Inhibitory effect of a selective thromboxane A₂ receptor antagonist, EP 092, on platelet aggregation in whole blood *ex vivo* and *in vivo*

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1 The inhibitory effect of a selective prostaglandin H₂ (PGH₂)/thromboxane A₂ receptor antagonist, EP 092, on platelet aggregatory responses in whole blood *ex vivo* (guinea-pig: Rhesus monkey) and intravascular aggregation *in vivo* (rabbit) has been investigated.

2 Collagen (0.1–10.0 µg ml⁻¹) caused a concentration-dependent decrease in single platelet count in samples of both guinea-pig and Rhesus monkey citrated whole blood incubated *ex vivo*. EP 092 administered to guinea-pigs by intravenous (0.1–3.0 mg kg⁻¹) or oral (1.0–10.0 mg kg⁻¹) routes significantly inhibited the platelet responses to collagen (ED₅₀ values 1.3 ± 0.2 and 1.4 ± 0.2 mg kg⁻¹ respectively). Similar potency against collagen-induced whole blood aggregation was observed in Rhesus monkey blood samples following EP 092 given orally (ED₅₀ 0.9 ± 0.3 mg kg⁻¹).

3 The duration of action of EP 092 against collagen aggregatory responses *ex vivo* in both guinea-pigs and Rhesus monkeys was between 3 and 6 h following oral administration at 3.0 mg kg⁻¹.

4 The inhibitory activity demonstrated by EP 092 against collagen-induced aggregation of Rhesus monkey whole blood *ex vivo* was not accompanied by any significant reduction in thromboxane A₂ formation except at the highest dose tested (10 mg kg⁻¹).

5 The intravascular aggregatory response induced by collagen or thrombin in the anaesthetized rabbit was significantly inhibited by an intravenous infusion of EP 092 (10 mg kg⁻¹). EP 092 appeared less potent and its effect was of shorter duration in this preparation compared with its inhibitory effect on *ex vivo* aggregation, being evident immediately after infusion of drug but not after a further 30 min.

6 It is concluded that collagen-induced platelet aggregatory responses in guinea-pig and Rhesus monkey whole blood *ex vivo* and rabbit *in vivo* exhibit a thromboxane-dependent component which can be inhibited in a dose-related fashion by pretreatment with the thromboxane antagonist EP 092. In the rabbit, moreover, the data support the possibility of a role for thromboxane in the intravascular aggregatory response to thrombin.

Introduction

The potent platelet aggregatory activity of the cyclic endoperoxide prostaglandin H₂ (PGH₂; Hamberg & Samuelsson, 1974) and its product thromboxane A₂ (TXA₂) formed by the enzyme thromboxane synthase (Svensson *et al.*, 1976) has been recognised for

more than a decade. Despite this, it is only relatively recently that the structure of TXA₂ has been unequivocally confirmed by total synthesis (Bhagwat *et al.*, 1985a), enabling the pharmacology of pure material to be finally described (Bhagwat *et al.*, 1985b; Bunting *et al.*, 1987). Recent evidence strongly suggests that TXA₂, PGH₂ and PGH₂-mimetics each bind with varying affinity to the same platelet receptor population (Armstrong *et al.*, 1983; Halushka *et al.*, 1987), and that TXA₂ is an order of magnitude more potent than PGH₂ at inducing aggregation of human platelet-rich plasma (PRP) *in vitro* (Bhagwat *et al.*, 1985b). Consequently,

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inhibition of the aggregatory activity of both potent endogenous arachidonic acid pathway products might be achieved by a single agent via selective blockade of the platelet PGH₂/TXA₂ receptor. This strategy has been widely viewed as therapeutically attractive, and the use of platelet radioligand binding assays in conjunction with aggregation experiments in washed platelet suspensions or PRP has been instrumental in the identification of several putative antagonists (Cross & Dickinson, 1987).

One such compound, EP 092 ((±)-5-endo-(6'-carboxyhex-2'-Z-enyl)-6-exo-[1'-[N-(phenylthiocarbonyl)-hydrazono]-ethyl]-bicyclo[2,2,1]-heptane), was initially identified amongst members of a chemical series first synthesized by Wilson *et al.*, (1982) and evaluated by Wilson & Jones (1985). The compound is a potent inhibitor of [³H]-15(S)-9,11-epoxymethano-PGH₂ ([³H]-U44069) binding to washed human platelets, and of aggregatory responses induced by TXA₂, PGH₂, or stable PGH₂-mimetics including 11,9-epoxymethano-PGH₂ and 9,11-azo-PGH₂ in diluted PRP or plasma-free platelet suspensions (Armstrong *et al.*, 1985a). EP 092 also causes a parallel shift to the right of log concentration-effect curves obtained in response to 15(S)-11,9-epoxymethano-PGH₂ (U46619) on isolated rabbit aorta, dog saphenous vein and guinea-pig trachea smooth muscle preparations *in vitro* (Jones, 1984).

In vivo, partial inhibition of U46619 and collagen-induced thrombocytopenia associated with elevated pulmonary inflation pressure in the guinea-pig has been noted (Jones *et al.*, 1983). Furthermore, administration of EP 092 prevents the marked rise in pulmonary artery pressure, the fall in cardiac output, and the respiratory distress which follows intravenous injection of *E. coli* endotoxin in the anaesthetized sheep (Armstrong *et al.*, 1985b). However, the inhibitory activity of this compound against platelet aggregatory responses in whole blood, rather than in PRP or washed cell suspensions, and the potency and duration of any such activity observed following parenteral or oral dosing to experimental animals, remains to be firmly established.

In the present study, we chose to investigate the ability of EP 092 to inhibit collagen-induced whole blood platelet aggregatory responses *ex vivo* using a single platelet counting technique (Lad *et al.*, 1987) in citrated blood samples obtained from guinea-pigs or Rhesus monkeys pretreated with EP 092. These experiments prompted an examination of the effect of EP 092 on collagen and thrombin-induced intravascular aggregation in the anaesthetized rabbit by continuous platelet count monitoring *in vivo* (Honey *et al.*, 1986). A preliminary account of these experiments has been given to the British Pharmacological Society (Lad *et al.*, 1988).

Methods

Collection of blood

EP 092 was administered to male Dunkin Hartley guinea-pigs (350–500 g) by either bolus intravenous injection (0.1–3.0 mg kg⁻¹) in sodium bicarbonate buffer pH 8.5 or oral gavage (1.0–10.0 mg kg⁻¹) as a PEG 400/2% carboxymethylcellulose (CMC) aqueous suspension (1:5 v/v). Treatment groups (*n* ≥ 5 animals in all cases) were bled at various time intervals after dosing as detailed in the text. Vehicle controls were included for both dosing routes and all time points, to allow comparison with drug-treated guinea-pigs. For blood collection, the guinea-pigs were anaesthetized with pentobarbitone sodium (Sagatal) by intraperitoneal injection. Blood samples (5.0 ml) were obtained from the abdominal aorta via a butterfly cannula, and drawn gently into a syringe containing 3.1% trisodium citrate anticoagulant at a final citrate: blood ratio of 1:9 v/v.

Male Rhesus monkeys (*Macaca mulatta*; 11–16 kg) were dosed by oral gavage with EP 092 (0.3–10.0 mg kg⁻¹) in PEG 400/2% CMC vehicle. Prior to collection of blood, each animal was lightly sedated with intramuscular ketamine. Blood samples were taken at the times specified in the text by venepuncture into a syringe containing 3.1% trisodium citrate anticoagulant as described above. A group of six animals were used to complete the study, with any one animal being dosed and bled on up to three occasions at one month intervals.

The values obtained for baseline citrated whole blood platelet count expressed as the mean (95% confidence limits) × 10⁶ μl⁻¹ were: guinea-pig 502 (270–734; *n* = 67) and Rhesus monkey 269 (102–436; *n* = 23). In any experiment where the correct volume of blood with respect to anticoagulant was not obtained, visible haemolysis was present, or a low baseline platelet count outside the normal range was recorded, the blood sample was discarded.

Platelet aggregation in whole blood

The method employed for monitoring platelet aggregatory responses in guinea-pig and Rhesus monkey whole blood was a modification of the single platelet counting technique first described by Nunn & White (1980) which we have described previously (Lad *et al.*, 1987). Briefly, each 0.5 ml aliquot of citrated blood was stirred in a 1 ml aggregation cuvette at 1000 r.p.m. and equilibrated to 37°C for 2 min before addition of either collagen at final concentrations in the range 0.1–3.0 μg ml⁻¹ or buffer alone as control (dose volume always 5.0 μl). Sequential 50 μl samples of blood were removed from each incubation immediately prior to, and at 1 min intervals up to 4 min following the addition of the aggregatory stimulus.

These samples were transferred to 5 ml centrifugation tubes containing 950 μ l of counting diluent (Coulter Isoton II), mixed, and immediately centrifuged (2 min at 150 *g*) in a Coulter Electronics Thrombofuge to obtain a dilute (1:19 v/v) platelet supernatant. This supernatant was then diluted a second time, 66 μ l being taken and added to 20 ml Isoton II diluent (1:303 v/v) with thorough roller mixing, before being counted in a Coulter Thrombo-counter (each sample was counted twice and the mean value used in subsequent data analysis).

The original blood platelet count in the aggregation cuvette at the time of sampling was derived by correction for the overall dilution factor in processing ($\times 6080$). Agonist concentrations and responses to collagen obtained in whole blood have not been corrected for haematocrit. All samples were diluted, processed and counted immediately. Aggregation experiments were completed within 2 h of obtaining a blood sample.

The results of the blood platelet aggregation experiments are expressed in the text as overall decrease in single platelet count (mean% $\pm$ s.e.mean) either with time or at the end of the incubation (4 min after addition of agonist). Attenuation of the aggregatory response to collagen in those samples from EP 092 treated animals is expressed as inhibition (mean% $\pm$ s.e.mean) at 4 min, compared to decreases in single platelet count observed at an identical collagen concentration in blood samples from vehicle (control) dosed animals. Data have been analysed by Student's *t* test with $P < 0.05$ being taken as indicative of a significant result. All determinations were performed in duplicate for each blood sample and at each agonist concentration.

Intravascular platelet aggregation

The effect of EP 092 on collagen and thrombin-induced intravascular platelet aggregation *in vivo* was determined in male NZW rabbits (2.5–4.0 kg) anaesthetized with a combination of 5,5'-diallyl barbituric acid and urethane administered by intraperitoneal injection (Banerjee *et al.*, 1985). Each animal preparation was artificially ventilated with air via a tracheal cannula throughout the experiment using a Harvard respirator. Indwelling cannulae in the left femoral artery and vein were used to monitor arterial blood pressure via a Statham pressure transducer, and to administer collagen or thrombin. Finally, a specially designed double lumen indwelling cannula (Smith & Freuler, 1973) was inserted into the right external carotid artery, and was connected to a Technicon continuous flow platelet auto-counter.

Anticoagulant (3.2% trisodium citrate; 0.015 ml min⁻¹) was pumped through the outer

lumen of the tube to the tip of the cannula, which on mixing with carotid arterial blood at the cannula tip, was drawn out of the vessel via the inner cannula sleeve at a flow rate of 0.1 ml min⁻¹ into the auto-counter. Following a two stage treatment of the blood with a mixture of ammonium oxalate (1% w/v) and saponin (0.002% w/v) during which the erythrocytes were lysed and the blood diluted by a factor of 2880, the number of single platelets present was determined by continuous passage of the diluted platelet suspension through a flow cell focussed in a photo-multiplier tube particle counter. Platelet count was corrected for dilution and continuously recorded on chart paper which had been precalibrated with a standard of known cell count immediately prior to each experiment.

The platelet agonists collagen (30 μ g kg⁻¹) or thrombin (10 units kg⁻¹) were administered as bolus intravenous injections via the femoral vein indwelling cannula, with a minimum period of 30 min being allowed to elapse between successive agonist doses. The first intravenous dose of agonist served to (a) ensure all dosing and monitoring cannulae were patent, (b) assess the robustness of the preparation on challenge (particularly with respect to mean arterial blood pressure, a fall in which accompanies the platelet response (Lad *et al.*, 1986)), and (c) confirm sensitive detection of platelet count changes with acceptably low background noise on the auto-counter. However, this initial platelet response was frequently found to be inconsistent between preparations, and atypical of those obtained subsequently in any one preparation, being variously of greater or lesser magnitude. It was not clear why this should be so except in those cases when it reflected problems with (a)–(c) above, but consequently, the data were not included for analysis. Thereafter, each agonist elicited reproducible successive platelet responses in controls (see text) and was administered 30 min prior to, immediately following, and finally at 30 min after infusion of EP 092.

The results in the text are derived from the maximum fall in circulating platelet count achieved in response to a particular dose of collagen or thrombin, and are expressed either as a percentage decrease relative to the mean platelet count determined over the 3 min period immediately before injection of agonist, or as the percentage inhibition of the decrease in circulating single platelet count when EP 092 was present compared to pre-drug controls.

EP 092 (3 or 10 mg kg⁻¹) or vehicle alone (sodium bicarbonate buffer, pH 8.5) were administered intravenously to separate groups of rabbits over a 10 min infusion period (1.0 ml min⁻¹) via a lateral ear vein butterfly cannula. Responses obtained to either thrombin or collagen at 0, 30 and 60 min after drug

were analysed both by comparison with the pre-drug control response in the same animal (paired Student's *t* test), and with responses obtained in the vehicle alone treated group (unpaired Student's *t* test).

Estimation of thromboxane formation

The effect of EP 092 on the formation of thromboxane A_2 (TXA₂) which accompanies collagen-induced platelet aggregation in citrated whole blood *ex vivo* was investigated in Rhesus monkey samples. Each aggregation incubation was terminated at 4 min after addition of collagen as stated above, by addition of indomethacin (10 μ M) and rapid cooling by immersion in an ice bath. The incubation mixture was transferred to an Eppendorf 1 ml capped tube, centrifuged (1 min, 10000 *g*), the plasma removed, and stored frozen (-20°C) until assay. Estimation of plasma TXA₂ formation was carried out by use of a specific radioimmunoassay procedure for TXB₂, the stable breakdown product of TXA₂. The radioimmunoassay contained commercially available [³H]-TXB₂ (150 Ci mmol⁻¹; Amersham International plc) and rabbit antibody to TXB₂ (Calbiochem-Behring, California). The presence of EP 092 in plasma samples did not interfere with measurement of TXB₂ in the radioimmunoassay. Cross reaction with the antibody was found to be <0.01% at final EP 092 concentrations in the assay between 3.2 and 405 μ M. The data shown in the text are expressed as specific collagen-induced increases in plasma TXB₂ (mean $\pm$ s.d.) derived by subtraction of basal (buffer incubated) control values.

Chemicals

Agents used in these studies were purchased from the following: collagen (Hormon-Chemie, Munich, F.R.G.), thrombin (Parke-Davis freeze dried material), urethane and 5,5'-diallylbarbituric acid (Sigma Chem. Co.). EP 092, ($\pm$)-5-endo-(6'-carboxyhex-2'-z'eryl)-6-exo-1'-[N-(phenyl thio carbamoyl)-hydrazono]-ethyl-bicyclo-(2,2,1)-heptane), was a generous gift from Drs R.L. Jones and N.H. Wilson (Department of Pharmacology, University of Edinburgh).

Results

Aggregatory responses to collagen in citrated whole blood

The collagen-induced platelet aggregatory response observed in samples of guinea-pig and Rhesus monkey citrated whole blood obtained from control animals is shown in Figure 1. In both cases, collagen (0.03–10.0 μ g ml⁻¹) caused a rapid, concentration-

Figure 1 The effect of collagen on single platelet count over a 4 min incubation period in samples of citrated (a) guinea-pig ($n = 18$) and (b) Rhesus monkey ($n = 22$) whole blood *in vitro*. Concentrations of collagen added at 0 min are shown in $\mu\text{g ml}^{-1}$; the vehicle alone treatment was glycine buffer (pH 3.5) at the standard dose volume (5 μ l). The data are expressed as the mean percentage decrease in count (s.e. mean shown by vertical bars) from baseline values obtained prior to the addition of collagen.

Figure 2 Concentration-effect curves for the platelet aggregatory response to collagen in guinea-pig (●) and Rhesus monkey (○) citrated whole blood *in vitro*. Results shown are decreases in single platelet count (mean % with s.e.mean shown by vertical bars) measured at 4 min after addition of agonist.

dependent decrease in single platelet count during the 4 min incubation period. Both the rate and extent of the decrease in platelet count were directly related to the initial collagen dose. In both species, the greatest decrease (approximately 90%) in platelet count was achieved at the higher collagen concentrations ($\geq 3 \mu\text{g ml}^{-1}$). Although concentration-effect curves plotted for the response at 4 min in blood from both species were parallel (Figure 2), guinea-pig platelets in whole blood appeared threefold more sensitive to collagen than those from the Rhesus monkey. EC_{50} values obtained were 0.14 ± 0.02 and $0.48 \pm 0.4 \mu\text{g ml}^{-1}$ (mean $\pm$ s.e.mean; $n > 15$) for guinea-pig and Rhesus monkey respectively. When vehicle alone was present, no consistent change in platelet count from baseline occurred, with an overall decrease in count being observed in guinea-pig blood samples of $8.7 \pm 3.1\%$ (509 ± 34 vs $465 \pm 32 \times 10^6 \mu\text{l}^{-1}$; NS) and an increase in Rhesus monkey blood samples of $2.0 \pm 2.3\%$ (248 ± 19 vs $253 \pm 20 \times 10^6 \mu\text{l}^{-1}$; NS) at 4 min after addition of collagen buffer. The addition of reagent vehicle and stirring of samples for the standard incubation period thus caused no significant non-specific platelet aggregation.

Effect of EP 092 on collagen-induced blood platelet aggregation ex vivo

Guinea-pig In these experiments EP 092 was administered either by intravenous injection (0.1–3.0 mg kg^{-1}) as its sodium salt or by oral gavage

Figure 3 Effect of EP 092 on collagen-induced aggregation of citrated guinea-pig whole blood *ex vivo*. Drug was administered as an intravenous bolus injection and the animal killed at 30 min (a) or by oral gavage and the animals killed at 1 h (b). The results are expressed as the mean percentage of the maximum control (●) response (vertical bars show s.e.mean) ($n = 4-6$ animals per group). Doses of EP 092 shown were 0.1 (○), 0.3 (■), 1.0 (□), 3.0 (▲) and 10.0 (△) mg kg^{-1} respectively, with statistical significance relative to controls indicated at the $P < 0.05$ (*) and $P < 0.01$ (**) levels.

(1.0–10.0 mg kg⁻¹) in PEG/CMC vehicle to groups of conscious male guinea-pigs. Initially, collagen-induced platelet aggregation was determined in individual samples of citrated blood obtained at 0.5 h (i.v.) and 1 h (orally) after dosing. EP 092 significantly inhibited the aggregatory response to collagen in a dose-related fashion when the compound was administered by either route (Figure 3). In both cases, the concentration-effect curves constructed to collagen were shifted in a progressive rightward manner with each increase in antagonist dose. EP 092 was a more potent inhibitor of the platelet responses to lower concentrations of collagen (0.03–0.3 µg ml⁻¹). Following intravenous (but not oral) administration of EP 092, a significant decrease in the maximum response achieved by collagen was observed at the higher antagonist doses (Figure 3a). ED₅₀ values (mean ± s.e.mean) for EP 092 against responses to a submaximal collagen concentration of 0.3 µg ml⁻¹ were 1.3 ± 0.2 mg kg⁻¹ (i.v.) and 1.4 ± 0.2 mg kg⁻¹ (orally) respectively.

In a second series of experiments, the duration of action of EP 092 (taken as the time after dose during which >50% inhibition of submaximal 0.3 µg ml⁻¹ collagen responses was maintained) was investigated following intravenous (1.0 and 3.0 mg kg⁻¹) or oral (3.0 and 10.0 mg kg⁻¹) administration. Again, onset of inhibition of platelet responses *ex vivo* was rapid with pronounced inhibitory activity evident at 30 min after dosing and peak inhibitory effects by 1 h with either route of administration (Figure 4). Duration of action of the compound was dose-dependent. Determination of an accurate pharmacodynamic half life was not attempted from the limited data, but whilst the effect of 1.0 mg kg⁻¹ i.v. was weak and transient, inhibitory activity following 3.0 mg kg⁻¹ i.v. was evident for at least 3 h. Indeed, although the onset of effect (30 min) was more rapid following i.v. dosing, thereafter, the time-response curves for the 3.0 mg kg⁻¹ dose level (i.e. approximately 2.0 × ED₅₀) by i.v. or oral route were remarkably similar (no significant difference at 1, 3 or 6 h time points). At the highest oral dose used (10 mg kg⁻¹) the duration of action was >6 h.

Rhesus monkey Platelet aggregation induced by collagen was monitored in citrated blood samples obtained at 1 and 3 h after dosing with EP 092 (0.3–10.0 mg kg⁻¹). EP 092 inhibited the aggregatory responses to collagen in a dose-dependent manner (Table 1). Estimated mean ED₅₀ values for the compound against a submaximal collagen concentration (0.5 µg ml⁻¹) were 0.9 ± 0.3 mg kg⁻¹ at 1 h and 1.8 ± 0.4 mg kg⁻¹ at 3 h. Duration of action appeared to be between 1 and 3 h at 1.0 mg kg⁻¹, and greater than 3 h at 3.0 and 10.0 mg kg⁻¹. In both the Rhesus monkey and the guinea-pig, the degree of

Figure 4 Duration of action of the inhibitory effect of EP 092 against collagen-induced guinea-pig citrated whole blood platelet aggregation *ex vivo*. Drug was administered either intravenously at 1.0 mg kg⁻¹ (●) and 3.0 mg kg⁻¹ (○) or orally at 3.0 mg kg⁻¹ (■) and 10.0 mg kg⁻¹ (□). Treatment groups (*n* = 4) were killed at the times shown, and blood platelet aggregation determined. The results are expressed as inhibition (mean % with s.e.mean shown by vertical bars) of the decrease in single platelet count induced by a submaximal collagen concentration (0.3 µg ml⁻¹) relative to the response in groups of control (vehicle alone) guinea-pigs killed at identical times.

inhibition achieved was comparable at 3.0 and 10.0 mg kg⁻¹ dose levels, with approximately 70% inhibition being observed at 3 h following either dose.

In the *ex vivo* experiments with Rhesus monkey whole blood, plasma samples were obtained at termination of the 4 min aggregation incubation period to determine the effect of EP 092 on TXB₂ synthesis. Collagen-induced whole blood aggregation was accompanied by an agonist concentration-dependent

Table 1 Effect of EP 092 on collagen-induced Rhesus monkey blood platelet aggregation *ex vivo*

EP 092 (mg kg ⁻¹)	(n)	Inhibition at time after oral EP 092 dose (h)	
		1	3
0.3	4	22 ± 8	20 ± 15
1.0	4	52 ± 7*	33 ± 9
3.0	5	60 ± 12*	76 ± 8*
10.0	5	87 ± 7*	73 ± 6*

Values are mean ± s.e.mean

Inhibition data are expressed relative to the aggregatory response induced by 0.5 µg ml⁻¹ collagen in blood samples from control (vehicle dosed) animals, where * = significance at the *P* < 0.05 level. Vehicle alone had no significant effect at either time interval after dosing.

Table 2 Collagen stimulated thromboxane B₂ (TXB₂) formation in Rhesus monkey blood

EP 092 (mg kg ⁻¹)	n	Sample Time	Increase in plasma TXB ₂ (ng ml ⁻¹) at collagen concentration:			
			0.5 µg ml ⁻¹	1.0 µg ml ⁻¹	3.0 µg ml ⁻¹	10.0 µg ml ⁻¹
1.0	4	Pre-Drug	6.3 ± 1.6	20.4 ± 3.9	68.5 ± 13.8	255.3 ± 24.2
		1 h	4.2 ± 2.1	15.6 ± 3.5	52.7 ± 14.2	255.2 ± 24.8
		3 h	5.3 ± 2.4	16.0 ± 6.0	66.9 ± 17.8	258.9 ± 41.6
3.0	5	Pre-Drug	3.4 ± 0.9	20.5 ± 5.6	61.3 ± 12.8	338.1 ± 57.1
		1 h	2.9 ± 0.9	16.8 ± 5.8	55.6 ± 15.0	281.9 ± 39.1
		3 h	3.8 ± 2.1	17.4 ± 5.2	45.6 ± 12.4	355.6 ± 65.2
10.0	5	Pre-Drug	8.5 ± 3.4	24.7 ± 5.4	80.2 ± 19.2	390.2 ± 56.4
		1 h	8.3 ± 2.8	27.5 ± 5.1	93.4 ± 18.3	347.7 ± 42.2
		3 h	7.1 ± 2.6	17.9 ± 2.3*	52.5 ± 18.8*	248.6 ± 50.5*

Platelet aggregation in citrated Rhesus monkey blood was induced by the addition of collagen at the concentrations stated. Basal levels of TXB₂ in collagen buffer-incubated samples for each treatment sample time ranged between 0.3–10.9 ng ml⁻¹ (mean ± s.d. 3.8 ± 0.5 ng ml⁻¹). The data shown in the table are specific collagen-stimulated increases in plasma TXB₂ (also mean ± s.d.) derived by subtraction of individual basal values, where * = $P < 0.05$ compared to pre-drug controls (paired t test).

increase in plasma TXB₂ levels. No significant effect of orally administered EP 092 on collagen-stimulated TXB₂ formation was observed at the 1.0 and 3.0 mg kg⁻¹ dose levels. Despite pronounced inhibition of the platelet aggregatory response to collagen (Table 1) plasma TXB₂ levels were unchanged compared to pre-dose samples (Table 2). However, at the highest dose of EP 092 (10 mg kg⁻¹) partial inhibition of TXB₂ formation was evident in the 3 h (but not 1 h) samples ranging 27–36% at 1.0–10.0 µg ml⁻¹ collagen concentrations in the incubation ($P < 0.05$; paired Student's t test).

Rabbit intravascular platelet aggregation in vivo

The effect of EP 092 on collagen and thrombin-induced transient thrombocytopenia (intravascular platelet aggregation) in the anaesthetized rabbit preparation was examined by the circulating platelet count continuous monitoring technique. Baseline circulating platelet counts observed at the start of each experiment were in the range 3.5–7.0 × 10⁸ ml⁻¹. In the case of collagen, the pretreatment control 3.0 µg kg⁻¹ i.v. dose (excluding the initial dose, see methods) caused decreases in platelet count ranging 19–34% (mean ± s.e.mean 29 ± 4%; $n = 22$). Further doses of collagen given immediately after and 30 min following administration of drug vehicle caused successive falls of 26 ± 7 and 25 ± 4% respectively (no significant difference from pre-dose controls). Similarly, for thrombin (10 units kg⁻¹ i.v.), successive doses administered to vehicle-dosed preparations induced decreases in platelet count of 54 ± 8%, 49 ± 6% and 51 ± 8% respectively ($n = 14$). Thus, for each agonist, three successive

responses to either collagen or thrombin were consistent for the duration of the experiment. However, the recovery in circulating platelet count which occurred in the period between agonist doses was slow (up to 15 min being necessary to establish a new baseline), and seldom complete, such that pre-dose counts decreased progressively throughout the experiment (Figure 5).

The administration of drug vehicle had no effect on platelet responses to collagen or thrombin. In

Figure 5 Effect of EP 092 on collagen-induced intravascular aggregation in the anaesthetized rabbit. The trace shows a representative experiment in which three successive responses to bolus i.v. injections of collagen (30 µg kg⁻¹) administered 30 min before, immediately following and a further 30 min after infusion of EP 092 (10 mg kg⁻¹) were observed. The recovery period between responses (30 min) is not shown in full and is represented by breaks in the trace.

Table 3 Effect of EP 092 on collagen and thrombin-induced intravascular platelet aggregation in the anaesthetized rabbit

Treatment	Dose (mg kg ⁻¹)	n	Inhibition of response to collagen	
			0 min	30 min
Vehicle	0	11	-7 ± 5	5 ± 5
EP 092	3.0	6	-12 ± 6	-13 ± 6
EP 092	10.0	5	46 ± 8*	20 ± 8
			Inhibition of response to thrombin	
			0 min	30 min
Vehicle	0	14	-6 ± 7	-4 ± 13
EP 092	3.0	6	12 ± 17	20 ± 17
EP 092	10.0	4	35 ± 7**	26 ± 8

Responses to collagen (30 µg kg⁻¹) and thrombin (10 units kg⁻¹) were determined in separate preparations (n) before and at 0 and 30 min after administration of EP 092. Drug was given by slow intravenous infusion over a 10 min period at rates of 0.3 and 1.0 mg kg⁻¹ min⁻¹ respectively. Data shown are mean ± s.e.mean; statistical significance with respect to pre-drug and vehicle alone controls as indicated: **P* < 0.05, ***P* < 0.01.

contrast (Table 3), immediately following the infusion of EP 092 at 10 mg kg⁻¹ but not 3.0 mg kg⁻¹, platelet responses to both collagen and thrombin were partially but significantly inhibited (Table 3). However, even at the highest EP 092 dose level, the inhibitory effect was of short duration, since no significant effect was evident after a further 30 min.

Discussion

Although the relative potency of PGH₂ and TXA₂ as platelet agonists *in vitro* has been clearly defined (Bhagwat *et al.*, 1985b), the respective contribution of each as secondary mediators in platelet aggregatory responses initiated by collagen or thrombin *in vitro*, and particularly *in vivo*, remains less clear. In PRP, collagen-induced aggregatory responses appear largely dependent on two pathways; ADP secretion from dense granules and the formation of PGH₂ and TXA₂ from platelet phospholipids following activation of phospholipase (Kinlough-Rathbone *et al.*, 1977; Vargaftig *et al.*, 1980; Lewis & Watts, 1982). TXA₂ synthase inhibitors such as dazoxiben or OKY1581 inhibit threshold responses to collagen in human PRP, but are ineffective against aggregation induced by higher concentrations of collagen (Randall *et al.*, 1981; Ito *et al.*, 1983). This latter observation coupled with the more pronounced inhibitory activity of cyclo-oxygenase inhibitors and PGH₂/TXA₂ receptor antagonists (Patscheke & Stegmeier, 1984; Ogletree *et al.*, 1985) at higher collagen concentrations implies that in these circumstances, PGH₂ may play an increasingly important role (Hornby & Skidmore, 1982). However, in whole blood, the activity of both agon-

ists and inhibitors may vary, and may also be influenced by other cells present and potentially also by other mediators such as prostaglandin I₂ or platelet activating factor (Morley *et al.*, 1979; Kennedy *et al.*, 1980; Abbate *et al.*, 1986).

In the *ex vivo* studies with guinea-pig and Rhesus monkey citrated whole blood, collagen caused concentration-dependent aggregatory responses reflected in a rapid decrease in single platelet count detectable within 1–2 min. The range of collagen concentrations at which an aggregatory response was observed was similar to that observed previously for both rodent and human blood (Saniabadi *et al.*, 1983; Lad *et al.*, 1987). Administration of EP 092 to guinea-pigs or Rhesus monkeys potently inhibited collagen-induced aggregatory responses in whole blood *ex vivo*, indicating a significant role for PGH₂ and TXA₂ in these responses. However, whilst a similar magnitude of inhibitory effect (between 70 and 80% inhibition) was achieved in both species at comparable oral doses of EP 092, full inhibition was not achieved in either case, indicating that additional mediators such as ADP are important in whole blood. The highest oral dose of EP 092 tested in the guinea-pig (10 mg kg⁻¹) merely extended the duration of the inhibitory effect of EP 092 to >6 h, rather than its magnitude. Also in the guinea-pig, the ratio of the intravenous and oral ED₅₀ values for EP 092 (1.3 ± 0.2 and 1.4 ± 0.2 mg kg⁻¹ at 30 min and 1 h after dosing respectively) was close to unity, which suggests that the compound had been rapidly and efficiently absorbed from the gastrointestinal tract following oral dosing.

In the Rhesus monkey, a similar oral ED₅₀ was obtained (0.9 ± 0.3 mg kg⁻¹), and the inhibitory effect of EP 092 on aggregation was not accompa-

nied by any significant reduction in collagen stimulated TXB₂ formation except at 3 h after the highest EP 092 dose (10 mg kg⁻¹). These data suggest that aggregation is not a prerequisite for collagen-induced TXB₂ synthesis in platelets, and equally, that EP 092 causes substantial inhibition of the platelet response to collagen, independent of any effect on TXB₂ formation until high doses of the compound (10 × ED₅₀) are administered. Our results closely mirror *in vitro* platelet data previously obtained for EP 092 (Armstrong *et al.*, 1985a), where a partial inhibitory effect on TXB₂ formation from arachidonic acid was noted only at higher *in vitro* concentrations of EP 092. The present data therefore support the earlier conclusion that with EP 092, inhibition of the endogenous thromboxane component of aggregation is likely to be due to thromboxane receptor antagonism rather than inhibition of biosynthesis. Furthermore, Darius *et al.* (1985) have also found that with the PGH₂/TXA₂ receptor antagonist SQ29548, an attenuation of the aggregatory response of rabbit platelets to arachidonic acid was not accompanied by any significant reduction in the formation of TXB₂.

In the dog, collagen-induced aggregatory responses in whole blood *ex vivo* have been shown to be potently inhibited by administration of the selective PGH₂/TXA₂ antagonists, AH23848 and GR32191 (Humphrey & Lumley, 1984; Lumley *et al.*, 1988). The present results demonstrate that platelet aggregatory responses to collagen can also be routinely monitored in citrated whole blood samples from a non human primate and that a selective PGH₂/TXA₂ antagonist is active in this species. Reports in the literature concerning platelet behaviour in blood or PRP samples obtained from Rhesus monkeys are few. It has been claimed that platelets in PRP from this species respond to agonists in a similar way to human platelets (Addonizio *et al.*, 1978). Rhesus monkey platelets synthesize TXB₂ in response to addition of arachidonic acid (Gorman *et al.*, 1983) or collagen (our data), and are susceptible to inhibition of U-44069-induced aggregation in PRP responses *ex vivo* by the PGH₂/TXA₂ receptor antagonist L-655240 (Hall *et al.*, 1987). In the present experiments, Rhesus monkey platelets in whole blood responded with a sensitivity to collagen (EC₅₀ 0.48 ± 0.4 µg ml⁻¹) almost identical to that of human whole blood under the same experimental conditions (EC₅₀ 0.53 ± 0.1 µg ml⁻¹; Lad *et al.*, 1987).

In the anaesthetized rabbit preparation, EP 092 also demonstrated inhibitory activity against collagen-induced intravascular aggregation (transient thrombocytopenia) assessed by continuous platelet count monitoring. EP 092 was, however, less effective in this model such that an intravenous dose

of 10 mg kg⁻¹ was necessary to achieve a significant inhibitory effect compared to an intravenous ED₅₀ of 1.3 mg kg⁻¹ in the guinea-pig *ex vivo* test. The apparent difference in the potency of EP 092 between species may be due to the different test design employed (*in vivo* or *ex vivo*), but this seems unlikely, since EP 092 has been reported to be as effective at 0.25 and 1.0 mg kg⁻¹ in the guinea-pig against collagen-induced intravascular aggregation and bronchoconstriction (Jones *et al.*, 1983). Furthermore, a close analogue, EP 045, is active at 1.0 mg kg⁻¹ against collagen responses in the guinea-pig (Mallarkey & Smith, 1985). It seems improbable that the rate of metabolism and excretion of EP 092 should differ to such an extent in the rabbit that the observed inhibitory potency of EP 092 against collagen-induced platelet responses *in vivo* is reduced by almost an order of magnitude, and its duration of action decreased from >6 h to <0.5 h at 10 mg kg⁻¹. More plausible is the possibility that the relative affinity of the antagonist for the platelet PGH₂/TXA₂ receptors varies between species due to receptor heterogeneity. Some support for this hypothesis is provided by the observation that EP 092 is significantly less active against 11,9-epoxymethano-PGH₂-induced aggregation of rabbit washed platelet preparations compared to those of rat or man (Jones *et al.*, 1987). Moreover, it has also been reported that rabbit PRP is relatively insensitive to the aggregatory action of the TXA₂ analogue STA₂ (Narumiya *et al.*, 1986). Clarification would be provided by comparative EP 092 receptor-binding studies with animal platelets, but these have yet to be carried out. Finally, it is conceivable that the amounts of intraplatelet PGH₂ and TXA₂ generated in response to collagen, and the relative contribution of these agonists to the observed aggregatory response varies between species. It is noteworthy that whereas up to 80% inhibition of the collagen platelet response was observed with EP 092 in the guinea-pig, the maximal inhibitory effect achieved *in vivo* in the rabbit was between 40 and 50%. A maximal inhibitory effect of approximately 50% has previously been reported in the rabbit collagen-induced intravascular aggregation model with indomethacin (Honey *et al.*, 1986) and with two other selective PGH₂/TXA₂ antagonists SQ29548 and BM13177 (Darius & Lefer, 1985).

Perhaps most surprising was the partial inhibitory activity observed with EP 092 against thrombin-induced intravascular aggregation in the rabbit. Although administration of intravenous bolus doses of thrombin causes a substantial increase in plasma TXB₂ levels in this model, PGH₂ and TXB₂ do not appear to contribute directly to the thrombin-induced aggregatory response since indomethacin is inactive *in vivo* (Honey *et al.*, 1986) and *in vitro*

(Kinlough-Rathbone *et al.*, 1977). Since thrombin is also a potent stimulant of PGI₂ formation in vascular endothelial cells (Weksler *et al.*, 1978), it is conceivable that in the presence of a selective inhibitor of the action of PGH₂ and TXA₂, a weak inhibitory effect on platelet aggregation *in vivo* due to release of vascular tissue derived PGI₂ may be unmasked.

In conclusion, the potent and selective platelet PGH₂/TXA₂ receptor antagonist EP 092 exhibited marked inhibitory activity against collagen-induced whole blood aggregation *ex vivo* in the guinea-pig and Rhesus monkey, and against collagen and thrombin-induced *in vivo* intravascular aggregatory responses in the rabbit. The present data and results

reported previously (Jones *et al.*, 1983) suggest that its activity as an inhibitor of PGH₂/TXA₂ dependent platelet responses varies between species with a relative potency order: guinea-pig $\approx$ Rhesus monkey $>$ rat $>$ rabbit. As the compound is also a potent inhibitor of human platelet aggregation *in vitro* (Armstrong *et al.*, 1985a), we suggest these findings support evaluation of its therapeutic potential as an anti-thrombotic agent in man.

The authors would like to thank Drs R.L. Jones and N.H. Wilson (Edinburgh University) for the generous gift of EP 092, Mr M. Chesterman for excellent technical assistance, and Mrs Joyce Brand for typing the manuscript.

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(Received June 8, 1988

Revised August 22, 1988

Accepted September 21, 1988)