombin, collagen, ADP and thromboxane A₂ (TxA₂). The initial event after receptor occupation is phospholipase C mediated breakdown of phosphatidylinositol 4,5-bisphos-

phate (PIP₂) to inositol 1,4,5 trisphosphate (IP₃) and diacylglycerol (Berridge & Irvine, 1984; Watson *et al.*, 1984), and these products may act synergistically to bring about platelet activation (Berridge & Irvine, 1984).

It has recently been shown that indomethacin (Siess *et al.*, 1983; Watson *et al.*, 1985) and aspirin (PPG, unpublished results) can substantially, though not totally, block the breakdown of inositol lipids in response to collagen and low concentrations of thrombin *in vitro*. It was therefore suggested (Watson *et al.*, 1985) that collagen and low doses of thrombin initially activate a small number of platelets, resulting in stimulated phosphoinositide turnover and phospholipase A₂ activation. Subsequent production of arachidonic acid and its metabolites would

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amplify the response in these platelets and initiate the aggregatory response in others (Lapetina, 1983). This amplification step would be blocked by cyclo-oxygenase inhibitors and aggregation prevented.

In these studies maximal doses of cyclo-oxygenase inhibitors were used, giving no information on the concentration range required for inhibition of these responses, which may be crucial when relating these effects to the *in vivo* actions of aspirin. We therefore carried out a study to investigate the *in vitro* concentrations and the *in vivo* doses of aspirin which would inhibit the collagen stimulated formation of inositol phosphates.

Methods

We studied eight healthy male volunteers (age 18–51 years), who had not taken any drug known to affect cyclo-oxygenase for 2 weeks. The study protocol was approved by the Oxford Health Authority Central Ethics Committee and subjects gave their consent before participating. Each volunteer was studied on five occasions, the first being a control in the absence of aspirin. Each then received a single dose of 10, 30, 100 or 600 mg of aspirin in solution (Aspro Clear) in a random order determined by a Latin square design, an interval of 2 weeks separating each dose. The aspirin was given 16 h before the removal of 60 ml of blood for analysis.

Blood was taken into 3.15% trisodium citrate (1 vol:9 vol blood), and platelet rich plasma was prepared by centrifugation at 200 g for 20 min at 20° C. Forskolin (final concentration 10 µM) was then added and platelets were separated by centrifugation at 800 g for 10 min at 20° C. The platelet pellet was then completely drained of plasma and resuspended in 1.5 ml of 5 mM HEPES/140 mM NaCl/4 mM KCl/1 mM MgCl₂/6 mM glucose/0.3% bovine serum albumin (BSA), pH 7.4. [³H]-myo-inositol was added at a concentration of 40 µCi ml⁻¹ and the samples were incubated at 37° C for 2 h with occasional mixing. The platelets were separated from extracellular label by elution off a Sepharose CL-2B column (6 cm × 2 cm; approx 15 ml gel) which had been equilibrated with buffer, and were diluted to a concentration of 3–5 × 10⁸ platelets ml⁻¹. 0.6 ml samples were then taken into mini-vials in a shaking water bath at 37° C and LiCl was added (final concentration 10 mM). Aspirin was also added at this time if required. After a further 15 min collagen (50 µg ml⁻¹) or thrombin (0.5 units ml⁻¹) was added. The reactions were stopped by the addition of 1.88 ml of chloroform:

methanol (1:2, v/v), and total inositol phosphates were estimated as described previously (Schachter *et al.*, 1985). When platelet aggregation was measured samples were taken through an identical isolation procedure, except that the [³H]-inositol was omitted. After gel-filtration platelets were diluted to 3 × 10⁸ ml⁻¹ and aggregation of 0.6 ml samples was determined in a Payton aggregometer at 37° C with stirring at 800 rev min⁻¹. In preliminary experiments the time course of formation of inositol phosphates was determined. These incubations were terminated with 1.2 ml ice-cold 4.5% perchloric acid and the individual inositol phosphates were extracted and separated as described by Godfrey & Putney (1984).

[³H]-myo-inositol (specific activity 15 Ci mmol⁻¹) was obtained from Amersham International, Amersham, UK; forskolin from Calbiochem-Behring, Cambridge, UK; Sepharose CL-2B, Dowex and Hepes from Sigma. All other reagents were of the highest grade available. Statistical analysis of the data was performed using the Mann-Whitney test.

Results

In preliminary experiments we found that the time course of formation of inositol phosphates in platelets stimulated with collagen (50 µg ml⁻¹) was identical to that described by Watson *et al.* (1985) though in the presence of LiCl inositol-l-phosphate levels did not peak until 30 min after addition of collagen (data not shown). Although IP₃ is regarded as the active second messenger it was not assayed in the remainder of this study because of the low amounts produced and the difficulty and expense of accurate measurements. In previous work measurement of total inositol phosphates (IP) in cells treated with LiCl was shown to provide a sensitive assay of phosphoinositide metabolism (Berridge *et al.*, 1982) and we have used this method for assaying the responses to collagen.

Before dosing with aspirin a sample of platelets from each volunteer was used to determine the concentration of aspirin required to inhibit aggregation and collagen stimulated IP formation *in vitro*. Figure 1 shows that collagen stimulated aggregation and IP formation can be maximally inhibited by 100 µM aspirin; the IC₅₀ for the inhibition of IP formation was approximately 1 µM, though at this aspirin concentration aggregation was similar to controls. Even at the maximal inhibitory concentration there was still a substantial increase in IP formation compared with controls (Figure 1b).

Figure 1 Effects of aspirin *in vitro* on aggregation and the formation of inositol phosphates in platelets stimulated with collagen (50 $\mu\text{g ml}^{-1}$). The upper panel shows representative aggregometer tracings; collagen was added at the arrow. The lower panel shows the levels of [^3H]-IP₁ after a 30 min incubation (mean $\pm$ s.e. mean; $n = 8$). Samples were prepared as described in the methods. Aspirin was added to the platelet suspension 15 min before the agonist.

In the experiments to determine the inhibitory effects of aspirin *in vivo* each platelet preparation was divided into four aliquots after the cells had been labelled with [^3H]-inositol; the first acted as control, the second was treated with 100 μM aspirin (to inhibit platelet cyclo-oxygenase maximally) before incubation with collagen, the third was incubated with collagen, and the fourth was incubated with 0.5 units ml^{-1} thrombin (the dose which gave maximal stimulation of IP formation in platelets treated with thrombin for 30 min; unpublished results). Because of variations in the platelet preparations and labelling the results were standardized by expressing the collagen responses as a percentage of the stimulation elicited by thrombin.

Maximal inhibition of the collagen response was obtained with a single dose of 100 mg or 600 mg aspirin (Figure 2). A single dose of 10 mg inhibited the collagen stimulated IP formation to

a small extent, and this effect was greater with the 30 mg dose, which was statistically significant from controls ($P < 0.05$). The ID_{50} for the inhibition by aspirin was 40–50 mg (Figure 2).

Discussion

Even though a high concentration of collagen was used in this study it was still substantially less efficacious than thrombin, increasing IP levels by only about 30% of the increase produced by thrombin (Figure 2), and in the presence of 100 μM aspirin this was reduced by a further two-thirds. Aspirin substantially, though never completely, inhibited the response to collagen (Figure 1). These data agree with previous work in which maximal doses of cyclo-oxygenase inhibitors did not fully inhibit protein phosphorylation, ATP release, or aggregation, though the rise in intra-

Figure 2 Effects of aspirin *in vivo* on formation of inositol phosphates in collagen stimulated platelets. Dosing regimens and sample preparation are described in the text. Results are means $\pm$ s.e. mean ($n = 8$) in samples stimulated with $50 \mu\text{g ml}^{-1}$ collagen (●) or $50 \mu\text{g ml}^{-1}$ collagen plus $100 \mu\text{M}$ aspirin (○). (*), Value significantly different from no aspirin control ($P < 0.05$).

cellular Ca^{2+} was fully blocked (Rink *et al.*, 1983; Watson *et al.*, 1985).

From the data in Figure 1 it appears that $1 \mu\text{M}$ aspirin can significantly inhibit IP formation without affecting aggregation. This discrepancy may be due to the phenomenon called receptor reserve (Michell *et al.*, 1981) which postulates that while only a small proportion of activated receptors are required for a full response, maximal PI turnover requires occupation of all receptors. This may explain why aspirin can significantly inhibit TxA_2 formation (and secondary activation of PI turnover) without any apparent effect on aggregation.

Previous work on *in vitro* inhibition of platelet phosphoinositide metabolism by indomethacin (Watson *et al.*, 1985) used only maximal inhibitory concentrations. We have extended these results to show that aspirin, both *in vitro* and *in vivo*, inhibits collagen stimulated IP formation in a dose dependent manner. The dose required for 50% *in vivo* inhibition of the response (ID_{50}) was about 45 mg (Figure 2). Assuming the bioavailability of the aspirin to be 50% and the distribution volume approximately 25 l (Pederson & FitzGerald, 1984) a maximum aspirin concentration of $5 \mu\text{M}$ could be anticipated with this dose. The ID_{50} for inhibition of IP formation in this study is of the same order as the dose of aspirin (20 mg enteric coated aspirin per day for 14 days) required to inhibit aggregation and

TxA_2 formation (Herd *et al.*, 1984). Furthermore the dose-response curve for the *in vivo* inhibition by aspirin of collagen induced IP formation (Figure 2) is similar to that for the *in vivo* inhibition of the formation of TxB_2 (Patrono *et al.*, 1980).

It is interesting to note that the IC_{50} for aspirin *in vitro* (Figure 1) was about $1 \mu\text{M}$, while the calculated ID_{50} *in vivo* (Figure 2) was higher ($5 \mu\text{M}$). There are several possible reasons for this apparent discrepancy. The calculated ID_{50} of $5 \mu\text{M}$ is a maximum possible value, and factors such as protein binding may reduce the free aspirin concentration. Whilst the protein binding of aspirin *in vivo* is not clear a figure of 60–70% has been suggested (Aarons *et al.*, 1980); in our experiments the buffer used for resuspending the platelets contained 0.3% BSA, whereas the plasma albumin concentration is around 4.0%. Alternatively, we may have overestimated the bioavailability of our aspirin preparation, or it may be that when aspirin is taken in solution a greater proportion is metabolized before entering the circulation.

It is currently unclear how the data in this report and other observations on the effects of aspirin on platelet functions may relate to the clinical effects of aspirin in treating cardiovascular disease. Considerable attention is currently being paid to the choice of a dose of aspirin low enough to spare vessel prostacyclin

production but high enough to inhibit platelet cyclo-oxygenase, in an effort to enhance the possible beneficial effects of aspirin in the prevention of thrombosis (Hirsch, 1985). Doses as low as 20 mg day⁻¹ for 7 days (FitzGerald *et al.*, 1983; Weksler *et al.*, 1985), while substantially inhibiting TxA₂ production, also inhibit PGI₂ formation, although to a lesser degree. Our data have shown that aspirin in single small doses can block a biochemical response which is thought to be intimately involved in platelet activation. Thus, measurement of PI metabolism can provide a further index of platelet reactivity, complement-

ing currently used techniques. From our results it is likely that activation of platelets by collagen will be substantially inhibited during chronic administration of aspirin in a dose range of 20–40 mg daily, and this is currently under investigation. Ultimately, however, the clinical usefulness of these low doses of aspirin, which have been shown to block various platelet functions, will have to undergo rigorous testing in the relevant clinical situations.

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References

- Aarons, L. J., Clifton, P., Fleming, G. & Rowland, M. (1980). Aspirin binding and the effect of albumin on spontaneous and enzyme catalysed hydrolysis. *J. Pharm. Pharmac.*, **32**, 537–543.
- Berridge, M. J. & Irvine, R. F. (1984). Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature*, **312**, 315–321.
- Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982). Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochem. J.*, **206**, 587–595.
- FitzGerald, G. A., Oates, J. A., Hawiger, J., Maas, R. L., Roberts, L. J., Lawson, J. A. & Brash, A. R. (1983). Endogenous biosynthesis of prostacyclin and thromboxane and platelet function during chronic administration of aspirin in man. *J. clin. Invest.*, **71**, 678–688.
- Godfrey, P. P. & Putney, J. W. (1984). Receptor-mediated metabolism of the phosphoinositides and phosphatidic acid in rat lacrimal acinar cells. *Biochem. J.*, **218**, 187–195.
- Herd, C. M., Rodgers, S. E., Duncan, E. M., Tunbridge, L. J., Imhoff, D. M., Lloyd, J. V. & Bochner, F. (1985). Platelet aggregation and thromboxane production after chronic low-dose enteric coated aspirin in humans. *Clin. exp. Pharmac. Physiol.*, Supplement 9, 59.
- Hirsch, J. (1985). The relationship between dose of aspirin, side effects and antithrombotic effectiveness. *Stroke*, **16**, 1–4.
- Lapetina, E. G. (1983). The role of phospholipase C in platelet responses. *Life Sci.*, **33**, 1011–1018.
- Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981). The stimulation of inositol lipid metabolism that accompanies calcium mobilisation in stimulated cells: defined characteristics and unanswered questions. *Phil. Trans. Roy. Soc. Lond. B.*, **296**, 123–137.
- Patrino, C., Ciabattini, G., Pinca, E., Pugliese, F., Castrucci, G., De Salvo, A., Satta, M. A. & Peskar, B. A. (1980). Low dose aspirin and inhibition of thromboxane B₂ production in healthy subjects. *Thrombosis Res.*, **17**, 317–327.
- Pederson, A. K. & FitzGerald, G. A. (1984). Dose-related kinetics of aspirin: presystemic acetylation of platelet cyclooxygenase. *New Engl. J. Med.*, **311**, 1206–1211.
- Rink, T. J., Sanchez, A. & Hallam, T. J. (1983). Diacylglycerol and phorbol ester stimulate secretion without raising cytoplasmic free calcium in human platelets. *Nature*, **305**, 317–319.
- Schachter, M., Godfrey, P. P., Minchin, M. C. W., McClue, S. J. & Young, M. M. (1985). Serotonergic agonists stimulate inositol lipid metabolism in rabbit platelets. *Life Sci.*, **37**, 1641–1647.
- Siess, W., Cuatrecasas, P. & Lapetina, E. G. (1983). A role for cyclo-oxygenase inhibitors in the formation of phosphatidic acid in stimulated human platelets. *J. biol. Chem.*, **258**, 4683–4686.
- Smith, J. B. & Willis, A. L. (1971). Aspirin selectively inhibits prostaglandin production in human platelets. *Nature New Biology*, **231**, 235–237.
- Watson, S. P., McConnell, R. T. & Lapetina, E. G. (1984). The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. *J. biol. Chem.*, **259**, 13199–13203.
- Watson, S. P., Reep, B., McConnell, R. T. & Lapetina, E. G. (1985). Collagen stimulates [³H]-inositol trisphosphate formation in indomethacin treated human platelets. *Biochem. J.*, **226**, 831–837.
- Weksler, B. B., Tack-Goldman, K., Subramanian, V. A. & Gay, N. A. (1985). Cumulative inhibitory effect of low-dose aspirin on vascular prostacyclin and platelet thromboxane production in patients with atherosclerosis. *Circulation*, **71**, 332–340.

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