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A comparative study of the involvement of the prostaglandin H_2/thromboxane A_2 pathway in intravascular platelet aggregation in guinea-pigs and rats

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1 The effects of indomethacin, dazoxiben and EPO45 on collagen-induced platelet aggregation *in vivo* were studied in guinea-pigs and rats to determine the involvement of the prostaglandin endoperoxide/thromboxane A_2 pathway in the aggregatory response.

2 Indomethacin and EPO45 (a thromboxane receptor antagonist) partially inhibited platelet aggregation in rats. It was concluded that only one third of the aggregatory response to collagen was mediated by the products of cyclo-oxygenase conversion of arachidonic acid.

3 In rats, dazoxiben was inactive although the conversion of the prostaglandin endoperoxides to thromboxane A_2 was inhibited (measured as thromboxane B_2). 6-keto $PGF_{1\alpha}$ was detected in plasma after collagen was injected into dazoxiben-treated rats. In this species therefore, the endoperoxides have significant aggregatory activity whilst the apparent increase in the level of prostacyclin was not sufficient to have any anti-aggregatory effect.

4 All three drugs were active in the guinea-pig. About 60% of the aggregatory response to collagen was due to the products of the cyclo-oxygenase pathway, the main mediator being thromboxane A_2.

5 In guinea-pigs, dazoxiben also elevated 6-keto $PGF_{1\alpha}$ in the plasma after an injection of collagen. However, this apparent increase in prostacyclin production did not contribute to the anti-aggregatory effect.

Introduction

Collagen is a constituent of sub-endothelial tissue. When platelets come into contact with collagen, as a result of vessel wall injury, they are activated to release their granule contents and mobilize arachidonic acid from a platelet phospholipid pool. The enzyme cyclo-oxygenase converts arachidonic acid to the prostaglandin endoperoxides, prostaglandin G_2 (PGG_2) then PGH_2 (Hamberg & Samuelsson, 1974). A further transformation in platelets produces the potent vasoconstrictor and platelet aggregatory substance, thromboxane A_2(TxA_2) (Hamberg *et al.*, 1975). Normally, platelets do not adhere to vessel wall endothelium (Saba & Mason, 1975). This is thought to be due to the endothelial production of prostacyclin (PGI_2), a vasodilator and anti-aggregatory product of PGH_2 transformation in the vessel wall (Moncada *et al.*, 1976). An upset in the balance of PGI_2 and TxA_2 production, in favour of the latter, may cause thrombus formation (Moncada *et al.*, 1976).

The production of TxA_2 and PGI_2 can be prevented by non-steroidal anti-inflammatory drugs through inhibition of the cyclo-oxygenase in platelets and vessel walls (Zimmermann *et al.*, 1980). Imidazole-type drugs however may preferentially inhibit thromboxane synthetase and Moncada & Vane (1978) have suggested that the resulting selective inhibition of TxA_2 may be utilised in antithrombotic therapy.

In animals and man given thromboxane synthetase inhibitors it appears that platelet PGH_2 is released and forms PGI_2 (Flower & Cardinal, 1979; Tyler *et al.*, 1981; Vermylen *et al.*, 1981; Defreyn *et al.*, 1982) which may provide an added antithrombotic effect. However, Hornstra (1980) suggests that arterial thrombosis formation depends primarily on the ability of platelets to produce TxA_2 and the possible antithrombotic effect of vascular PGI_2 is not as important. Also, the increased level of PGH_2 may activate platelet aggregation directly (Hamberg *et al.*, 1974) or be

catabolised into PGE_2 (Defreyn *et al.*, 1982), PGD_2 and $PGF_{2\alpha}$ (Blair *et al.*, 1983).

Bertele & De Gaetano (1982) found that, in man, inhibition of TxA_2 synthesis does not modify the platelet response to aggregating stimuli *in vitro* and concluded that combination treatment of a thromboxane synthetase inhibitor and a TxA_2 receptor antagonist may be an appropriate clinical approach to thrombosis prevention.

Previous work has shown that the modified Technicon Autocounter (Smith & Freuler, 1973) can be used for studying intravascular platelet aggregation where the collagen-induced fall in platelet count can be monitored continuously without the use of an intravenous anticoagulant (Smith, 1981). When this technique was used in the rat it appeared that extracellular divalent cation is essential for normal intravascular platelet aggregation, that the endoperoxide/thromboxane pathway is responsible for part of the aggregation response and that the involvement of this pathway is dependent upon the amount of collagen exposed to the platelets (Mallarkey & Smith, 1984a).

The aim of this study was to compare the effect of a cyclo-oxygenase inhibitor (indomethacin), a thromboxane synthetase inhibitor (dazoxiben) and a thromboxane receptor antagonist (EPO45) on collagen-induced platelet aggregation, *in vivo*, in the guinea-pig and in the rat in an attempt to predict which antithrombotic therapy may be beneficial clinically. A preliminary report of this work was given to the British Pharmacological Society (Mallarkey & Smith, 1983).

Methods

Anaesthesia

Male Sprague-Dawley rats (350–450g) were anaesthetized with pentobarbitone (60 mg kg^{-1} i.p.). Duncan-Hartley guinea-pigs (400–600g) of either sex received a mixture of urethane (280 mg kg^{-1}) and allobarbitone (70 mg kg^{-1}) given intraperitoneally.

Continuous platelet counting

After anaesthesia, the trachea was cannulated to ensure a free airway and a jugular vein was cannulated for injection of drug solutions. A carotid artery was cannulated with a double cannula to allow continuous withdrawal of a sample (0.1 ml min^{-1}) of circulating blood. Only at this point was blood anticoagulated with 3.15% tri-sodium citrate (0.015 ml min^{-1}). Using the Technicon Autocounter (Smith & Freuler, 1973) the anticoagulated blood was diluted with ammonium oxalate (1%) and saponin (0.002%) to lyse the erythrocytes. The platelets were counted optically and the number of platelets continuously recorded on precalibrated chart paper.

Pharmacological intervention of collagen-induced platelet aggregation in vivo

When a continuous platelet count had been achieved, collagen (40 $\mu g\,kg^{-1}$ i.v.) was injected at 15 min intervals. The fall in platelet count was measured by calculating the maximum % fall in platelet count that occurred after each injection of collagen. Five minutes before the third injection of collagen, a dose of drug or vehicle was injected. The subsequent % fall in platelet count, due to injection of collagen was compared with the mean % fall in platelet count of the two preceding collagen injections. The difference was expressed as a % inhibition of the mean control response.

Blood sampling from anaesthetized animals

A carotid artery was cannulated 10 min before injection of collagen and a total volume of 1 ml was collected in a 1 ml syringe containing 120 μl EDTA/indomethacin solution. The syringe was centrifuged at 1500 *g* for 6 min and the supernatant collected and frozen at − 20°C. Basal levels of the metabolites (TxB_2 and 6-keto $PGF_{1\alpha}$) were determined from samples collected before injection of collagen. All other samples were collected 1 min after injection of collagen. Any materials in contact with blood were siliconised.

Radioimmunoassay of thromboxane B_2 and 6-keto prostaglandin $F_{1\alpha}$

The frozen plasma samples were thawed and the content of TxB_2 and 6-keto $PGF_{1\alpha}$ was determined by accurate and precise assays without use of extraction or thin layer chromatography (Mallarkey & Smith, 1984a,b). The final concentration of both antisera was 1:2000. The sensitivity of the assays was 30 pg 100 μl^{-1} (TxB_2) and 20 pg 100 μl^{-1} (6-keto $PGF_{1\alpha}$), defined as the amount of added ligand which displaced 10% of bound labelled ligand. Cross reactivities for TxB_2 antiserum were: TxB_2, 100%; PGD_2, 0.9%; $PGF_{2\alpha}$, 0.1%; PGE_2, 0.02%; PGE_1, 0.1%; 6-keto $PGF_{1\alpha}$, $<0.01\%$ and for 6-keto $PGF_{1\alpha}$ were: 6-keto $PGF_{1\alpha}$, 100%; PGD_2, $<0.01\%$; $PGF_{2\alpha}$, 1.14%; PGE_2, 0.1%; PGE_1, 0.13%; TxB_2, $<0.01\%$.

Statistical analyses

The difference between two means was calculated using Student's *t* test (two-tailed). The F-test was used to compare the variance associated with each mean. In all cases the tabulated value of F(2.5% points) was not exceeded. Therefore each *t* test was deemed valid.

If $P > 0.05$, then the difference between two means was not significant.

Drugs and reagents

Drugs used were: collagen, bovine achilles tendon, type I (Diamed Diagnostics); dazoxiben (gift from Pfizer, dissolved in saline); EP045 (5-endo (6'-carboxyhex-2'Z-enyl)-6-exo (N-phenylcarbamoyl) hydrazonomethyl)-bicyclo (2,2,1) heptane (gift from R.L. Jones, University of Edinburgh, dissolved in Na_2CO_3 solution); indomethacin (gift from MSD, dissolved in Na_2CO_3 solution); pentobarbitone (Sagatal); urethane and allobarbitone (both from Sigma) were dissolved in Na_2CO_3 solution.

Reagents used were: ammonium oxalate (Fisons); saponin (BDH); EDTA (BDH)/indomethacin solution was prepared by adding 1 ml indomethacin (1 mg ml^{-1}) to 4 ml EDTA (1%) in normal saline; [^{3}H]-6-keto $PGF_{1\alpha}$ and [^{3}H]-TxB_2 (specific activity, 120 ci mmol^{-1} and 139 Ci mmol^{-1} respectively, both from Amersham); 6-keto $PGF_{1\alpha}$ (gift from J.B. Smith, Thomas Jefferson University, Philadelphia); prostaglandin used to determine cross-reactivities were gifts from Dr J. Pike (Upjohn); TxB_2 and antisera were provided by Dr J.A. Salmon, Wellcome Research Laboratories.

Results

Collagen, 40 μg kg^{-1}, was found to produce reproducible falls in platelet count in guinea-pigs and rats. Radioimmunoassay of 6-keto $PGF_{1\alpha}$ and TxB_2 showed that neither metabolite was present in the plasma (below the level of detection) prior to injection of collagen. After injection of collagen, TxB_2 was detected in the plasma of guinea-pigs and rats but 6-keto $PGF_{1\alpha}$ was not detected (Table 1).

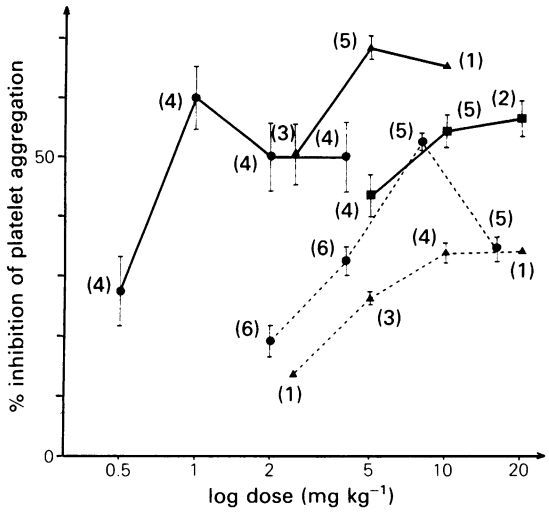

Figure 1 The effect of indomethacin (●), dazoxiben (■) and EPO45 (▲) on collagen-induced aggregation in guinea-pigs (solid lines) and rats (interrupted lines). The number of observations is shown in parentheses.

Indomethacin partially inhibited collagen-induced aggregation in both species (Figure 1). In rats, indomethacin (4 and 16 mg kg^{-1}) produced about 30% inhibition whilst 8 mg kg^{-1} caused 52.5% inhibition. In guinea-pigs only 1 mg kg^{-1} was required to produce 59.9% inhibition and higher doses (2 and 4 mg kg^{-1}) were just as effective at inhibiting collagen-induced

Table 1 The effect of indomethacin, dazoxiben and EPO45 on TxB_2 and 6-keto $PGF_{1\alpha}$ formation during collagen-induced platelet aggregation *in vivo*

Dose of drug (mg kg^{-1})		*Control*	*Indomethacin* 1	8	*Dazoxiben* 10	40	*EPO45* 5	10
TxB_2 levels in plasma (ng ml^{-1})	Rat	4.2 ± 1.0(5)		ND(5)		ND(4)		4.1 ± 0.8(5)*
	G-pig	5.4 ± 1.1(5)	ND(4)		ND(4)		3.8 ± 1.0(5)*	
6-keto $PGF_{1\alpha}$ levels in plasma (ng ml^{-1})	Rat	ND(5)		ND(5)		0.8 ± 0.2(6)		ND(5)
	G-pig	ND(5)	ND(3)		0.7 ± 0.1(4)		ND(3)	

ND = not detected; number of experiments shown in parentheses; values are mean ± s.e.mean; * = not significantly different from respective control value.

aggregation. Table 1 shows that neither TxB_2 nor 6-keto $PGF_{1\alpha}$ was detected in indomethacin-treated guinea-pigs and rats.

Dazoxiben produced a dose-dependent inhibition of collagen-induced aggregation in guinea-pigs (Figure 1). A dose of 40 mg kg^{-1} was ineffective in the rat. In both species, plasma TxB_2 was reduced below the level of the detection but 6-keto $PGF_{1\alpha}$, previously undetected, was found in plasma from guinea-pigs and rats (Table 1).

Dazoxiben 80 mg kg^{-1} inhibited collagen-induced platelet aggregation in rats (22.6 ± 2.8%, $n = 6$). This dose of dazoxiben produced respiratory distress in the rats and subsequent injections of collagen, 20 or 35 min after injection of dazoxiben, killed a majority (4) of the animals.

EPO45 produced a dose-dependent inhibition of collagen-induced platelet aggregation in guinea-pigs (Figure 1). The inhibition produced by 5 mg kg^{-1} EPO45 was significantly greater ($P < 0.01$) than the inhibition achieved by 10 mg kg^{-1} dazoxiben, but was similar to the inhibition caused by 1 mg kg^{-1} indomethacin (no significant difference, Figure 1). In the rat, EPO45 produced a dose-dependent inhibition of collagen-induced platelet aggregation (Figure 1). In both species EPO45 did not reduce the level of TxB_2 (not significantly different from control level), and 6-keto $PGF_{1\alpha}$ was not detected (Table 1). The maximal inhibition of collagen-induced platelet aggregation by EPO45, in the guinea-pig, was greater than the maximal inhibition observed in the rat ($P < 0.001$, Figure 1).

Discussion

Previous work in this laboratory showed that the quantity of pentobarbitone which was required to produce surgical anaesthesia in guinea-pigs caused severe respiratory depression. For this reason, the anaesthetic was changed to the urethane/allobarbitone mixture as detailed in Methods. This mixture did not alter the extent of collagen-induced platelet aggregation, nor did it change the inhibition of collagen-induced aggregation by indomethacin, from the results obtained in rats anaesthetized with pentobarbitone (Mallarkey, 1984). It was therefore assumed that results obtained in the guinea-pig could be compared with those obtained in the rat, without suspecting that any differences were due to the different anaesthetics received by each species.

Dazoxiben had no effect on *in vivo* collagen-induced platelet aggregation in rats at non-toxic doses. However, TxB_2 production was completely inhibited and the level of 6-keto $PGF_{1\alpha}$ was raised by a factor of at least three. EPO45 did inhibit aggregation but the level of TxB_2 was not reduced and 6-keto $PGF_{1\alpha}$ was not detected. Therefore, if dazoxiben is a selective thromboxane synthetase inhibitor and EPO45 is a competitive antagonist at TxA_2 receptors (Armstrong *et al.*, 1981), then it can be concluded that conversion of endoperoxides to TxA_2 to elicit the aggregation response is not necessary in the rat. Needleman *et al.* (1976) showed that addition of endoperoxides to human PRP produced aggregation without conversion to TxA_2. Therefore, the inhibition of thromboxane synthetase in the rat does not prevent aggregation though the results do suggest that some of the platelet PGH_2 is converted into PGI_2 by prostacyclin synthetase, the source of which may be in the vessel wall or leukocytes. This apparent increase in PGI_2 levels (also observed in samples subjected to extraction/t.l.c. (Mallarkey, 1984)) did not have any anti-aggregatory effect.

In this study EPO45 produced a maximum inhibition of 33% in rats. It has previously been reported (Mallarkey & Smith, 1984b) that both piroxicam, a selective cyclo-oxygenase inhibitor (Carty *et al.*, 1980), and 4 mg kg^{-1} indomethacin produced about 30% inhibition of collagen-induced aggregation *in vivo*. This previous study concluded that the additional inhibitory effect of 8 mg kg^{-1} indomethacin must be due to some non-specific component. These results suggest that the endoperoxide/TxA_2 pathway plays a minor role in collagen-induced intravascular platelet aggregation in the rat and that 70% of the aggregation response is independent of activation by the arachidonic acid cascade.

Contrary to the results obtained with the rat, dazoxiben was found to inhibit collagen-induced aggregation in the guinea-pig. Similar to the results with the rat, it reduced the level of TxB_2 below the limit of detection and elevated 6-keto $PGF_{1\alpha}$ levels. The doses of indomethacin and EPO45 which caused maximal inhibition of aggregation in the guinea-pig were lower than those required to cause maximal inhibition in the rat. The inhibition of aggregation by EPO45 in the guinea-pig was not associated with a reduction in the level of TxB_2 and 6-keto $PGF_{1\alpha}$ was not detected. Therefore, unlike the situation in the rat, the conversion of PGH_2 to TxA_2 to produce platelet aggregation is required in the guinea-pig. Also, the % inhibition caused by each drug shows that the endoperoxide/TxA_2 pathway in guinea-pig platelets is responsible for over 60% of the collagen response.

In vitro studies have shown that collagen can cause aggregation by activating at least two independent pathways in platelets (Kinlough-Rathbone *et al.*, 1980). These pathways include mobilisation then conversion of arachidonic acid to the endoperoxides/TxA_2 and the release of adenosine diphosphate (ADP) and 5-hydroxytryptamine (5-HT) from dense granules in the platelet cytoplasm. Preliminary studies in rats have failed to show any involvement of ADP or 5-HT

during *in vivo* collagen-induced platelet aggregation (Duncan *et al*, 1980). Collagen may also cause the formation of platelet activating factor (Paf-acether) though rat platelets do not respond to Paf-acether challenge *in vitro* (Vargaftig, 1980). Unpublished results have confirmed that the rat platelets are also refactory to Paf-acether *in vivo*. Further experiments are in progress to elucidate further the mechanisms involved in collagen-induced platelet aggregation *in vivo*.

It was noted that the maximal inhibition achieved by EPO45 was significantly greater than that achieved by dazoxiben, which may indicate that, in the guinea-pig, the prostaglandin endoperoxides are capable of some aggregation without the need for conversion to TxA_2. The increase in the level of PGI_2 (measured as 6-keto $PGF_{1\alpha}$) as a result of dazoxiben pretreatment, indicates that, in the guinea-pig some platelet PGH_2 is used as a substrate for conversion by prostacyclin synthetase. In this acute model of intravascular platelet aggregation, the small increase in PGI_2 did not produce any observable anti-aggregatory effect. It may be that an increase in the levels of PGI_2 has no beneficial effect in guinea-pigs. A recent study by Gorman *et al.* (1983) in an *in vivo* model of thrombosis in dogs, has shown that the high efficacy of thromboxane synthetase inhibitors, compared with cyclo-oxygenase inhibitors, may be due to the increase in endogenous PGI_2 levels. Burke *et al.* (1983) have shown that platelets from guinea-pigs and cats most closely resemble those from human in responsiveness to agonists and antagonists of the arachidonic acid cascade, whereas dog platelets show fewer similarities. Therefore, an *in vivo* study of platelet aggregation in the guinea-pig may be of more use in predicting clinical activity of a drug than an *in vivo* study in the dog.

In conclusion, this paper confirms that rat platelets are particularly refractory to drugs that cause inhibition of arachidonic acid metabolism (Mallarkey & Smith, 1984b). This comparison has also shown that there are considerable differences in the involvement of the endoperoxide/TxA_2 pathway in guinea-pig and rat platelet aggregation *in vivo*. It is evident therefore that potential antithrombotic drugs should be studied in a number of different species until it is clear which species can predict clinical activity.

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