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SHORT COMMUNICATIONS

Inhibition of human platelet thromboxane generation by aspirin in the absence of measurable drug levels in peripheral blood

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It has been known for many years that aspirin inhibits the formation of the aggregatory thromboxane A_2 (TxA_2) in platelets and the antiaggregatory prostaglandin I_2 (PGI_2) in vascular cells [1]. On the basis of *in vitro* data, it was suggested that lower doses of aspirin are required to prevent platelet TxA_2 generation than vascular PGI_2 synthesis [2]. Studies in animals and man, however, have failed to determine the single dose(s) of aspirin which completely suppress platelet TxA_2 generation without affecting PGI_2 formation [3–8]. TxA_2 synthesis was fully inhibited by single oral doses of aspirin as low as 80–100 mg, while the simultaneous effect on vascular PGI_2 varied widely from one study to another, and even within subjects in the same study. Little attention has been paid to aspirin pharmacokinetics with regard to the different pharmaceutical formulations employed [9–12]. We show here that after administration of a compressed (320 mg) or an enteric-coated (800 mg) formulation of aspirin to six healthy volunteers platelet TxA_2 generation was almost completely inhibited, even on five occasions when aspirin levels were not measurable in the peripheral circulation. *In vitro* studies showed that concentrations of aspirin at least 100 times the detection limit (0.5 μM) of the HPLC assay used were required to inhibit TxA_2 generation in blood by more than 80%. We suggest that effective acetylation of the platelet enzyme occurs on exposure to aspirin in the portal circulation. Following variable first-pass de-acetylation in the entero-hepatic circulation [13] variable amounts or even no aspirin may be delivered to the peripheral blood. The inhibitory effect on platelet TxA_2 generation would be the same in all cases, but vascular PGI_2 synthesis would only be affected if intact aspirin escaped the entero-hepatic circulation. A better knowledge of the kinetic parameters of aspirin for each of the formulations used in thrombosis prevention trials might help in solving the "aspirin dilemma".

Methods and results

Six healthy male volunteers, 26–39 years old, weighing 65–75 kg, abstained from any drugs for 15 days before this study. In a random cross-over design they received a single oral dose of either a compressed (320 mg) or an enteric-coated (800 mg) aspirin preparation. Each subject received both preparations, with an interval of 10 days.

Aspirin was undetectable (less than 100 ng/ml) in plasma from three subjects given compressed aspirin and from two subjects who had taken enteric-coated aspirin. All had fasted overnight.

Figure 1 shows the typical time course of aspirin and salicylate in plasma of subjects where aspirin was detectable after either formulation. Blood samples were collected from an antecubital vein on 0.1% cold Na_2EDTA with 0.3% KF to inhibit *in vitro* hydrolysis of ASA by plasma esterases. Blood was drawn before and 5, 10, 15, 20, 30, 40, 50, 60, 90 min, 2, 3, 4, 6, 8 and 24 hr after compressed ASA, and 10, 60, 90 min, 2, 2.5, 3, 4, 6, 9, 12, 16 and 24 hr after enteric-coated ASA. During the first hour after compressed ASA, blood was drawn through a butterfly needle left in the vein. The line was kept open by slowly dripping sterile saline. All other samples were taken by venipuncture.

Aspirin and salicylate were assayed by HPLC as described elsewhere [22] modified in several details. Briefly, 2.5 ml of 0.1 N HCl and 100 μl of 10 μg/ml theophylline-1-propyl as internal standard were added to 2 ml plasma as described [23]. The samples were shaken for 15 min with 5 ml of diethyl-ether. After centrifugation, the organic phase was transferred and evaporated under a gentle nitrogen stream. The residue was dissolved in 100 μl of the chromatographic mobile phase and injected into the liquid chromatograph. Using a Perkin-Elmer (Norwalk, CT, U.S.A.) series 2/2 liquid chromatograph equipped with a LC 55 spectrophotometer (226 nm) and reversed phase column (Hibar RP-8, 5 μm particle size, 250 mm × 4 mm, Merck), the samples were eluted in isocratic conditions with a mobile phase of 25% (v/v) acetonitrile in diluted phosphoric acid (0.03%, pH 2.2) at 1.4 ml/min flow rate. Reagents (LiChrosolv, Merck) were u.v. grade. Internal calibration curves of aspirin and salicylate were prepared for each set of samples. Linearity was found over the investigated concentration range. No interference with other aspirin metabolites was observed. The detection limit was 100 ng/ml for both compounds.

Aspirin was undetectable in all plasma samples from three subjects given 320 mg of the drug and reached a peak concentration of 2.91 ± 0.65 μg/ml (about 16 μM) in the

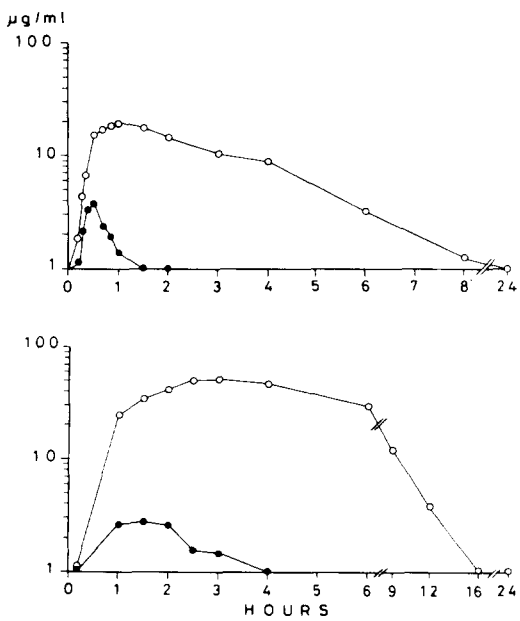

Fig. 1. Typical time course of aspirin (closed circles) and salicylate (open circles) plasma levels in two subjects given an oral dose of 320 mg compressed aspirin (upper panel) or 800 mg enteric-coated aspirin (lower panel).

other three. Aspirin was also undetectable in all plasma samples from two subjects given 800 mg (enteric-coated formulation) and reached a peak concentration of 3.30 ± 0.27 μg/ml (about 18 μM) in the other four individuals. Table 1 reports the area under the curve of aspirin and salicylate plasma levels separately for the subjects in whom aspirin was detectable and not. No significant difference in salicylate kinetic parameters could be found between the two groups after either formulation.

Inhibition of serum TxB_2 generation by both aspirin formulations was similar in the six subjects irrespective of whether plasma levels of aspirin could be detected or not.

In vitro experiments showed that incubation of human whole blood from three different donors with 0.5 μM aspirin for 1 hr at 37° did not result in any significant reduction of serum TxB_2 generation. This concentration of aspirin corresponds to the detection limit of the HPLC assay method for aspirin. Concentrations of aspirin 10 times (5 μM) or 100 times (50 μM) higher than this detection limit induce respectively 51–59% and 82–98% TxB_2 inhibition.

Discussion

On account of the potential for erratic absorption patterns of aspirin (especially from enteric-coated formulations), detectable plasma levels of aspirin may depend on fortuitous blood collection times [9]. In this case, however, it was unlikely, since as many as eight blood samples were collected within the first hour after compressed aspirin and six during the first 3 hr after enteric-coated aspirin. Moreover salicylate was detectable in all blood samples collected from the six subjects after both aspirin preparations.

It has been suggested that the delayed, prolonged release achievable with enteric-coated aspirin formulations allows extensive, even complete first-pass de-acetylation of aspirin [10]. Our data indicate that ingestion of compressed aspirin may also result in complete first-pass hydrolysis of the drug.

Inhibition of serum TxB_2 generation in the absence of measurable circulating aspirin might result from acetylation of platelet cyclo-oxygenase by exposure to the drug in the portal circulation [9, 12]. Inhibiting platelet function in the portal circulation but sparing cyclo-oxygenase in the peripheral vasculature could represent a pharmacological goal for solving the so-called "aspirin dilemma" [14, 15]. This dilemma emerged when it was shown that aspirin prevented the formation of two compounds with opposite biological effects—namely the aggregatory TxA_2 in platelets and the anti-aggregatory prostaglandin I_2 (PGI_2) in vascular cells. Simultaneous inhibition of TxA_2 and PGI_2 synthesis by aspirin is considered one possible reason for the deceptive results from clinical trials on the anti-thrombotic effect of ASA [14]. The use of formulations that deliver little or no aspirin to the peripheral circulation might be an effective means to avoid inhibition of vascular PGI_2 synthesis [12].

Possibly the current dispute [14–18] on the difference in sensitivity to low doses of aspirin between platelet and vascular cyclo-oxygenase arises from the wide inter-individual variability of aspirin pharmacokinetics. Indeed, as occurred in our experiments, the same dose or formulation may or may not deliver aspirin to the systemic circulation; the inhibitory effect on platelet cyclo-oxygenase would be the same but vascular PGI_2 synthesis would only be spared in the absence of detectable peripheral aspirin levels.

Since salicylate was detectable in all instances in the peripheral circulation, its accumulation during repeated aspirin administration might prevent the inhibitory effect on platelet vascular cyclo-oxygenase of aspirin escaping hydrolysis within the portal circulation [19, 20]. It has in fact been shown that salicylate can prevent the inhibitory effect of aspirin on platelet cyclo-oxygenase activity in man [21].

In conclusion, after oral administration of a compressed (320 mg) or an enteric-coated (800 mg) formulation of aspirin to six healthy volunteers platelet thromboxane generation was inhibited irrespective of whether peripheral plasma levels of the drug could be detected or not. *In vitro* experiments showed that concentration of aspirin 100 times higher than the detection limit of the HPLC assay method used were required to inhibit thromboxane generation by more than 80%. The current debate on "low" or "high" dose aspirin for thrombosis prevention should take into account the possibility that some formulations of aspirin may deliver no intact drug to the systemic circulation (thus sparing vascular prostacyclin) while acetylating circulating platelet within the entero-hepatic district.

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Table 1. Area under the curve of aspirin and salicylate plasma levels versus time and TxB_2 generation after oral aspirin

Preparation of aspirin (oral dose)	N	(AUC 0→∞) (nmol·ml^{-1}·hr) Aspirin	Salicylate	TxB_2 (% of control)
Compressed	3	17.3 ± 1.4	544 ± 42	2.1 ± 1.7
(320 mg)	3	n.d.	830 ± 40	1.8 ± 0.6
Enteric-coated	4	31.9 ± 3.3	2680 ± 153	0.4 ± 0.2
(800 mg)	2	n.d.	2190 – 2851	0.5 – 0.4

Plasma levels were fitted to a one compartment open model by non-linear regression analysis (NL-FIT) [24]. Serum was obtained by incubating native whole blood collected 1 hr after aspirin at 37° for 30 min [25, 26] and immunoreactive TxB_2 was measured by a specific radioimmunoassay [27]. The antibodies against TxB_2 were kindly supplied by J. B. Smith (Cardeza Foundation, Philadelphia, PA, U.S.A.).

AUC, area under the curve.

TxB_2, Thromboxane B_2 (control values = 655 ± 80 pmole/ml, N = 12).

n.d., not detectable.

Figures are means ± S.E.M.

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Paraquat toxicity is enhanced by iron and reduced by desferrioxamine in laboratory mice

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Desferrioxamine, a specific iron chelator [1, 2] used clinically to treat cases of iron overload [3, 4], successfully increased the survival of laboratory mice poisoned by paraquat. On the other hand, loading the mice with iron prior to the poisoning led to a severe reduction in their life span. We therefore conclude that iron plays a major role in paraquat toxicity.

Paraquat (methyl viologen; 1,1′-dimethyl-4,4′-bipyridinium (cation), di-chloride; PQ) is a widely used herbicide [5, 6]. Since the early sixties, when its use spread all over the world, hundreds of cases of death from paraquat poisoning have been reported [7–9]. This has prompted extensive research using animal studies and other models, both *in vivo* and *in vitro*. Using bacterial systems, it has been shown that the manifestation of paraquat toxicity requires molecular oxygen and a readily metabolizable electron source [10, 11]. However, despite voluminous research, the exact mechanisms of paraquat toxicity in mammals is still not fully understood [12]. It is known that paraquat causes its toxicity through a pulmonary injury [13], regardless of the method of contact [14]. Clinical manifestations of paraquat toxicity include edema (higher water content) of the lungs [15], fibrosis of the lungs, and change in activity of lung enzymes [16], all leading to mortality.

Metal chelators such as desferrioxamine have been shown to reduce and even prevent paraquat toxicity in *E. coli* (R. Kohan and M. Chevion, submitted), where a catalytic amount of copper ion (1 μM) dramatically enhanced the rate of inactivation of the cells by paraquat. These findings are similar to results in other systems, indicating the protective effect of chelators against biological damage induced by redox cycling compounds such as streptonigrin [17, 18, 10], bleomycin [20] and ascorbate [21]. Consequently, we studied the effects of desferrioxamine on paraquat poisoned mice.

Three parameters were studied: survival; activity of an enzyme in the lungs; water content of the lungs in paraquat intoxicated mice.

Male mice (Balb/C; 30 g in weight) were used. All the injections were given intraperitoneally (i.p.). Each experiment was repeated three times. The reported results are an average of the replications.

In the survival experiment, four groups of mice were tested (10 mice in each group). Mice that received a single dose of paraquat (17 mg/kg) (Sigma) began to die 24 hr after the injection, 50% of the mice were dead by 63 hr after the injection (Fig. 1), and no survivors were left by the fourth day (Fig. 1). In contrast, mice that received the repeated injections of desferrioxamine (5 mg/inj) (Desferal, Ciba–Geigy) once 24 hr prior to, and twice daily, for two days following the same dose of paraquat, began to die 40 hr after injection, 50% of these mice were dead only 90 hr after injection, and 20–30% of the mice survived for at least three more months (Fig. 1). Thus, desferrioxamine therapy significantly increased the life span of poisoned mice. Two other groups were used as controls; one group was given only desferrioxamine twice daily for five days, and the second group received injections of water only; both control groups survived.