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The Inhibitory Effect of Human Plasma Albumin and Haptoglobin on Platelet Aggregation and 5-HT Release

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Abstract: Platelet aggregation contributes to the pathogenesis of cardiovascular diseases. After activation it leads to dense granule secretion and 5-HT release. The question arises; how platelet aggregation is endogenously controlled during blood circulation. In preliminary studies, we observed that human platelets aggregate more rapidly when suspended in buffer as compared to those suspended in plasma (PRP). These observations point to the presence of an endogenous substance that may inhibit arachidonic acid–induced platelet aggregation. An analysis of plasma Cohn fractions demonstrated that most of the plasma inhibitory activity was associated with albumin–rich and α-globulin rich protein fractions. The identity of plasma endogenous inhibitors of platelet aggregation (EIPA) was established by affinity chromatography on Cibacron Blue F3G-A for specific removal of albumin. The association of α-globulins to EIPA activity was recognized as due to haptoglobin by affinity chromatography on a column of hemoglobin-sepharose. In addition, we also found that the distribution of EIPA activity varies according to sex and physiological state. These findings reveal that EIPA may act by modulation of arachidonic acid metabolism or sequestering the fatty acid substrate.

Keywords: human plasma; endogenous inhibitor of platelet aggregation (EIPA); arachidonic acid metabolism; affinity chromatography; albumin; haptoglobin.

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Introduction

Platelets are prime modulators of haemostasis. They play a fundamental role in preventing hemorrhage through their ability to adhere to a damaged vessel wall and to aggregate into large clumps in response to a number of stimuli resulting in shape change and dense granule secretion [1]. There are several possible biochemical and physiological pathways of platelet aggregation [2]. The three most widely studied pathways are the release of adenosine-5-phosphate (ADP), liberation and metabolism of arachidonic acid (AA) and platelet-activating factor (PAF) [3]. In platelets, ADP is an autocrine agonist and is stored in the form of granules. Upon platelet stimulation via ADP, PAF and TXA2 the stored granules fuse with the platelet membrane at different points and release alpha and dense granules via exocytosis. This induces platelet shape change, secretion and amplifying platelet aggregation [4]. Therefore, hyperactivity of ADP and platelet aggregation has been associated in the pathogenesis of thrombosis and stroke [5].

AA metabolism by way of prostaglandin (PG) endoperoxide synthase pathway in platelets leads to the generation of PG endoperoxides (PGG2, PGH2) and thromboxane-A2 (TXA2) which are strongly pro-aggregatory of platelets [6]. TXA2 has been thought to contribute to the development of stroke, myocardial infarction and angina pectoris [7]. The balance between pro-aggregatory TXA2 produced by platelets and anti-aggregatory prostacyclin (PGI2) produced by blood vessel wall may be an important factor in controlling hemostasis and thrombosis [8].

PAF is a unique phospholipid which is known to carry out a wide variety of action which include the ability to aggregate human platelets at very low concentrations and enhance pulmonary and vascular permeability [9]. Because of these important actions, PAF has been considered an important mediator of thrombosis and shock [10].

Aspirin, an inhibitor of PG synthesis is known to inhibit platelet aggregation [11] and its use as an anti-thrombotic drug has been investigated. [12, 13]. Therefore, the effect of PG synthesis inhibitors on synthesis of TXA2 and PGI2 is of considerable significance. The question arises, how the production of PG endoperoxides and thromboxane is regulated during circulation?

To answer this question, we investigated the effect of plasma on AA induced platelet aggregation. The results of this study indicated that human plasma or serum contains one or more circulating endogenous inhibitors of platelet aggregation named EIPA. The objective of the present investigation was to determine the identity and possible functions of EIPA associated with the inhibition of platelet aggregation and release reaction.

Material and Methods

The following materials were used from Sigma Aldrich, plasma Cohn fractions, arachidonic acid (AA) (Type 1, 99% pure), pronase, plasma haptoglobin (≥95% pure). Plasma albumin levels were determined using the kit supplied by Bio-Rad, USA. Platelet counts were performed by phase contrast microscopy (Leica microsystem). Solutions of arachidonic acid were prepared by mixing a 10 mg ampoule in 20 µl ethanol and diluting with 730 µl of 0.2% w/v sodium carbonate in water. All other drugs and chemicals used were obtained from Tocris and were of the highest purity grade available.

Platelet aggregation

Blood was drawn from human volunteers after written and informed consent. All procedures with volunteers were performed according to local policy and all needed agreements were obtained. The human volunteers were free of any aspirin-like drugs for at least 7 days. Blood was gently mixed with heparin in siliconized glass tubes to give final heparin concentration of 10 units/ml. The blood was centrifuged at 200 g for 10 minutes at 20°C, giving a supernatant platelet-rich plasma (PRP). An aliquot of PRP was taken for testing, and the remainder was centrifuged at 1200 g for 15 minutes. The resulting platelet pellet was resuspended in half the original plasma volume of calcium-free Krebs solution containing NaCI (118 mM), KCI (4 mM), NaHCO3 (25 mM), KH2PO4 (1 mM), MgSO4 (1 mM) and glucose (10 mM). Aggregation was monitored with Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using aliquots (0.45 ml) of PRP incubated at 37°C for 1 minute before treatment with the aggregating agent. The resulting aggregation was recorded by Lumi-aggregometer and expressed as percent inhibition as shown in Fig. 1A. The results obtained using washed platelet suspension in buffer at 4minutes after treatment are shown in Fig. 1B. In further experiments, effect of human plasma on AA-induced platelet aggregation using washed platelet suspension at two doses (1.4 & 2.8% V/V) is shown in Fig. 2A. Washed platelets suspended in buffer were used to test Cohn fractions and the commonly used plasma expanders dextran T40 and T70 as shown in Fig. 2B where fibrinogen (14.3 mg/ml) and dextran T40 and T70 had no effect. Significance level between different human plasma samples were determined by Student's T-test.

5-HT release from platelets

The method of platelet aggregation and 5-HT release was adapted from that described previously by Heptinstall *et al.* (1985) [14] and Groenewegen, Heptinstall (1990) [15]. Blood platelets have a rapid and active transport system for 5-HT, and they can be

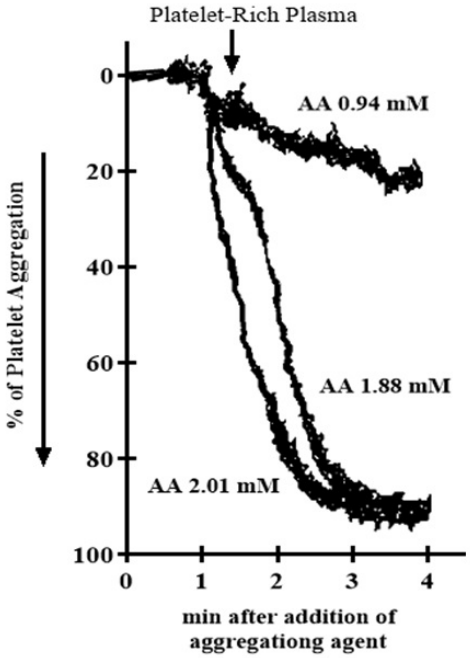

Fig. 1A. Effect of arachidonic acid (AA) on human platelet rich-plasma.

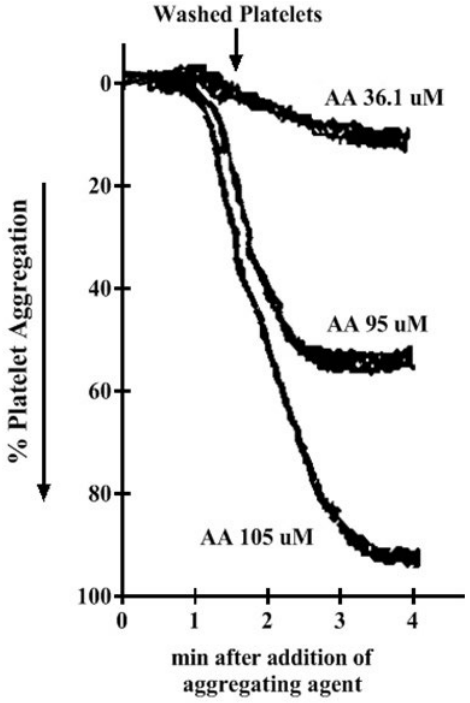

Fig. 1B. Effect of arachidonic acid (AA) on washed platelets suspended in buffer.

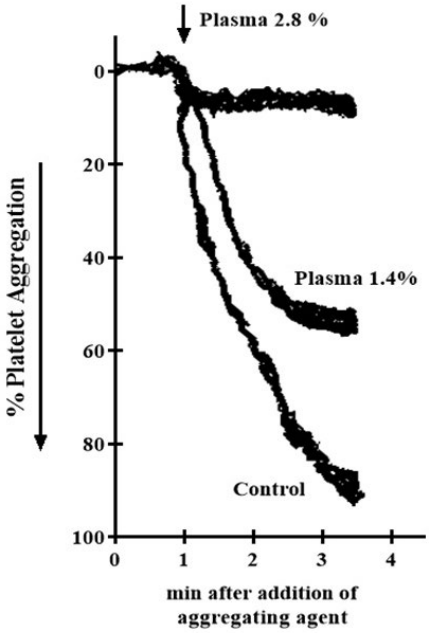

Fig. 2A. Effect of human plasma on AA-induced platelet aggregation in washed platelet suspension.

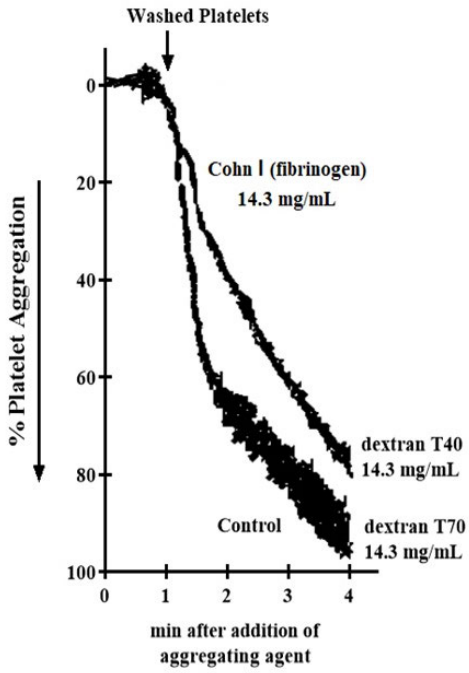

Fig. 2B. Effect of human fibrinogen (Cohn I) and dextran T40 and T70 on arachidonic acid induced aggregation of human washed platelets.

readily loaded with [14C] 5-HT (Nishio *et al.*, 1995) [16]. 10 ml of PRP and washed platelets were suspended in buffer, incubated with 10 ˜l of 14C-5HT (CPM = 18×10^5) for 30 min at 37°C. Non incorporated 14C-5-HT was removed by washing the platelets twice with Krebs buffer and resuspended in the same buffer containing imipramine (1×10^6 M) to prevent the reuptake of 5-HT during aggregation reaction. In order to determine the time course of uptake, samples (200 ˜l) were taken at 5 min interval, centrifuged and radioactivity determined both in the supernatant and platelets resuspended in fresh Krebs buffer. Since no difference was observed in 5-HT uptake between platelets in plasma and platelets in buffer in subsequent experiments PRP was used.

Platelet activation occurs in two stages following the addition of aggregating agent such as AA. After activation by AA, the primary phase of platelet activation is characterized by platelet shape change and platelet aggregation. This is followed by the secondary phase, which includes release of the dense granule contents such as 5-HT, AA; that potentiates the aggregation further (Puri and Colman, 1997) [17] (Fig. 3). In this study, platelet aggregation and 5-HT release were measured simultaneously, monitored after activation with AA.

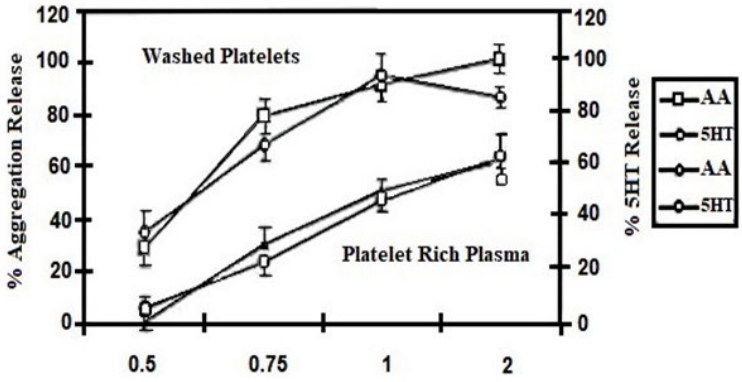

Fig. 3. Concentration-dependent activation by AA of platelet rich plasma and washed platelet suspension with corresponding value of 5-HT release.
Washed platelets were pre-labelled with [^{14}C] – 5HT and resuspended in their own PPP containing imiprmine to prevent reuptake. Reaction was stopped after 4 min by centrifugation and radioactivity released to the supernatant was determined. Results were expressed as mean % aggregation and 5HT release for 3–4 × 108 ml-1 platelets and as % of total platelet activity. To measure the total platelet [+14+C] – 5HT, 100 µL sample of the whole pre-labelled suspension was counted without centrifuged (content = 13473 ± 1897 cpm, n = 5).

In our preliminary experiments we found that EIPA activity is associated with plasma proteins (°-globulin and albumin). In addition to this, it was also observed that EIPA is non-dialyzable and labile to heat for 10 min at 98°C or to incubation at 37°C for 30 min, indicating that EIPA is either proteinaceous or closely related with plasma proteins. A study of various Cohn fractions of human plasma showed that

several had little or no EIPA activity. However, activity was high in Cohn IV, especially IV-4 (haptoglobin) and in Cohn V which is pure albumin. The results are summarized in Table 1.

Table 1. Effect of plasma Cohn fractions on washed platelets suspended in buffer.

Test material human	Concentration (%V/V)	Mean percent inhibition ± S.E.M.
Plasma	0.3	36 ± 4
	1.4	69 ± 4
	2.8	89 ± 6
Serum	0.3	12 ± 2
	1.4	65 ± 4
	2.8	80 ± 10
Fibrinogen (Cohn I)	14.3*	12 ± 11
γ-globulin (Cohn II)	14.3*	14 ± 5
-globulin (Cohn III)	14.3*	25 ± 4
-globulin (Cohn IV-4)	1.2*	53 ± 2.5
	2.8*	85 ± 2
Albumin	0.29*	24 ± 7
(Cohn V)	0.71*	63 ± 9
	1.42*	92 ± 4
Dextran T40	14.3*	8 ± 5
Dextran T70	14.3*	15 ± 3

*Concentration expressed in mg/ml. Results are mean ± S.E.M. of 4–6 determinations. Aggregation in test suspension was compared with that in control, 4 min. after treatment with a 95 μM arachidonic acid. Purified Cohn fractions were obtained from Sigma, USA.

Identification of EIPA by affinity chromatography

Removal of haptoglobin

We found that pure haptoglobin inhibited platelet aggregation and that this activity of haptoglobin was completely abolished by heamoglobin. We, therefore, prepared and measured EIPA activity in albumin and haptoglobin-depleted plasma in an effort to analyse the plasma EIPA activity (Table 2). Haptoglobin was removed from human plasma by affinity chromatography on Sepharose to which human haemoglobin was linked using the method of David and Liang (1973) [18, 19], with slight modifications as: 15 g of activated CH (6-aminohexanoic acid)-Sepharose 4B was swollen in 1 mM-HCl for 5 min, then washed with 3 litres of the same solution over a coarse sinter and finally rinsed with 500 ml of 0.1M Sodium bicarbonate. The resulting slurry was

Table 2. Inhibitory effects of normal human plasma on haptoglobin depleted and albumin depleted plasma or both constituents on aggregation using washed platelets suspension.

Serum	[Haptoglobin] (mg of protein/ml)	[Albumin] (mg of protein/ml)	Relative [protein] (%)	Relative EIPA Activity (%)
Whole Plasma	1.20	21.34	100	100
Haptoglobin-depleted	<0.03	20.56	91.3	67
Albumin-depleted	0.85	0.88	46.4	15
Haptoglobin- and albumin-depleted	<0.03	0.18	46.4	4

treated with 75 ml of fresh haemolysate prepared from 75 ml of blood. This haemoglobin-Sepharose preparation was incubated by inversion with 45 ml of human plasma containing approx. 100 mg of haptoglobin. After 30 min at room temperature (21°C), the suspension was filtered on a Buchner funnel and the filtrate dialysed against several changes of 0.5% NaCl and finally concentrated by freeze-drying.

This process removed 98% of haptoglobin from plasma. Haptoglobin was determined by the peroxidase method of Connell and Smithies in 1959 [20], and protein was estimated by the method of Lowry in 1951 [21].

Removal of albumin

Albumin was removed from normal or haptoglobin-depleted plasma by affinity chromatography on Cibacron Blue F-3-GA conjugated with Sepharose by the method of Travis *et al.* (1978) [22]. For the present experiments, we applied 480 mg of protein to a 20.0 cm × 2.5 cm column filled with Cibacron Blue-Sepharose and eluted with the standard eluent, 0.05M-Tris/HCl buffer, pH 8.0, containing 0.05 M NaCl at a flow rate of 50 ml/h. The passage of plasma through this column removed over 95% of albumin.

The absence of both haptoglobin and albumin from the depleted plasma was confirmed by single radial immunodiffusion in agar gel (LC-Partigen plates obtained from Hoechst) containing specific antibodies [23, 24].

Results and Discussion

Arachidonic acid (AA) has been shown to produce aggregation of human platelets suspended in plasma. In this study we found that AA is several fold more potent in inducing aggregation of human platelets suspended in buffer than in plasma (PRP) (Fig. 1A). Maximal aggregation in PRP was obtained with 1.8 mM AA whereas maximal aggregation of washed platelets suspended in buffer was obtained with 0.19 mM AA. This suggests that plasma inhibits the action of AA on platelets.

Since PG endoperoxides and TXA2 are known to induce aggregation [25], the inhibitory effect of AA may indicate that plasma is probably inhibiting platelet PG endoperoxide synthase and TXA2 formation. Therefore, for further experiments, washed platelets suspended in buffer were used to test Cohn fractions and the commonly used plasma expanders dextran T40 and T70 [26]. Fig. 2A shows a tracing from a typical experiment where human plasma inhibited AA-induced platelet aggregation in a concentration related manner. Plasma Cohn fractions V (albumin) (Fig. 4A) and IV (°-globulins) (Fig. 4B) have inhibited platelet aggregation in a concentration-related manner. Cohn I (fibrinogen), Cohn II, Cohn III (¸-globulin) and dextran T40 and T70 were ineffective. Complete results are summarized in Table 1. The present findings indicate that dextran and fibrinogen have no effect on platelet aggregation.

These results demonstrate an important finding that human blood plasma or serum potently inhibits AA-induced platelet aggregation. We conclude that human blood plasma or serum contains endogenous factors that inhibit platelet aggregation (EIPA). The mechanism by which EIPA inhibits AA induced aggregation is not fully understood. It is known that platelet aggregation can also be caused by other physiological substances such as AA, ADP and phosphalipase-A2. These prostanoids and TXA2 are formed during blood clotting [27] and platelet aggregation induced by AA, ADP, and collagen and phospholipase A2 is greatly enhanced by AA that by itself does not cause aggregation.

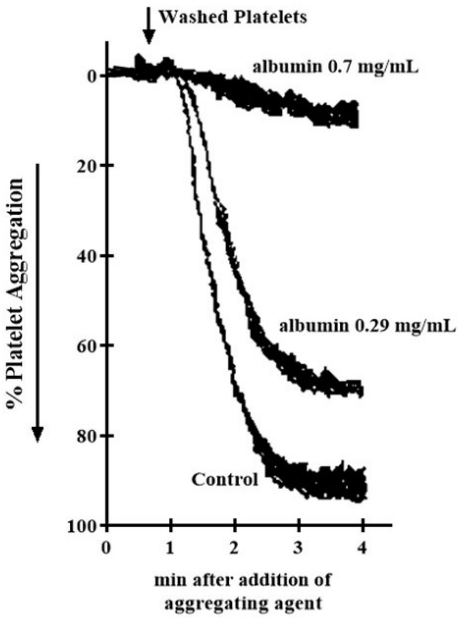

Fig. 4A. Inhibition of arachidonic acid induced platelet aggregation of human washed platelets by plasma albumin (Cohn V).

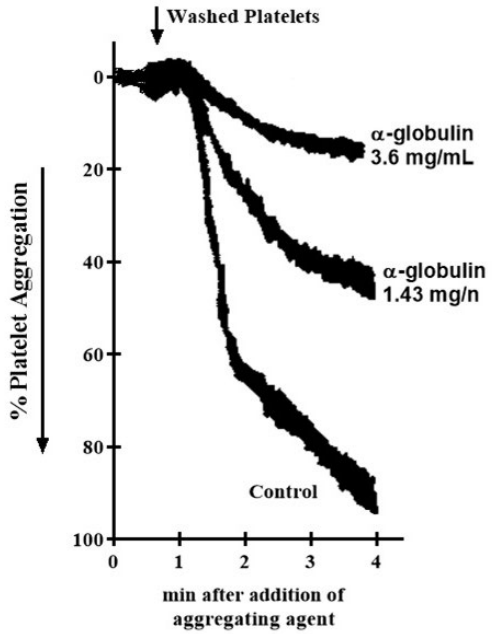

Fig. 4B. Inhibition of arachidonic acid induced platelet aggregation of human washed platelets by alpha globulins (Cohn IV-4).

Inhibitors of prostaglandin synthesis, such as aspirin inhibit platelet aggregation in response to collagen or epinephrine or AA [28]. Esterified AA is present in phospholipids, cholesterol esters and triglycerides of blood and other tissues [29]. Non-esterified AA is known to be bound to plasma albumin [30], a part of the plasma EIPA activity, and therefore, is an important factor in controlling haemostasis. Since anti-aggregatory effect of albumin has a ceiling aggregation due to massive liberation of AA, as might occur (in thrombosis) with damage to the vessel wall, would be unimpaired [31].

Human plasma albumin is the most abundant protein in the vascular compartment where it represents 60% of the total in plasma. This globular protein of 66 kDa consists of non-glycosylated single chain polypeptide containing 585 amino acids. Albumin also serves antioxidant functions in vascular compartment. It shows inverse relationship between concentration of plasma albumin and total mortality. Plasma albumin has also cardioprotective role where low plasma albumin level is associated with cardiovascular diseases such as myocardial infarction, atherosclerosis and thrombosis. Albumin decreases the platelet aggregation, has antithrombotic effect and acts as a primary anti-inflammatory constituent of plasma. An acute increase in tissue albumin exerts beneficial effects, for example, by binding metal ions and providing antioxidant protection. It inhibits endothelial apoptosis, modulates arachidonic acid release and membrane fluidity, affects cellular redox signaling and protects against myocardial and neuronal injury from ischemia and reperfusion [32–36].

On the other hand, haptoglobin (Hp), the other component of EIPA is present in normal plasma at a concentration of approximately 1 mg/ml [37]. It has been proposed that the function of Hp is concerned with the prevention of loss of iron through the kidney [38]. Hp possesses significant cathepsin B inhibitory activity [39] and also inhibits the hemagglutinating ability of influenza virus for chicken erythrocytes [40]. The results of these and subsequent studies cited in this report suggest yet another function for haptoglobin, i.e., endogenous inhibition of platelet aggregation and 5-HT release [41]. The plasma concentration of Hp increases several fold in the event of an inflammatory stimulus such as infection.

In human populations three common phenotypes are present i.e., Hp1-1, Hp2-2 and the heterozygous Hp2-1 [42] which is determined by two alleles HP1 and HP2 (Smithies and Walker, 1955) [43]. The HP2 allele contains a partial duplication of approximately 1.7 kb from an unequal crossing over event between HP1 alleles (Maeda *et al.*, 1984) [44]. The Hp gene transcript yields α and β protein subunits interconnected by disulfide bridges after post-translational processing. Inherited variations in the α-subunit give rise to the EIPA activity of various plasma samples was concentration dependent.

The IC50 values of all four groups and albumin contents are given in Table 3. It is shown that EIPA activity in plasma, derived from adult females and males, was significantly ($p < 0.05$) higher than in plasma from pregnant females and foetal umbilical cord (Table 3). These results demonstrate significant differences between the EIPA activities according to sex and physiological state. The greater potency for inhibition of AA induced platelet aggregation displayed by plasma from non-pregnant women when compared with men or pregnant women provides source for some speculation. Doll *et al.* [45] have recently shown that among non-smokers, men are six times more likely to suffer a thrombotic episode. Such a causal relationship is unlikely but we would like to speculate that EIPA may contribute to the development or outcome of these events. The relatively poor ability of umbilical cord plasma to inhibit AA-induced platelet aggregation when compared with adult plasma is suggestive that the endogenous inhibitor(s) of platelet aggregation (EIPA) do not cross the placenta from mother to the foetus to any significant extent. The significance of the

Table 3. Inhibition of arachidonic acid-induced platelet aggregation (EIPA) by various human plasmas.

Subject	EIPA activity (IC_{50} 50% v/v)		
A. Males	0.03 ± 0.03	(12)	$p < 0.05$ vs B
B. Females	0.02 ± 0.01	(13)	$p < 0.05$ vs C
C. Pregnant females	0.44 ± 0.06	(11)	$p < 0.05$ vs A
D. Foetal/umbilical cord	0.57 ± 0.03	(6)	$p < 0.001$ vs A

low EIPA activity in cord plasma is uncertain. This finding, however, is consistent with the high level of plasma AA metabolites such as prostaglandin (PG) E and F that exist in healthy preterm infants as reported by Mitchell *et al.* [46]. Further, these two PG's do not induce aggregation of human platelets.

Regarding to the mode of action of EIPA, it is evident that Hp inhibits COX. Deby *et al.* [47] found a direct correlation between Hp levels and response to arachidonic acid induced hypotension in rabbits. Previously, we reported [48, 49] that foetal calf plasma, which does not contain Hp, was completely devoid of COX inhibitory activity. This and other evidences, where glucocorticoids increase Hp levels and COX inhibitory activity [50] to similar extent with comparable time courses, provide further support that Hp inhibits COX.

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Conflict of interest

None declared.

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