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Thrombolytic action of ticlopidine: possible mechanisms

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Abstract

Ticlopidine (Ticlide), an anti-platelet drug with a broad scope of clinical applications, is claimed to be an antagonist of adenosine diphosphate on platelet receptors. In vitro this antagonism cannot be demonstrated. Ex vivo it is detectable many hours after oral administration of the drug, perhaps subsequently to its biotransformation to an unknown metabolite. Here, we report for the first time that in patients with peripheral arterial disease and in cats with extracorporal circulation ticlopidine evokes instantaneous thrombolytic or fibrinolytic effects which are not associated with inhibition of platelet aggregation. Shortening of euglobulin clot lysis time and increase in plasma levels of tissue plasminogen activator were observed 1–2 h after oral ingestion of ticlopidine at a single dose of 500 mg. In cats ticlopidine produced instantaneous anti-thrombotic and thrombolytic effects at doses of 0.3–1 mg/kg and 10–15 mg/kg i.v., respectively. Thrombolysis by ticlopidine (10 mg/kg i.v.) was comparable to that by prostacyclin at a dose of 0.3 μg/kg i.v. Ticlopidine at a concentration of 100 μM increased endothelial thromboresistance in vitro. The drug did not inhibit the activity of cyclooxygenase-1 or 12-lipoxygenase while it inhibited lipid autooxidation (IC_{50} = 18 μM) in rat liver microsomes. Our data point to a possibility that the therapeutic efficacy of ticlopidine might be associated not only with its delayed anti-platelet effects but also with its immediate thrombolytic action which is likely to be mediated by endothelial prostacyclin and tissue plasminogen activator rather than by platelet mechanisms.

Keywords: Ticlopidine; Thrombolysis; Anti-platelet potency; Prostacyclin; Nitric oxide (NO); Tissue plasminogen activator

1. Introduction

Ticlopidine hydrochloride, i.e. [5-(2-chlorophenyl)methyl]-4,5,6,7-tetrahydro-thieno[3,2-*c*]pyridine hydrochloride (Ticlide) is an anti-platelet drug which is used to prevent myocardial infarction (Verstraete, 1994), restenosis after coronary angioplasty (Bertrand et al., 1990), platelet consumption during extracorporal circulation in heart surgery (Installe et al., 1981), thromboembolism in patients with prosthetic heart valves (Hayashi et al., 1994), ischemic stroke (Gent, 1993; Albers, 1995), peripheral arterial obstructive disease (Blanchard et al., 1994), thrombosis of arterio-venous shunts in hemodialyzed patients with renal failure (Milutinovic et al., 1993) and complications of diabetic retinopathy (TIMAD, 1990; Guillausseau, 1994). The mechanism of the beneficial action of ticlopidine is assigned to a selective blockade by its unknown and unstable metabolite of the methylthio-adenosine diphosphate sensitive receptors for adenosine diphosphate on the platelet membrane. (Gachet et al., 1990; Defreyn et al., 1991; Mills et al., 1992; Savi et al., 1994). Stimulation of this particular type of receptors by adenosine diphosphate is associated with inhibition of adenylate cyclase and a fall in levels of intraplatelet cyclic 3′,5′-adenosine monophosphate. An unstable metabolite of ticlopidine is thus assumed to preserve cyclic adenosine monophosphate in platelets. It is no wonder that ticlopidine potentiated the anti-aggregatory effect of endogenous prostacyclin on platelets (Bruno, 1983). Other than this, ticlopidine influences neither activity of enzymes (e.g. cyclooxygenase-1, thromboxane synthase, cyclic adenosine monophosphate phosphodiesterases) nor functioning of any other type of receptors in platelets (Harker and Bruno, 1993). Unlike aspirin, ticlopidine has to be given orally for several days before its ex vivo irreversible anti-platelet effect develops fully (Gent et al., 1989). The concentrations of ticlopidine

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which are required to inhibit adenosine diphosphate-induced platelet aggregation in vitro (0.1–3.0 mM) are by far higher than those attainable in blood (1–10 μM) after its oral administration (Maffrand et al., 1988). The pharmacodynamics and pharmacokinetics of ticlopidine have been thoroughly studied (Teitelbaum, 1993) and no correlation was found between the ex vivo antiaggregatory action of ticlopidine and the plasma levels which followed its ingestion at doses of 500 or 750 mg daily.

In the light of the above we were struck by an observation that, in our cat model for assaying thrombolysis in vivo (Gryglewski et al., 1978; Gryglewski, 1995), an intravenous administration of ticlopidine produced an immediate thrombolytic response. Following this observation we looked for possible mechanisms of this unexpected, instantaneous action of ticlopidine in vivo. We cannot avoid putting an embarrassing question: is it really the anti-aggregatory effect of ticlopidine ex vivo which is responsible for its therapeutic efficacy in vivo?

2. Materials and methods

2.1. Fibrinolytic action of ticlopidine in man

2.1.1. Euglobulin clot lysis time

12 male patients aged 45–67 years (mean 56 years) with peripheral arterial disease entered this study. Plasma fibrinolytic activity was measured by the method of Von Kaulla and Schultz (1958). Briefly, venous blood which contained tri-sodium citrate (3.8% w/v) at a ratio of 4 : 1 was centrifuged at room temperature at 2000 × *g* for 5 min to produce platelet poor plasma. Distilled water was added (14 ml/ml of platelet poor plasma) and the pH was adjusted to 5.4 by bubbling with CO_2 gas. This procedure causes precipitation of the euglobulin fraction, while the acidity destroys the biological activity of plasminogen activator inhibitor-1. After a second centrifugation at 2000 × *g* for 5 min the supernatant was discarded and the euglobulin precipitate was dissolved in 1 ml of phosphate buffer (13.4 mM KH_2PO_4/53.6 mM Na_2HPO_4). To induce clotting 10 μl of solution of 200 U thrombin/ml in 0.05 M $CaCl_2$ was added. The clot was incubated at 37°C and the time required for complete lysis was recorded.

2.1.2. Plasma levels of tissue plasminogen activator and plasminogen activator inhibitor-1

The activity of tissue plasminogen activator and of plasminogen activator inhibitor-1 was assayed in plasma, using commercially available kits (Biopool, Sweden), according to the kit manual.

2.2. Platelet aggregability in man

2.2.1. Adenosine diphosphate-induced platelet aggregation

Venous blood which contained tri-sodium citrate (3.8% w/v) at a ratio of 9 : 1 was centrifuged at room temperature at 200 × *g* or at 2000 × *g* for 10 min. In this way platelet rich plasma or platelet poor plasma was obtained, respectively. The platelet count in platelet rich plasma was adjusted with homologous platelet poor plasma to a count of 2×10^8 platelets/ml.

The threshold pro-aggregatory concentrations of adenosine diphosphate were determined in a Born aggregometer (Born, 1962). They were expressed as EC_{50} of adenosine diphosphate-induced aggregation.

2.2.2. Spontaneous platelet aggregability

Blood samples (0.5 ml) were drawn from the antecubital vein directly into two separate polypropylene syringes. One contained 2 ml of buffered ethylenediaminetetraacetic acid (EDTA) formalin solution and the other 2 ml of isotonic buffered EDTA solution at pH 7.4. After thorough mixing the contents were transferred to two polypropylene tubes and centrifuged at 200 × *g* for 10 min at 22°C to obtain platelet rich plasma. Platelet counts in both samples were determined in a Bürker chamber and the results were expressed as the ratio of platelet aggregates, i.e. the ratio of the platelet count in a mixture of EDTA-formalin-platelet rich plasma to the platelet count in a mixture of EDTA-platelet rich plasma (Wu and Hoak, 1974).

2.3. Antithrombotic and thrombolytic actions of ticlopidine in cats

We used our in vivo model for studying thrombolytic or anti-thrombotic properties of drugs (Gryglewski et al., 1978; Gryglewski, 1995). In the extracorporal circulation of anaesthetized (sodium pentobarbital 30 mg/kg i.p.) and heparinized (2500 U/kg i.v.) cats a collagen strip was superfused with arterial blood (2 ml/min). The gain in weight of the strip was monitored continuously and after 20 min of superfusion it levelled at 200–500 mg of the freshly made thrombi which adhered to the collagen surface. Microscopically, the thrombi consisted mainly of platelet aggregates, a few erythrocytes, leukocytes and scan patches of fibrin. Intravenous injections of thrombolytic agents produced a loss in weight of the preformed thrombi which were attached to the blood-superfused strips. In this model thrombolytic action was induced by prostacyclin at doses of 0.1–1.0 μg/kg i.v. and by nitric oxide donors (e.g. glyceryl trinitrate, 30 μg/kg i.v.). Aspirin (1–50 mg/kg i.v.) had no thrombolytic effect of its own, although it exerted an antithrombotic action, preventing the build up of clots when administered intravenously before blood was allowed to superfuse the collagen strip. Recombinant tissue plasminogen activator (5–15 μg/kg i.v.) or streptokinase (3000 U/kg i.v.) produced a biphasic thrombogenic/thrombolytic response (Gryglewski, 1995).

Ticlopidine at doses from 0.3 to 30 mg/kg i.v. was tested for its thrombolytic and anti-thrombotic properties in 28 mongrel cats of either sex, 2–3 kg body weight in which arterial blood pressure was also recorded.

2.4. Thromboresistance of rabbit aortic endothelium in vitro

A tubular segment of rabbit thoracic aorta was turned inside out and its endothelial surface was superfused (2 ml/min) with citrated (3.8%) rabbit blood at room temperature (Korbut et al., 1990). A gain in its weight occurred partly as a result of thrombogenesis and partly because of soaking with plasma. When this last was eliminated endothelial thromboresistance was inversely proportional to the net weight of thrombi formed. Basal thromboresistance was decreased by blocking the production of prostacyclin and nitric oxide. To this purpose rabbit aortic tubes were soaked for 10 min in solutions of N^G-monomethyl-L-arginine (300 μM) and aspirin (100 μM). Ticlopidine (30–100 μM) was preincubated for 1 h with blood before the blood was used for superfusion. Streptokinase (100–2000 U/ml) was used as a standard thrombolytic agent. Streptokinase produced a biphasic response, similar to that observed in cats in vivo (Gryglewski et al., 1995), i.e. paradoxical thrombogenesis was followed by protracted thrombolysis. A thromboxane synthase inhibitor (camonagrel, 30 μM) when added to superfusing blood abolished the first phase of the action of streptokinase, while its thrombolytic phase was augmented. Aspirin (100 μM) did not influence the profile of action of streptokinase

2.5. Antioxidant properties of ticlopidine in vitro

2.5.1. Generation of malondialdehyde by rat liver microsomes

A buffered suspension of rat liver microsomes (1.5 mg/ml) was incubated with ferric chloride (100 μM) and ascorbic acid (100 μM) at 37°C for 20 min with or without ticlopidine (5–50 μM). The reaction was stopped with 1 ml of 20% trichloracetic acid and the mixture was centrifuged at 1000 × *g* for 5 min. The supernatant with 1 ml of 0.67% thiobarbituric acid was kept in boiling water for 20 min, cooled and the extinction was read at 535 nm to assay for malondialdehyde. The IC_{50} for inhibition of malondialdehyde formation by ticlopidine was calculated (Robak and Gryglewski, 1993).

2.5.2. Scavenging of superoxide anions generated by the xanthine / xanthine oxidase system

Superoxide anions (O_2^-) were generated by xanthine oxidase (8 mU/ml) from xanthine (100 μM) and detected with ferricytochrome *c* (25 μM) in 0.1 M phosphate buffer pH 7.4 at 25°C as superoxide dismutase-sensitive reduction. Catalase (370 U/ml) was added to prevent the re-oxidation of ferrocytochrome *c*. Δ_E was measured for 5 min at 550 nm. The effect of ticlopidine (100 μM) on the generation of O_2^- was measured (Robak and Gryglewski, 1993).

2.5.3. Assay of activity of lipoxygenases and cyclooxygenase

The effects of ticlopidine (up to 1000 μM) on the activity of soybean 15-lipoxygenase, horse platelet 12-lipoxygenase and bovine seminal vesicle microsome cyclooxygenase-1 were assayed with methods described previously (Duniec et al., 1983).

2.6. Statistical analysis

All data are expressed as the means ± S.D. of *n* experiments. Statistical analysis was performed using the paired Student's *t* test.

3. Results

3.1. Fibrinolytic action in man

Ingestion of ticlopidine at a dose of 500 mg by 12 patients with peripheral arterial disease with a mean euglobulin clot lysis time of 96 ± 24 min resulted in lysis time shortening after 1 h by 23% ($t = 5.90$, $P < 0.01$), after 2 h by 43% ($t = 9.77$, $P < 0.001$), after 3 h by 39% ($t = 8.84$, $P < 0.001$), and after 6 h by 26% ($t = 3.87$, $P < 0.05$). Fig. 1 shows actual values of the euglobulin clot lysis time and their mean (bold line) for each patient.

In another group of five patients, 2 h after ingestion of ticlopidine at a dose of 500 mg, the plasma activity of tissue plasminogen activator rose from the control level of 0.78 ± 0.20 to 1.32 ± 4.0 IU/ml ($t = 2.58$, $P < 0.05$), whereas the plasma levels of plasminogen activator inhibitor did not change – control levels 19 ± 4.2 compared with 18 ± 4.0 IU/ml after ticlopidine ($t = 0.20$, $P > 0.1$)

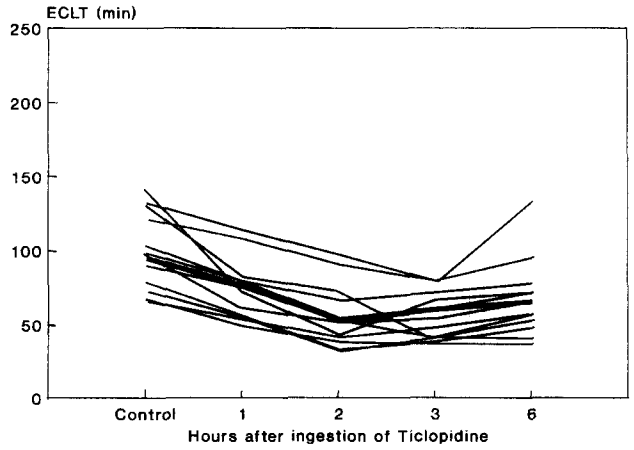

Fig. 1. Euglobulin clot lysis time (ECLT) in 12 patients with peripheral arterial disease 1–6 h after ingestion of ticlopidine (500 mg p.o.). Bold line represents the mean value of ECLT.

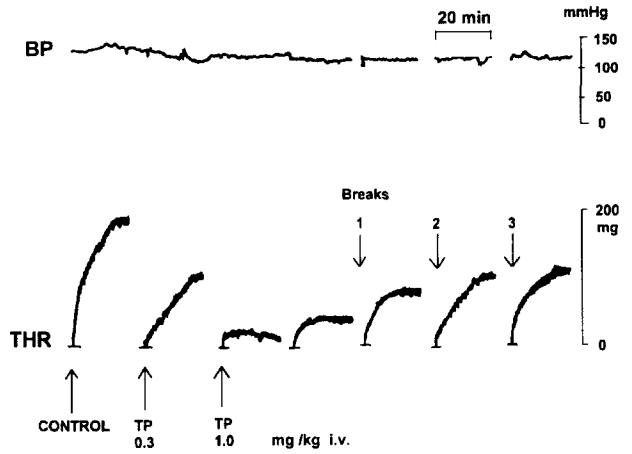

Fig. 2. Antithrombotic action of ticlopidine in a cat. Breaks numbered 1, 2 and 3, lasted 30 min each; the last assay was recorded 3 h after injection of the drug at a dose of 1 mg/kg i.v. BP: mean arterial blood pressure; THR: weight of the thrombus formed on the surface of a collagen strip which was continuously superfused with heparinized arterial blood.

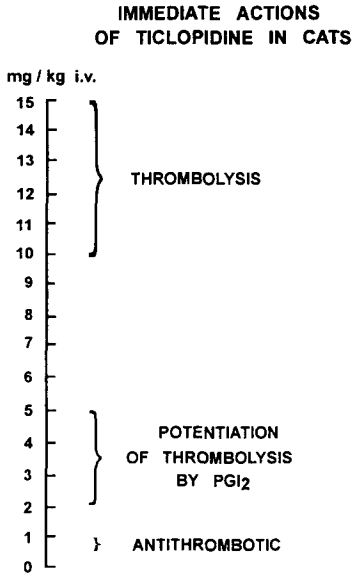

Fig. 4. A scheme of dose dependence of immediate actions of ticlopidine on haemostasis in cats in vivo.

3.2. Platelet aggregability in man

In a group of ten patients with peripheral arterial disease, 2 h after ingestion of ticlopidine at a dose of 500 mg, neither the threshold pro-aggregatory concentration of adenosine diphosphate (4.4 ± 0.8 vs. 4.7 ± 1.1 μM) nor spontaneous platelet aggregability (0.65 ± 0.1 vs. 0.67 ± 0.1) was changed.

3.3. Antithrombotic and thrombolytic actions in cats

In cats, ticlopidine at a dose of 0.3 mg/kg i.v. produced an immediate, weak and short-lasting (< 1 h) antithrombotic effect. Ticlopidine at a dose of 1 mg/kg prevented the formation of thrombi by 70%. Half of this antithrombotic potency was still recorded after 3 h (Fig. 2). Ticlopidine required high doses, 10–15 mg i.v., to produce thrombolysis of $31 \pm 5\%$ ($n = 7$) with a delay of 2–6 min as compared to $30 \pm 4\%$ ($n = 12$) thrombolysis by prostacyclin at a dose of 0.3 μg/kg i.v. (Fig. 3). Ticlopidine at a dose of 2 mg/kg i.v. had no thrombolytic action of its own, however, it potentiated the thrombolytic response to prostacyclin (0.3 μg/kg, i.v.) up to $47 \pm 6\%$ ($n = 8$) (Fig. 4). Pretreatment with a high dose of aspirin (50 mg/kg, i.v.) reduced the immediate thrombolytic response to ticlopidine (10 mg/kg i.v.) to $12 \pm 4\%$ ($n = 4$).

3.4. Thromboresistance of rabbit aortic endothelium

Natural endothelial thromboresistance allowed aortic specimens to gain in weight by 120 ± 10 mg ($n = 25$). A

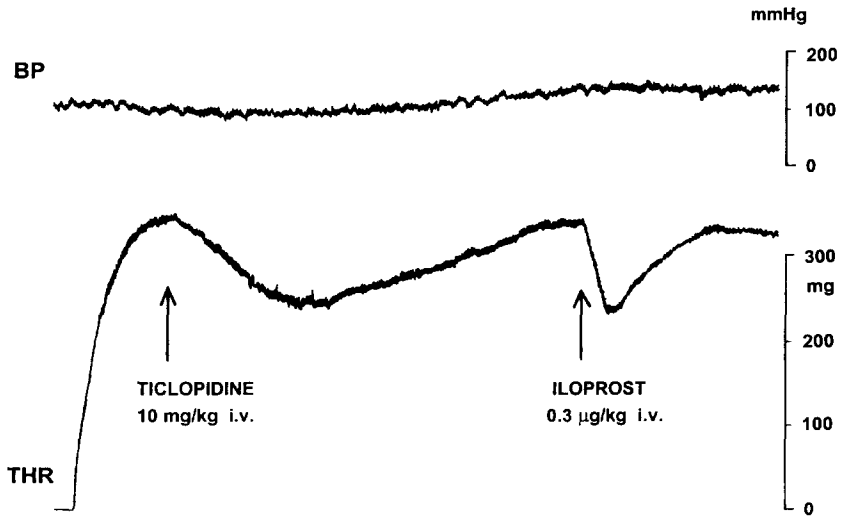

Fig. 3. Comparison of thrombolytic action of ticlopidine (10 mg/kg i.v.) with that of iloprost (0.3 mg/kg i.v.) in a cat. BP: mean arterial blood pressure; THR: weight of the thrombus formed on the surface of a collagen strip was continuously superfused with heparinized arterial blood.

decrease of 119 ± 6% ($n = 4$) in their thromboresistance was achieved after pharmacological inactivation of endothelial cyclooxygenase and nitric oxide synthase. Ticlopidine then (30 or 100 μM) inhibited thrombogenesis by 17 ± 5% ($n = 7$) or by 72 ± 3% ($n = 7$), respectively. The anti-thrombotic potency of ticlopidine resembled that of aspirin.

3.5. Antioxidant properties in vitro

Ticlopidine inhibited the formation of malonedialdehyde by rat liver microsomes ($IC_{50} = 18 \pm 4$ μM, $n = 8$). At higher concentrations (100 μM) ticlopidine also scavenged by 34 ± 13% ($n = 6$) superoxide anions which were generated by xanthine oxidase.

Ticlopidine at a concentration of 1000 μM had no effect on the activity of cyclooxygenase-1 ($n = 6$) and platelet 12-lipoxygenase ($n = 6$), while soybean 15-lipoxygenase was very slightly inhibited by ticlopidine ($IC_{50} = 416 \pm 53$ μM, $n = 6$).

4. Discussion

Unlike aspirin, ticlopidine develops its clinical action a few days after ingestion (Gent et al., 1989). Only then does ticlopidine show its full anti-aggregatory action on platelets ex vivo. It is accepted that beneficial effects of ticlopidine depend on its delayed platelet-suppressant potency. We have now shown that, in humans and cats, ticlopidine evokes fast anti-thrombotic, thrombolytic or fibrinolytic effects which are not accompanied by suppression of platelets. Depending on dosage this prompt in vivo effect tends to vanish before any effect on platelets has a chance to appear. In the light of the above one may wonder if the ex vivo assay of the anti-aggregatory effect is the best index of antithrombotic or thrombolytic actions of ticlopidine.

In our cat model (Gryglewski et al., 1978; Gryglewski, 1995) ticlopidine showed a prompt but weak and transient thrombolytic action similar to that of prostacyclin or iloprost, although ticlopidine was weaker by five orders of magnitude (Fig. 3). Actually, ticlopidine at a non-thrombolytic range of doses doubled the thrombolytic effect of exogenous prostacyclin and, at still lower doses, exerted an immediate anti-thrombotic effect. In our cat model the effects of aspirin, ticlopidine or prostacyclin on haemostasis differed greatly. Ticlopidine and prostacyclin were thrombolytic and anti-thrombotic whereas aspirin was only anti-thrombotic.

The above data prompted us to seek for fibrinolytic effects of ticlopidine in patients with atherosclerosis. Indeed, in humans, ticlopidine activated fibrinolysis rapidly, as was shown by the shortening of the euglobulin clot lysis time and a rise in plasma levels of tissue plasminogen activator. At the same time, ticlopidine showed almost no effect on indices of platelet activation such as adenosine diphosphate-induced aggregation, spontaneous platelet aggregation or plasma levels of platelet activator inhibitor-1. In humans and in cats, ticlopidine perhaps exerts its brisk effect on haemostasis through endothelial mechanisms such as the release of prostacyclin, nitric oxide or tissue plasminogen activator, rather than through platelet mechanisms.

Indeed, prostacyclin is thrombolytic in experimental animals (Gryglewski et al., 1978) and in humans (Szczeklik et al., 1983; Loosemore et al., 1994), sharing this property with nitric oxide (Gryglewski, 1995). The release of prostacyclin and that of nitric oxide are coupled (De Nucci et al., 1988), and although the mechanisms of their fibrinolytic actions are not necessarily the same (Gorman et al., 1977; Moore et al., 1988; Gryglewski, 1995) they supplement each other. The releasers of prostacyclin such as nicotinic acid, defibrotide or kallikrein are thrombolytic in our cat model (Grodzińska et al., 1993; Gryglewski, 1995). Like these agents, ticlopidine augmented the thrombolytic action of exogenous prostacyclin in cats while its own thrombolytic action was reduced by the pretreatment with a high dose of aspirin. Anti-oxidant properties of ticlopidine may contribute to the protection of endogenous prostacyclin and/or nitric oxide from destruction by oxygen free radicals (Moncada et al., 1976; Gryglewski et al., 1986). Our data on the increase of tissue plasminogen activator levels in plasma of patients treated with ticlopidine may point to the endothelial release of tissue plasminogen activator, especially as a fall in plasminogen activator inhibitor-1 was not observed. This line of reasoning is supported by a finding that clopidogrel, a new congener of ticlopidine, prevents reocclusion of injured canine coronary arteries following therapy with tissue plasminogen activator (Yao et al., 1994).

Lack of an immediate anti-aggregatory effect of ticlopidine in vivo (Gent, 1993; present data) does not prevent speculation that the drug may still exert its thrombolytic action through platelets. Platelets have the potency to inhibit fibrinolysis by releasing plasminogen activator inhibitor-1, α_2-anti-plasmin and factor XII. Platelets may also inhibit the binding of tissue plasminogen activator to fibrin (Keijer et al., 1991). On a more long-term basis the release of growth factors such as TGFβ from platelets may modify fibrinolytic components of endothelial cells (Fujii et al., 1989).

Some of these platelet-mediated fibrinolytic mechanisms were claimed to be responsible for thrombolytic properties of nitric oxide donors (Lidbury et al., 1990; Corell et al., 1994; Korbut et al., 1995). However, our present results do not support this line of reasoning for ticlopidine.

In our in vitro studies, we failed, as have many others, to elucidate the mechanisms of in vivo actions of ticlopidine. Ticlopidine did not interfere with the activity of cyclooxygenase and of lipoxygenases. Its scavenging ef-

fect on superoxide anions was rather weak. Ticlopidine at concentrations of 10–20 μM inhibited lipid autooxidation in rat liver microsomes, however, it increased endothelial thromboresistance only at much higher concentrations.

In summary, in humans and cats, ticlopidine shows immediate anti-thrombotic, fibrinolytic and thrombolytic actions, and in vitro it increases endothelial thromboresistance and inhibits lipid oxidation. Our data point to a possibility that in vivo thrombolysis by ticlopidine may be mediated by the endothelial release of prostacyclin and/or tissue plasminogen activator.

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