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European Journal of Pharmacology 460 (2003) 59–62
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Short communication

Anti-thrombin action of low-dose acetylsalicylic acid

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Received 30 September 2002; received in revised form 6 December 2002; accepted 17 December 2002

Abstract

It is known that low-dose aspirin is effective in coronary artery therapy, although it has not yet been clarified how it exerts its action. Here, we report that treatment of coronary artery patients with 100 mg/day of aspirin does not attenuate thrombin generation, but reduces free thrombin by favouring the formation of thrombin/antithrombin (TAT) complexes. Antithrombin hyperactivation is mediated by inhibition of platelet factor 4 release from α-granules, leading to higher heparin availability.

Keywords: Acetylsalicylic acid; Aspirin; Anti-thrombin action; Coronary heart disease; Platelet factor 4

1. Introduction

It is known that a variety of stimuli induce plaque rupture in atheromatous arteries, which touches off a cascade of enzymatic and cellular responses that frequently culminate in artery occlusion via increased thrombin generation (Hathaway and Goodnight, 1993). It was suggested that this knowledge can be translated into clinical practice adopting longstanding anti-thrombotic mainstays for coronary syndromes, like heparin and aspirin (acetylsalicylic acid) (Turpie, 1999). Large-scale clinical trials have demonstrated that patients with chronic coronary insufficiency benefit from therapy with low-dose aspirin. On the other hand, those with acute coronary insufficiency need association of anti-thrombin drugs to improve outcome. However, it is not sufficiently clear as to which specific mechanism acetylsalicylic acid exerts its action against thrombin (Eisenberg, 1995).

Acetylsalicylic acid remains the first choice for anti-thrombotic therapy, due to its relative safety and well-documented efficacy. It is maximally effective at daily doses much lower than those required for other actions. It is generally assumed that its anti-thrombotic as well as anti-platelet action is mediated by irreversible acetylation of platelet cyclooxygenase, which leads to impairment of platelet function through inhibition of thromboxane A_2 synthesis (Patrono, 1994). Other studies point to an anti-thrombotic effect of acetylsalicylic acid by depression of thrombin generation (Szczeklik et al., 1992; Kessels et al., 1994; Szczeklik, 1994). Overall, it is generally believed that the mechanism through which acetylsalicylic acid exerts its therapeutic action involves diminution of thrombin formation, although it was shown that acetylsalicylic acid treatment does not have any effect on dilution-induced enhancement of coagulation (Ruttmann and James, 1999). It seems therefore worth investigating along which specific pathway acetylsalicylic acid exerts its action against platelets and thrombin at the low doses suggested for artery occlusion therapy.

2. Materials and methods

2.1. Subjects

Four groups of subjects were recruited after informed consent was procured. Two control groups comprised of either (i) 20 healthy non-smoker young volunteers who are

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doi:10.1016/S0014-2999(02)02925-4

not related to anyone with a history of thrombotic disease and who are currently not undergoing any pharmacological therapy, or (ii) twenty 60- to 70-year-old non-coronary males without any history of thrombosis. This second group acted as a control for atherogenesis, which is generally present in elderly subjects. Two groups of patients were selected on the exclusive basis of altered electrocardiogram and myocardial scintigraphy, both at rest and under stress, but without angina and/or other clinical signs. This allowed us to exclude subjects with acute coronary insufficiency, who are usually under therapy with other anti-thrombotic and fibrinolytic drugs. Thus, these groups were made up of either 20 age- and gender-matched subjects with chronic coronary disease, who could not be treated with acetylsalicylic acid because of allergy or asthma and other similar respiratory diseases (Szczeklik et al., 2001), or 20 age- and gender-matched subjects affected by chronic coronary insufficiency, who were under therapy with 100 mg/day of aspirin for 1 month. All patients selected had levels of plasma prothrombin activation peptide (F1.2) comparable with those of elderly controls, so that we could exclude the occurrence of any thrombotic episode.

2.2. Protocol

Blood samples were drawn after 12 h fasting, provided subjects were at rest for at least 30 min prior to bleeding. Plasma or serum samples were obtained by centrifugation, using 0.11 M sodium citrate as anti-coagulant, or allowing blood clotting for 30 min at 37 °C, respectively. For each group of subjects, we evaluated both the plasma and serum concentration of antithrombin (AT III), and the serum concentration of F1.2. The serum concentration of thrombin/antithrombin (TAT) complexes was determined as well, both in the absence and in the presence of anti-platelet factor 4 antibody (aPF4), which was mixed with blood immediately after bleeding whenever necessary.

2.3. Reagents

All reagents and kits were obtained from Behring (Marburg, Germany), except for aPF4, which was purchased from Cabru (Milan, Italy). The concentration of AT III was measured either by immunodiffusion using NorPartigen AT III plates or by a colorimetric assay based on modification of absorbance at 405 nm upon hydrolysis of the substrate tosyl-gly-pro-arg-5-amino-2-nitrobenzoic acid isopropylamide. Enzygnost F1.2 or Enzygnost TAT enzyme-linked immunosorbent assay kits were employed according to manufacturer's instructions to evaluate the concentrations of F1.2 or TAT complexes, respectively.

2.4. Statistical analysis

Data were expressed as means ± S.D. The means were compared using the Bonferroni *t*-test method after analysis of variance. A P value of less than 0.01 was considered significant.

3. Results

3.1. AT III concentration and thrombin generation

Effects of low-dose aspirin (100 mg/day) are summarized in Table 1. The total amount of thrombin, as measured by the serum concentration of F1.2, is almost similar among normal young, non-coronary elderly, aspirin-untreated coronary, and aspirin-treated coronary subjects. This indicates that thrombin formation is not modified in coronary patients, irrespective of acetylsalicylic acid treatment. In other words, acetylsalicylic acid does not display any anti-thrombin action because it does not alter thrombin generation. On the other hand, it can be appreciated that acetylsalicylic acid causes significant attenuation of the serum concentration of AT III in coronary subjects. Considering that the anti-thrombin action of acetylsalicylic acid cannot be ascribed to the diminution of thrombin generation, we have hypothesized that consumption of AT III reflects the formation of TAT complexes.

3.2. Concentration of TAT complexes

Fig. 1 shows that the amount of thrombin associated into complexes with AT III is almost the same in young and elderly non-coronary volunteers, and in aspirin-untreated coronary patients, being roughly 20% of generated thrombin (~ 25 nM, see Table 1). Instead, the concentration of TAT complexes nearly doubles in aspirin-treated coronary subjects, thus lowering the concentration of free thrombin. It can be appreciated that inhibition of platelet factor 4 by a specific antibody (aPF4), which was mixed with blood immediately after bleeding, is effective in increasing the concentration of TAT complexes in both normal and aspirin-untreated coronary subjects to levels that are quite close to those observed in aspirin-treated patients. On the other hand, the addition of a-PF4 to the blood of subjects under treatment with acetylsalicylic acid did

Table 1
F1.2 generation and AT III concentration[a]

	Serum F1.2 (nmol/l)	Plasma AT III (μmol/l)	Serum AT III (μmol/l)
Young volunteers	116 ± 15	27.8 ± 2.4	24.1 ± 3.9
Non-coronary elderly subjects	118 ± 13	26.3 ± 2.4	23.7 ± 1.7
Untreated coronary patients	124 ± 15	27.0 ± 2.7	23.7 ± 2.0
Aspirin-treated coronary patients	123 ± 14	27.3 ± 2.5	15.4 ± 1.9[b]

[a] Data are presented as mean ± S.D.
[b] $P < 0.01$ (Bonferroni's *t*-test) vs. the respective value of each of the other groups.

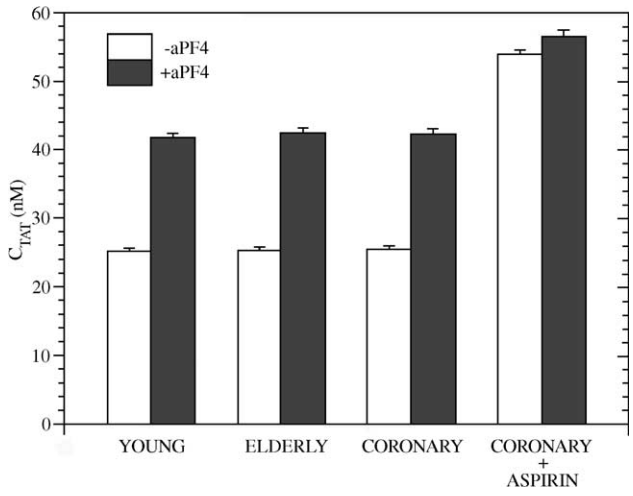

Fig. 1. Molar concentration (mean ± S.D.) of TAT complexes in sera before (empty bars) and after (solid bars) treatment with aPF4. For each group, the concentration of complexes in plasma was insignificant (data not shown). aPF4: Anti-platelet factor 4 antibody; TAT: thrombin/antithrombin complexes.

not significantly modify the serum concentration of TAT complexes.

4. Discussion

The aim of this study was to clarify the molecular mechanism underlying low-dose acetylsalicylic acid treatment. An ambivalent behaviour has been recently observed in 1-week-long aspirin treatment of healthy volunteers, depending on whether 75 or 500 mg/day aspirin dosage was used. It was observed that thrombin generation, as measured by the serum concentration of F1.2, was not significantly attenuated in low-dose treatment, but the reverse occurred at high dose. On the other hand, anti-platelet effects, as monitored by collagen-induced platelet aggregation in whole blood, were already complete at 75 mg of aspirin, whereas no further inhibition of platelet function was found at 500 mg (Wallen and Ladjevardi, 1998). It was then suggested that different mechanisms lie behind this dose-dependent behaviour, with the anti-thrombin action requiring much higher amounts of acetylsalicylic acid than the anti-aggregating effect. It was not clarified, however, as through what mechanism low-dose aspirin exerts its action against thrombin.

It is known that high-dose aspirin exerts its anti-thrombin action by acetylating plasma proteins (Villanueva and Allen, 1986; Pinckard et al., 1968) or even prothrombin (Szczeklik, 1994). On the other hand, low-dose aspirin is not expected to depress thrombin formation (Drouet, 1990). In fact, platelets participate in this process by providing a catalytic surface for prothrombinase through a thrombin/ADP-induced shape change involving the anionic phospholipids of the membrane. After binding to membrane, factor V interacts with factor X and prothrombin, leading to formation of thrombin and F1.2 (Rosing et al., 1980; Kane and Davie, 1988; Tracy and Mann, 1986). Any treatment or condition that inhibits the procoagulant power and exposition of anionic membrane phospholipids would certainly impair thrombin generation. Such an effect is caused by annexin V, iloprost, glycoprotein IIb/IIIa receptor blockers, and inhibitors of binding between glycoprotein Ib and von Willebrand factor, which prevent platelet internal signalling and activation (Eschwege et al., 1995). A pathological impairment of such a procoagulant action is extraordinarily rare (Weiss et al., 1979) and occurs in severe thrombocytopenia, as proven by the increase of residual serum prothrombin in this pathology. Low-dose aspirin does inhibit thromboxane A_2 and prostacyclin formation (Bode-Boger et al., 1998), but does not at all interfere with the shape change leading to platelet aggregation. In fact, platelets retain their ability to expose membrane phospholipids, cooperating in prothrombinase activation.

We have already shown that the procoagulant action of seminal vesicle protein IV occurs, through its capacity to prevent thrombin association into complexes with AT III (Di Micco et al., 1994, 1997). Furthermore, as a proof of the thrombin-modulating effect displayed by TAT complexes, it has been recently found that the onset of serious haemorrhage in haemophilic patients also depends on the fact that the percentage of total thrombin associated into TAT complexes is higher in these subjects than in controls (Di Micco et al., 2000). In this paper, we provide evidence that in coronary patients under low-dose aspirin therapy for 1 month, thrombin attenuation takes place through TAT complex formation. This is better understood considering that during the shape change platelets also secrete the contents of their organelles (the ‘‘release reaction’’), which causes them to adhere to one another, finally leading to primary haemostasis through the formation of a platelet plug. In this context, the anti-heparin agent platelet factor 4, which is contained in α-granules, controls heparin availability and thereafter thrombin association with AT III. If this action of platelet factor 4 is impaired, thereby hindering α-granules release, it follows that a higher amount of TAT complexes can form, because increased heparin availability hyperactivates AT III. In controls and aspirin-untreated coronary patients, inhibiting platelet factor 4 with a specific antibody may remarkably increase the basal concentration of TAT complexes. This effect is absent in aspirin-treated patients, which allows us to infer that low-dose aspirin inhibits the thromboxane A_2-dependent release of platelet factor 4 from α-granules. As a consequence, there is increased heparin availability leading to AT III hyperactivation and binding to larger amounts of thrombin. The anti-thrombin effect produced by this mechanism may be mild, but sufficient to inhibit the amount of thrombin produced by atherosclerotic subjects. When the blood concentration of F1.2 is high, implying conspicuous thrombin generation, a therapeutic approach aiming at thrombin inhibition may take advantage of acetylsalicylic acid association with a direct inhibitor.

Finally, it might be argued that the action of acetylsalicylic acid does not depend on the age and the health status of people. Thus, we could have checked the effects of low-dose aspirin in healthy volunteers, thereafter inferring that a similar mechanism is expected to occur in patients affected by chronic coronary insufficiency. However, this may give rise to criticism. Moreover, our study also aims at showing that the shadow of scepticism spread on the use of low-dose aspirin as a first low-cost aid in anti-thrombotic therapy is likely unjustified.

Acknowledgements

This study was financed by L. 41/94 funds from the Regione Campania, Italy.

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