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LOW DOSE ASPIRIN DOES NOT PREVENT FIBRINOLYTIC RESPONSE TO VENOUS OCCLUSION

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Abstract—Interest in the antithrombotic potential of low-dose aspirin is based on its ability to inhibit thromboxane (Tx)A2-related platelet function with concomitant sparing of vascular prostacyclin (PGI2) production. The aim of this study was to investigate the effect of low-dose aspirin (20 mg daily for 7 days) on the increase in fibrinolytic activity in healthy volunteers after venous occlusion. We also tested the effect of high-dose aspirin (650 mg × 2), of salicylate (569 mg × 2) and of indobufen (200 mg × 2), a new cyclo-oxygenase inhibitor unrelated to salicylates. Low-dose aspirin reduced serum TxB2 generation by about 90% and suppressed arachidonate-induced platelet aggregation. In contrast, fibrinolytic activity, measured by the euglobulin lysis area and the euglobulin lysis time, was not significantly affected. Both high-dose aspirin and indobufen significantly inhibited TxB2 generation and the rise in fibrinolytic activity induced by venous occlusion, without affecting the pre-occlusion values. Salicylate did not significantly affect any parameter studied. Besides offering a favorable solution to the "aspirin dilemma" related to the TxA2/PGI2 balance, low-dose aspirin might leave intact the fibrinolytic capacity of the vessel wall.

Aspirin prevents the formation of two compounds with opposite biological effects namely, the aggregatory thromboxane (Tx)A2 in platelets and the antiaggregatory prostaglandin (PG) I2 in vascular cells [1, 2]. The "aspirin dilemma" derives from the assumption that simultaneous inhibition of TxA2 and PGI2 synthesis might reduce the antithrombotic potential of aspirin [1, 2]. In the past few years pharmacological studies in normal volunteers [3, 4] and in patients with atherosclerosis [5] or cerebrovascular disease [6] or after myocardial infarction [7] have suggested that low-dose aspirin may inhibit TxA2-related platelet function with concomitant sparing of vascular PGI2 production. Other studies, however, both in animals [8] and humans [9, 10] have failed to completely dissociate the effect of low-dose aspirin on the TxA2-PGI2 balance.

A recent report [11] showed that high-dose aspirin may have an anti-fibrinolytic effect in man, since it inhibited the release of tissue-type plasminogen activator (t-PA) after venous occlusion. The ability of vascular cells to release t-PA is considered the major determinant of physiological fibrinolysis and of its role in thrombosis prevention [12].

The aim of this study was to investigate the effect of low-dose aspirin on fibrinolytic activity in normal individuals before and after venous occlusion. In comparison we also tested the effect of high-dose aspirin, of salicylate and of indobufen, a new platelet aggregation inhibitor. This compound is structurally unrelated to salicylate, but inhibits platelet TxA2 generation by suppressing cyclo-oxygenase activity [13].

METHODS

Experimental design

Eighteen healthy male volunteers (23–34 years old) having taken no drugs during the previous two weeks, entered the study, giving informed consent.

In the first study, six volunteers ingested 20 mg aspirin (tablets kindly provided by Prof. C. Patrono, Catholic University, Rome, Italy) daily for seven consecutive days in the morning, the last dose being taken 2 hr before blood collection. The venous stasis test was performed in the morning before and after the weekly aspirin treatment.

In the second study, following a double blind randomized cross-over design, six volunteers ingested aspirin (Aspro, Bouty, Milan, Italy) or indobufen (Ibustrin, Farmitalia Carlo Erba, Milan, Italy) or placebo, with two weeks between each treatment. Aspirin (650 mg) or indobufen (200 mg) were given 18 and 2 hr before testing under the control of one of the investigators.

In the third study, six volunteers ingested either sodium salicylate (569 mg), a dose equimolar to 650 mg of aspirin, or placebo, following a double blind cross-over design, 18 and 2 hr before testing.

Venous stasis and blood collection

Venous stasis was performed in the morning in fasting subjects after 15 min supine rest. Venous blood was collected from antecubital vein without stasis into plastic syringes and added to 3.8% sodium citrate (9:1, v/v) in precooled plastic tubes, and kept on ice until centrifuged. Blood was then collected from the opposite arm after venous occlusion (v.o.) of the forearm by a sphyngomanometer cuff inflated at a pressure intermediate between the systolic and diastolic for 10 min. Plasma was obtained by centrifugation at 4000 rpm for 20 min at 4° and aliquots were frozen at −20° for subsequent analysis. Serum was obtained by incubating a fixed amount of native blood in glass tubes for 1 hr at 37° and subsequent centrifugation (at 4000 rpm for 20 min). At each

blood collection hematocrit was measured by a standard method.

Fibrinolysis tests

Fibrinolytic activity was measured on the euglobulin fraction. Briefly, euglobulins were prepared by acidification at pH 5.9 of diluted plasma (1:10 with b.d. water) with 0.25% (v/v) of acetic acid at 4°. The precipitate obtained after centrifugation (5 min at 3000 rpm at 4°) was dissolved in the original plasma volume in an appropriate buffer.

Euglobulin lysis time (ELT). The euglobulin precipitate was dissolved in a borate buffer pH 7.6 (0.18 M H_3BO_3; 0.01 M $Na_2B_4O_7 \cdot 10\,H_2O$; 0.038 M NaCl) 250 μl, clotted with the same amount of $CaCl_2$ (0.025 M) in a glass test tube in duplicate and incubated in a water bath at 37°. The complete dissolution of the clot was followed by continuous visual inspection and expressed as time of lysis (min).

Euglobulin lysis area (ELA). The precipitate was dissolved in EDTA–gelatin–barbital buffer pH 7.8 (0.05 M Na diethyl-barbiturate, 0.1 M NaCl, 0.25% gelatin, 0.1% Na_2 EDTA) and two aliquots of 30 μl each were placed onto the surface of two different fibrin plates.

After 18 hr incubation at 37° on a levelled shelf, the fibrin plates were coloured by 0.1% amidoschwarz to evidentiate the lysis zones better; two perpendicular diameters were measured by means of a millimeter paper and the results expressed as area of the circular lysis zones (mm^2). Fibrin plates were prepared as described [14], with the exception that human plasminogen-rich fibrinogen (grade L, Kabi, Sweden) was used instead of bovine.

Measurements of TxB2 and 6-ketoPGF 1α

TxB2 was measured in serum at appropriate dilutions (between 20 and 10,000 times) by radioimmunoassay (RIA) [15]. 6-KetoPGF1α was measured in undiluted plasma by RIA [16]. Reference standard curves of 6-ketoPGF1α were prepared in charcoal-treated human plasma.

Both specific antisera against TxB2 or 6-keto PGF1α were kindly provided by Prof. C. Patrono.

Statistical analysis

Statistical analysis of the data was performed by two or three ways analysis of variance (ANOVA) followed by Tukey's multiple comparison test for randomized blocks [17]. All values are given as means ± SEM.

Table 1. Fibrinolysis in placebo and aspirin (20 mg × 7 days) treated subjects before and after venous occlusion (v.o.)

	Placebo	Aspirin	P_2
	Euglobulin lysis area (mm^2)		
Before v.o.	168.4 ± 15.2	182.3 ± 33.1	n.s.
After v.o.	482.2 ± 75.4	466.3 ± 60.3	n.s.
P_1	<0.01	<0.01	
	Euglobulin lysis time (min)		
Before v.o.	210.0 ± 19.1	187.5 ± 23.8	n.s.
After v.o.	77.5 ± 32.7	71.6 ± 17.3	n.s.
P_1	<0.01	<0.01	

Statistical analysis: P_1, before vs. after v.o.; P_2, placebo vs. aspirin.

RESULTS

Seven daily oral doses of 20 mg aspirin reduced serum TxB2 generation by 83–93% (from 263.0 ± 55.8 ng/ml to 28.6 ± 3.2 ng/ml). Platelet aggregation induced by 1 mM arachidonic acid was completely suppressed (data not shown). Table 1 shows the effect of low-dose aspirin on the rise in vascular fibrinolytic activity induced by venous occlusion. Fibrinolytic activity (measured as euglobulin lysis area and euglobulin lysis time) was not significantly affected by aspirin ingestion either before or after venous occlusion.

Ingestion of high-dose aspirin (650 mg × 2) or indobufen (200 mg × 2) significantly reduced serum TxB2 production respectively by 99.9 and 97.6% (from 330.6 ± 15.2 to 0.25 ± 0.07 and to 7.87 ± 2.17 ng/ml).

Table 2 shows the effect of high-dose aspirin and indobufen on vascular fibrinolytic activity induced by venous occlusion. Aspirin and indobufen significantly inhibited the rise in vascular fibrinolytic activity induced by venous occlusion, but had no significant effect on pre-occlusion values.

In the six volunteers who received salicylate (569 mg × 2, a dose equimolar to aspirin) serum TxB2 levels were unaltered (219.8 ± 37.1 and 208.4 ± 35.9 ng/ml in control and salicylate-treated

Table 2. Fibrinolysis in placebo, aspirin (650 mg × 2), and indobufen (200 mg × 2) treated subjects before and after venous occlusion (v.o.)

	Placebo	Aspirin	P_2	Indobufen	P_3
		Euglobulin lysis area (mm^2)			
Before v.o.	81.2 ± 14.5	101.8 ± 28.2	n.s.	88.0 ± 26.2	n.s.
After v.o.	400.5 ± 37.0	235.8 ± 66.7	<0.01	216.3 ± 54.8	<0.01
P_1	<0.01	<0.01		<0.01	
		Euglobulin lysis time (min)			
Before v.o.	130.8 ± 9.6	143.3 ± 13.3	n.s.	129.2 ± 7.4	n.s.
After v.o.	70.8 ± 9.7	135.0 ± 30.9	<0.01	132.5 ± 19.5	<0.01
P_1	<0.01	n.s.		n.s.	

Statistical analysis: P_1, before vs after v.o.; P_2, placebo vs aspirin; P_3, placebo vs indobufen.

Table 3. Fibrinolysis in placebo and salicylate (569 mg × 2) treated subjects before and after venous occlusion (v.o.)

	Placebo	Salicylate	P_2
	Euglobulin lysis area (mm^2)		
Before v.o.	139.9 ± 28.2	136.5 ± 23.2	n.s.
After v.o.	442.9 ± 47.5	400.4 ± 45.1	n.s.
P_1	<0.01	<0.01	
	Euglobulin lysis time (min)		
Before v.o.	175.0 ± 14.3	197.5 ± 19.1	n.s.
After v.o.	97.5 ± 18.5	127.5 ± 18.1	n.s.
P_1	<0.01	<0.01	

Statistical analysis: P_1, before vs. after v.o.; P_2, placebo vs. salicylate.

respectively). As Table 3 shows, salicylate had no significant effect on fibrinolytic activity. Plasma levels of 6keto-PGF1α were below the detection limit of our method (<4 pg/ml) before and after venous occlusion.

The mean hematocrit values ranged between 38.3 and 40.7% before and between 47.0 and 51.2% after venous stasis for all the treatments studied. Hemoconcentration (ranging between 21 and 27%) occurring during the 10 min venous occlusion was not significantly different between the various treatments. Postocclusion values of the parameter measured were corrected according to a standard formula [11]:

$$(\text{post V} = 1 - \text{postocclusion hematocrit} / 1 - \text{preocclusion hematocrit}).$$

However, since it is not clear whether this is a valid reflection of the partition of t-PA between vascular and extra-vascular spaces [11], we also analysed the data without correction. The results were not different, so we report only uncorrected values.

DISCUSSION

Daily oral doses of 20 mg aspirin to normal subjects failed to prevent the rise in fibrinolytic activity after venous occlusion. In contrast high dose aspirin (650 mg × 2) had a significant inhibitory effect. Similarly release of fibrinolytic activity was reduced by indobufen (200 mg × 2), a newly developed inhibitor of platelet TxA2 synthesis. In no instance was the pre-occlusion fibrinolytic activity affected. This suggests that high-dose aspirin or indobufen acted by inhibiting increase of fibrinolytic activity from the vessel wall and had no other effect on the test system used [11].

The mechanism by which high-dose aspirin inhibits fibrinolytic activity after venous occlusion is still unknown. Venous occlusion reportedly induces a rise of PGI2 [18], but we are unable to confirm this in our subjects using a highly sensitive and specific RIA [16, 19].

Our findings, however, do not exclude the possibility that the rise in fibrinolytic activity after venous occlusion is mediated by *local* PGI2 generation and that inhibition of fibrinolytic activity is due to inhibition of PGI2 synthesis. This hypothesis is consistent with the suggestion that repeated low-dose aspirin while significantly reducing platelet TxA2 synthesis, leaves unaltered the PGI2 synthesizing activity of vascular cells [3, 4]. Moreover salicylate, which has no effect on human vascular PGI2 generation [20], did not alter fibrinolytic activity after venous occlusion.

Levin *et al.* [11] found no significant relationship between inhibition of t-PA activity and salicylate plasma levels after aspirin administration and suggested that the inhibitory effect of aspirin might be related to its acetylating capacity rather than to its salicylate moiety. In addition several studies point to a causal relationship between prostaglandins—particularly PGI2- and t-PA release [21, 22].

Although both low and high-dose aspirin significantly reduced platelet TxA2 generation in serum, the inhibition by high-dose regimen was virtually complete, while that by low-dose averaged 87.6%. Whether this difference could account for the different effect of the two aspirin regimens on the release of fibrinolytic activity remains to be established.

Some recent reports [23–25] failed to confirm the effect of high-dose aspirin on t-PA release. The discrepancy between these findings and those reported by Levin *et al.* [11] and in the present study may derive from the small number of subjects tested, differences in statistical analysis and wide individual variability in the fibrinolytic response to venous occlusion, in the pharmacokinetics of aspirin or some other unknown variables. Previous studies of the effect of high doses of aspirin on blood fibrinolytic activity also gave controversial results [26–28].

While further investigation is required to establish the extent of high-dose aspirin's effect on fibrinolytic activity increase, it seems safe to conclude that low-dose aspirin's does not interfere with vascular fibrinolysis. Besides offering a favorable solution to the "aspirin dilemma" related to the TxA2–PGI2 balance [3–5], low-dose aspirin might leave intact the fibrinolytic capacity of vascular wall.

Last, but not least from a practical point of view, a careful pharmacological history should always be taken before laboratory tests are made on fibrinolytic activity. It is likely indeed that ingestion of aspirin or other non steroidal anti-inflammatory drugs could contribute to the broad individual variability observed in the fibrinolytic activity of normal individuals after venous occlusion [29].

It cannot be excluded that patients classified as "non responders" to venous occlusion could simply have taken aspirin or other similar drugs.

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