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SHORT COMMUNICATIONS

Modulation of the inhibitory action of prostaglandin E_1 on platelet aggregation in rats: a study with ticlopidine, aspirin, dipyridamole and sulfinpyrazone

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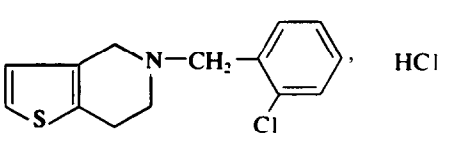
Ticlopidine is a potent long lasting inhibitor of platelet aggregation in animals [1, 2]. It inhibits the nucleotide release and displays antithrombotic action [3]. In humans the inhibitory action of ticlopidine was evidenced on the platelet aggregation [4–9] and also on the release reaction [8]. Its antithrombotic action was clinically demonstrated by the prevention of blood clotting in patients with chronic hemodialysis [10, 11]. Aspirin ingestion induced significant variations in the functions of human platelets due to interference with arachidonic acid metabolism at the cyclooxygenase step [12] and deprived the cell of endoperoxides, precursors of thromboxane A_2, a potent stimulus of platelet aggregation. Dipyridamole is thought to act through its inhibition of phosphodiesterase [13] and on the formation of thromboxane A_2 [14]. Sulfinpyrazone has been shown to inhibit the prostaglandin synthesis *in vitro* [15]. However, in clinical trials as AMIS (Aspirin Myocardial Infarction Study) and PARIS (Persantine Aspirin Reinfarction Study) the efficiency of aspirin and dipyridamole in preventing cardiac death was not apparent [16]. The Anturane Reinfarction Trial Research Group showed, on the other hand, that sulfinpyrazone did reduce cardiac mortality but only during the first six months after a heart attack [16].

As prostaglandins may be physiological regulators of platelet functions [17], it is important to be aware of the effects of drug treatments on the reactivity of platelets towards prostaglandins. Prostaglandin E_1 (PGE_1) and prostacyclin (PGI_2) are strong inhibitors of platelet activation and have been shown to bind to the same receptor [18]. Prostaglandin E_2 (PGE_2) competed for the same binding site [19] and at low concentrations enhanced the aggregating action of ADP [20].

The aim of the present work was to examine the influence of ticlopidine, aspirin, dipyridamole and sulfinpyrazone on the ADP-induced platelet aggregation and on the reactivity of platelets towards PGE_1 and PGE_2 in the rat.

Materials and methods

Ticlopidine, 5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno [3,2-*c*] pyridine hydrochloride [21]



was supplied by the Chemistry Department of Parcor–Sanofi (Toulouse, France). Aspirin, dipyridamole, sulfinpyrazone were from commercial origin; ADP, PGE_1, PGE_2, and human serum albumin (HSA) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade. A Tris–saline–albumin (TSA) buffer had a concentration of 5 mM glucose, 0.134 M NaCl and 15 mM Tris–HCl (pH 7.4) and 2% HSA. An ADP solution was made of 100 μM ADP, 10 mM $CaCl_2$ and 25 mM Tris–HCl (pH 7.4); $CaCl_2$ included in the ADP solution afforded better reproducibility to the aggregation test.

Male Sprague–Dawley rats weighing about 300 g were used. Drugs were orally administered in a suspension of 0.5% gum arabic at 150 mg/10 ml/kg for all drugs except sulfinpyrazone at 100 mg/10 ml/kg, twice a day for 2 days. Control rats received the vehicle alone (10 ml/kg).

Eighteen hours after the last dose, blood was taken from the jugular vein of animal under mild ether anaesthesia into 0.1 volume of 3.8% citrate solution, pH 7.4. After centrifugation at 240 *g* for 20 min the platelet-rich plasma (PRP) fraction was separated and stored at room temperature and diluted just before the aggregation test with the TSA buffer to give a final concentration of 400,000 platelets/μl.

Platelet aggregation was monitored photometrically by Born's method [22]. The extent of aggregation was indicated by the increase of the optical transmission (O.T.) 3 min after addition of ADP. A transient decrease in O.T. at low concentration of ADP was indicative of the shape change of platelets. For testing the reactivity to prostaglandins, the platelet suspensions were preincubated for 1 min with PGE_2, if any, then with PGE_1 for another minute before challenging with equipotent concentrations of ADP previously determined. Changes in the extent of aggregation, in the presence of prostaglandins, were then credited to their proper action.

Statistical analysis of data was done by a non-parametric U test of Mann and Whitney [23].

Results and discussion

PRP from each group of animals were pooled and samples of platelet suspensions were challenged with increasing concentrations of ADP.

None of the drugs was active against the shape change induced by ADP (Fig. 1). Platelets from sulfinpyrazone and dipyridamole treated animals did not differ from that of control at both 5 μM and 8 μM ADP. Dipyridamole and sulfinpyrazone have short half-lives, but a prolonged action of sulfinpyrazone in man has been described recently [34]. A single dose (400 mg) inhibited sodium arachidonate induced platelet aggregation during 72 hr, a duration much longer than the half-life of the drug or any of its known metabolites in plasma. Platelets from aspirin treated animals showed a 20% inhibition against 5 μM ADP but no significant difference from the control at higher ADP concentration, while the aggregation of platelets from ticlopidine treated rats was significantly inhibited at both concentrations of ADP, 69% and 27% inhibition at 5 μM and 8 μM of ADP, respectively (Table 1). The extent of aggregation of platelets from ticlopidine treated rats at 8 μM of ADP was equivalent to that obtained at 5 μM of ADP with platelets from control animals. The reactivity of platelets to prostaglandins after different drug treatments is illustrated by Fig. 2:

(a) Addition of PGE_1 at a final concentration of 6 nM resulted in 50% inhibition of the aggregation in control platelets. No significant difference was obtained with platelets from aspirin, dipyridamole or sulfinpyrazone treated rats whereas those from ticlopidine treated animals displayed a significant ($P < 0.002$) enhanced inhibition of 34% with respect to the control.

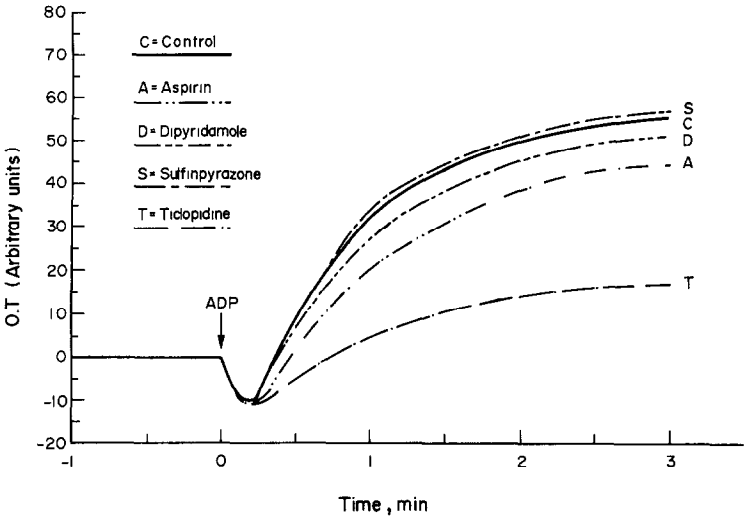


Fig. 1. Effect of drug administration on ADP-induced platelet aggregation. Drugs were orally administered in a suspension of 0.5% gum arabic at 150 mg/10 ml/kg for all drugs (except sulfinpyrazone at 100 mg/10 ml/kg), twice a day for 2 days. Blood was taken 18 hr after the last dosing. Platelet suspensions were prepared as described in Materials and Methods. 0.5 ml of platelet suspensions, stirred at 1000 rpm, at 32° were challenged with different final concentrations of ADP. The figure shows typical curves of platelet aggregation induced by 5 μM of ADP. The height of the curves at 3 min are noted and taken as arbitrary units of optical transmission (O.T.) Aggregation was indicated by an increase in O.T. The transient decrease in O.T. soon after the addition of the aggregating agent was indicative of the shape change of platelets. Control, (————); aspirin, (· · — · · —); dipyridamole, (– – — – – —); sulfinpyrazone, (– — – —); ticlopidine (· — · —).

(b) Addition of PGE_1 at a final concentration of 12 nM resulted in 70% inhibition of aggregation in control platelets. Again, significantly ($P < 0.002$) enhanced additional inhibition (+28%) was obtained only with platelets from ticlopidine treated animals.

(c) Preincubation first with 60 nM of PGE_2 largely impaired the inhibitory action of PGE_1, i.e. 15% inhibition of aggregation in control platelets at 6 nM of PGE_1. Aspirin, dipyridamole and sulfinpyrazone did not improve the extent of inhibition, while ticlopidine provided a 31% additional inhibition ($P < 0.002$).

(d) After preincubation with 60 nM of PGE_2, 12 nM of PGE_1 induced 35% inhibition of aggregation in control platelets. Here again, only ticlopidine was effective (41% additional inhibition, $P < 0.002$).

A scheme where four functional states of the platelets designated P1 through P4 was proposed by Marguerie *et al.* [24]:

(1) Platelet shape change from discoid to spheroid form,

$$P1 + ADP \rightleftharpoons P2$$

(2) Expression of fibrinogen (fg) receptor in presence of divalent ion,

$$P2 \xrightleftharpoons{ADP,\ Ca^{2+}\ or\ Mg^{2+}} P3\ Ca^{2+}\ or\ P3\ Mg^{2+}$$

(3) Reversible binding of fibrinogen to its receptor,

$$P3\ Ca^{2+}\ or\ P3\ Mg^{2+} + fg \rightleftharpoons P3\ fg$$

(4) Irreversible binding of fibrinogen to receptor,

$$P3\ fg \rightarrow P4\ fg$$

Table 1. Effect of drug treatment on the ADP-induced platelet aggregation

ADP		Control	Aspirin	Dipyridamole	Sulfinpyrazone	Ticlopidine
5 μM	m	55.6	44.6	51	56.8	17.3
	S.D.	5.4	9.7	10.3	6.7	12.3
	n	10	5	6	6	10
	P		0.05	N.S.	N.S.	0.002
	%		20	8	−2	69
8 μM	m	69.8	63	68	72.8	51
	S.D.	8.9	12	11	10.2	10.5
	n	10	5	6	6	10
	P		N.S.	N.S.	N.S.	0.002
	%		10	3	−4	27

Platelets from control, aspirin, dipyridamole, sulfinpyrazone and ticlopidine treated rats. m, mean of the extent of platelet aggregation in arbitrary units of optical transmission; S.D., standard deviation of the mean; *n*, number of experiments each experiment was performed with pooled platelets from 6 animals for each group; P, probability value of statistical analysis of data by U test of Mann and Whitney; %, per cent inhibition compared to control.

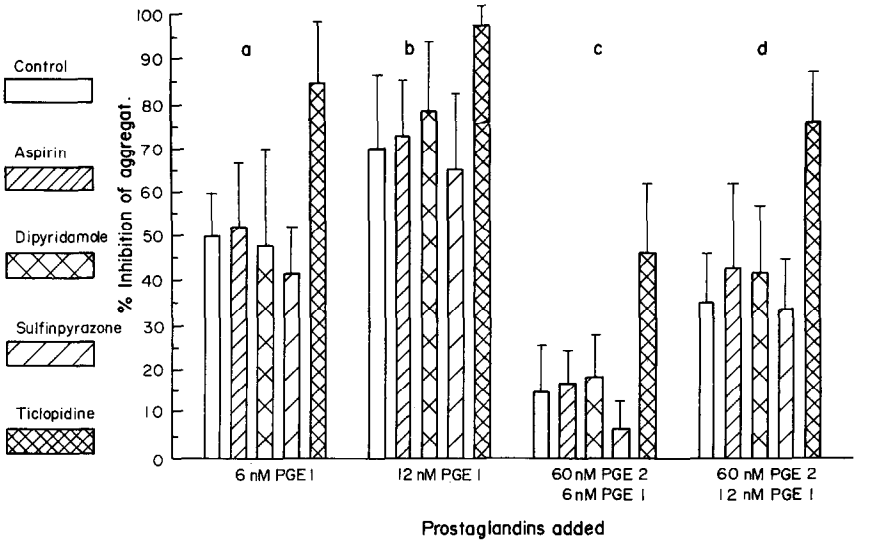


Fig. 2. Effect of drug treatment on platelet reactivity to prostaglandin E_1, and the combination prostaglandin E_2 and prostaglandin E_1. Rats were treated orally by aspirin, dipyridamole, sulfinpyrazone and ticlopidine, and control by vehicle along. Final concentrations of ADP were 6 μM, 5 μM, 5 μM, 8 μM and 5 μM, respectively. ADP additions followed: (a) Preincubation with 6 nM (final concentration) PGE_1 for 1 min in the aggregometer. (b) Preincubation with 12 nM PGE_1 during 1 min. (c) Preincubation with 60 nM PGE_2 during 1 min then 6 nM PGE_1 during another 1 min. (d) Preincubation with 60 nM PGE_2 during 1 min then 12 nM PGE_1 during one additional minute. Vertical bars indicated 1 S.D. Control, ▭; aspirin, ▨; dipyridamole, ▩; sulfinpyrazone ▨; ticlopidine, ▩.

It is then tempting to suggest that ticlopidine treatment might inhibit either the unmasking of fibrinogen receptor or the binding of fibrinogen to its receptor. In this context, it is relevant to mention that Lips *et al.* [6] have demonstrated in humans that the inhibition of platelet aggregation by ticlopidine was without influence upon the shape change and that the binding of [^{14}C]ADP to platelets consisted of two classes: high and low affinity, the last one being suppressed following ticlopidine ingestion, leaving untouched the high affinity binding.

With regard to platelet reactivity to prostaglandins, our findings that ticlopidine administration potentiated the effect of PGE_1 on the platelet are in agreement with those of Ashida *et al.* [20] who found that ticlopidine activated basal and PGE_1-stimulated activity of adenylate cyclase through increase in affinity of the cyclase in platelet membrane to PGE_1. However, experiments of Ashida *et al.* were performed with micromolar concentrations of PGE_1 whereas our results were obtained with nanomolar concentrations of PGE. In most studies done with rats including the present one, blood samples were collected under anaesthesia. Concerning ticlopidine, although rats were anaesthetised, the synergism with PGE_1 was in accordance with the result obtained by Johnson *et al.* [25] in man without anaesthesia. The action of dipyridamole is on the other hand somewhat controversial. *In vitro* it provided a clear synergism with PGI_2 [13, 26]. On the contrary, ingestion of dipyridamole is reported to reduce the inhibitory effect of PGI_2 on human platelets [27]. The present data show that, dipyridamole did not enhance the anti-platelet action of PGE_1 and neither did aspirin nor sulfinpyrazone in contrast to ticlopidine which was still efficient even in presence of proaggregating concentration of PGE_2. It is pertinent to note that Knudsen *et al.* [8] showed that ticlopidine ingestion was efficient in controlling the platelet aggregation in patients with hyperaggregability *in vitro* defined by a low threshold concentration of ADP. Although the reason for this hyperactivity was unknown, decreased platelet sensitivity to PGI_2 has been described in patients with myocardial ischaemia [28]. An elevated level of cAMP has been associated with an inhibition of platelet aggregation. On the other hand aggregating agents, through different pathways are thought to involve in some common steps the release of Ca^{2+} from storage bodies (mitochondria, dense tubular system, dense bodies) to the cytosol. Collagen, ionophore A 23187 [29], thrombin [30] or platelet activating factor (PAF) [31] activate Ca^{2+}-dependent kinases which phosphorylate a number of specific proteins. One kinase has now been identified as that of the light chain of myosin and its activation requires in a fore-step the interaction of Ca^{2+} with calmodulin [32]. On the other hand, inhibitors of platelet aggregation and release reaction like dibutyryl-cAMP, PGE_1 induce phosphorylation of another group of specific proteins, in rabbit [31] and human [29] platelets. One of these proteins could be identical to the phospholamban-like membrane protein demonstrated by Kaser-Glanzmann *et al.* to be phosphorylated in presence of cAMP and ATP. This protein appears to play an essential role in the regulation of the intra-cellular Ca^{2+} by taking up the divalent ion into accumulating vesicles [33].

As ticlopidine is active against a variety of agonists of platelet stimulation (aggregation as well as release) its synergism with PGE_1 may be due to the elevation of basal and PGE_1-induced cAMP level in the platelets as demonstrated by Ashida *et al.* [20] at high concentrations of PGE_1 but an alternative to this mechanism or complementary to it, an inhibition by enhancement of cAMP-dependent phosphorylation (e.g. of phospholamban-like proteins) and/or by impairment of Ca^{2+}-dependent phosphorylation (e.g. of light chain myosin) would also be in agreement with the suggested site of action of ticlopidine in the scheme of Marguerie *et al.*, where divalent ions are required. Further investigations are needed to clarify this point.

In summary, ticlopidine administration to rat resulted in a strong long-lasting inhibition of ADP-induced platelet aggregation with a normal shape change. Among the other drugs tested: dipyridamole, sulfinpyrazone and aspirin, only the latter was active at low concentration of ADP. Specific to the action of ticlopidine was a synergism with PGE_1 against platelet activation. This synergism still persisted in the presence of a proaggregating concentration of

PGE_2. This fact may explain its efficiency even in pathological conditions of hyperaggregability with decreased sensitivity of platelets to PGI_2 or PGE_1. Aspirin, dipyridamole and sulfinpyrazone treatments were without effect. The mechanism of action of ticlopidine was discussed.

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Hydroxylation of hexobarbital and benzo[*a*]pyrene by hepatic microsomes isolated from the fetal stumptailed monkey (*Macaca arctoides*) A developmental study

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It is now well documented [1–3] that liver obtained from human fetuses as well as from fetuses of nonhuman primates possesses relatively well developed oxidative, hydrolytic and conjugative drug-metabolizing enzyme systems early in gestation. However, only one study has been published which follows the development of human fetal hepatic drug metabolism as a function of gestational age [4]. Due to legal and ethical restrictions, this study was limited to the first 20 weeks of gestation. To gain insight into the development of fetal hepatic drug metabolism and its significance to the developing human, we proposed the nonhuman primate, specifically the stumptailed macaque (*Macaca arctoides*), as an appropriate animal model for such studies [5–9]. The purpose of this investigation was to follow the development of benzo[*a*]pyrene and hexobarbital hydroxylase in microsomes isolated from the fetal stumptailed mon-