Decrease of platelet intracellular pH and adhesion by ticlopidine in patients with vascular disease

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Background. Ticlopidine inhibits platelet aggregation by preventing the binding of fibrinogen to its platelet receptor. We examined whether this inhibition involved platelet transduction system such as Na^+/H^+ pump and platelet intracellular calcium.

Methods. Platelet adhesion in 13 patients with peripheral vascular disease treated with ticlopidine, 250 mg b.i.d for 30 days, was measured in culture microplates before and after therapy. The microplate wells were coated with human plasma, fibrinogen or collagen, and platelet adhesion was studied in the resting condition and after stimulation with 1 and 10 μM ADP. At the same time, platelet intracellular calcium and ADP-induced calcium increases were measured with the fluorescent indicator Fura 2. In addition, intracellular pH and thrombin-induced pH variations were measured with the fluorescent probe BCECF.

Results. Platelet adhesion to plasma and fibrinogen was significantly reduced (about 50%) after treatment with ticlopidine, while adhesion to collagen was not modified. Basal calcium and ADP-induced calcium increase were not significantly different before and after ticlopidine. Platelet basal intracellular pH was reduced (from 7.44±0.009 to 7.41±0.017, $p<0.05$), but agonist-induced alkalinisation was not significantly different. Early acidification, not dependent on Na^+/H^+ exchange, was also reduced ($p<0.05$).

Conclusions. These data do not seem to support the hypothesis that ticlopidine-induced reduction of platelet adhesion depends on alteration of the mechanisms determining signal transduction, at least as far as basal and post-stimulation intracellular calcium is concerned. On the contrary, the possibility that ticlopidine inhibits the Na^+/H^+ antiport remains open to consideration.

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Key words: Platelet adhesiveness - Ticlopidine - Platelet aggregation inhibitors - Calcium metabolism - Sodium hydrogen antiporter metabolism.

In *ex vivo* studies on healthy volunteers and patients with vascular disease, ticlopidine elicits selective inhibition of ADP-induced aggregation and also inhibits aggregation induced by other agonists, preventing amplification of the activating effects of released ADP. It has been shown that ticlopidine inhibits binding of fibrinogen to its platelet receptor, glycoprotein GP IIb/IIIa, inducing a "functionally thromboasthenic" state [1] and altering the receptor function of the glycoprotein for adhesive molecules. The receptor function of GPIIb/IIIa is triggered by stimulus response-coupling during platelet activation by soluble agonists. Moreover, in intact platelets, the binding of adhesive proteins to the GPs triggers a series of additional events involved in the transduction signal, which indicates that conformational changes and changes in cell metabolic activity are interconnected.[2]

Little is known about the action of ticlopidine on transduction systems for the post-receptor signal. In particular, no consistent and unequivocal data are available on biochemical events, such as intracellular calcium change and the function of the Na^+/H^+ pump, which play an essential role in platelet activation.[3-6] Ticlopidine has been reported to have different effects on intracellular calcium.[3-5,7] The influx of calcium across the membrane, measured in man and in the rat by the Quin 2 method, does not seem to be affected.[3,4,7] Mobilisation of intracellular stores in response to ADP and thrombin has been found to be inhibited in rabbit platelets loaded with Fura 2 [5] and rat platelets loaded with Quin 2,[7] though some A. have not reported any such inhibition.[3,4] In the literature, there are no reports on Na^+/H^+ exchanger activity in patients treated with ticlopidine. All that has been demonstrated is that epinephrine can remove ticlopidine-induced inhibition of ADP-induced aggregation, by a mechanism involving Na^+/H^+ exchanger activation.[6]

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The aim of the present study was to evaluate the effect of ticlopidine, administered for one month to patients with peripheral arterial disease, on the cytosolic calcium concentration and cytosolic pHi of platelets, basally as well as after stimulation with ADP and thrombin. In the same patients we tested the effect of the treatment on platelet function by a sensitive new assay of adhesion to various protein substrates.[8] The results show that the drug-induced decrease in platelet adhesion is associated with the decrease of basal pHi and of the thrombin-induced acidification phase, while no modifications of intracellular calcium were found.

Materials and methods

Patients

We studied 13 patients (11 males, 2 females; mean age 65.5 yrs, range 44-80 yrs), with Fontaine stage II peripheral vascular disease which had been stable for at least 6 months. Patients received ticlopidine 250 mg b.i.d. for 30 days, following a 20-day wash-out period. One patient also had ischaemic heart disease, and 2 had aneurysms of the abdominal aorta. All patients gave informed consent to participation in the study. Blood samples were taken at 8.00 a.m., following overnight fasting, before and after 30 days of oral administration of the drug.

Preparation of platelet suspension

A sample of 40 ml blood was drawn by venipuncture and 8.4 ml aliquots of blood were added to 1.6 ml of ACD (citric acid 15 g/L, 20 g/L dextrose, 25 g/L sodium citrate) in plastic test tubes. Platelet-rich plasma (PRP) was prepared by centrifugation at about 120 g for 10 min.

For adhesion studies, PRP was re-centrifuged at 700 g for 15 min and platelets were gently suspended (5×10^7/ml) in a buffer consisting of 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM Na_2HPO_4, 6 mM glucose and 0.2% human serum albumin, pH 7.4. The platelet suspension was kept at room temperature and used within one hour.

Assay of adhesion

Platelet adhesion was measured as previously described [8] in culture microplates, precoated by overnight incubation at +4°C with one of the following solutions: (a) human plasma, diluted 1/1 in phosphate-buffered saline (PBS); (b) 0.2 mg/ml human fibrinogen, in PBS; (c) 20 µg/ml collagen, diluted in 0.9% NaCl. According to preliminary experiments, these doses gave the optimal coating on the plastic surface.[8] Immediately before use, the plates were washed twice with 0.9% NaCl, using an automatic plate washer (Easy Washer 2, SLT Labs Instruments).

Incubation was carried out for 60 min, using a humidified thermostat (37°C, 5% CO_2), in platelet suspension buffer supplemented with 1 mM $CaCl_2$, and 1 mM $MgSO_4$, both with and without the indicated agonist (ADP) concentration. Each well contained 2.5×10^6 platelets.

At the end of incubation, the plate was transferred to an automatic washer and subjected to 2 washing cycles with PBS at room temperature. Adherent platelets were measured by the acid phosphatase assay.[8] The percentage of cells was calculated on the basis of a standard curve, obtained with a known number of platelets from the same subject. The interassay reproducibility and intraindividual variability were respectively 7% and 4%.

Cytosolic monitoring of platelet pHi

Intracellular pH was measured with a BCECF fluorescent probe, according to the method described by Grinstein *et al.*[9] The platelet suspension ($20\text{-}40\times10^6$/ml) in the same buffer as that for adhesion with a final concentration of $CaCl_2$, 1 mM was placed in quartz cuvettes in a Hitachi F 2000 spectrophotometer (Hitachi LTD, Tokyo, Japan) and stirred with a magnetic rod. The BCECF fluorescence (λ excitation: 490 nm; λ emission: 530 nm; slit width: 10 nm) was recorded on a chart recorder. The measurements were performed at 37°C. First, intraplatelet pH of platelets incubated with BCECF-AM, both alone and with 0.1, 1, 10 µg/ml of ticlopidine, was assayed. No ticlopidine-related changes were observed. Thrombin was used as an agonist for the study of the Na^+/H^+ pump because in our experimental conditions, ADP gave far weaker alkalinisation partly masked by acidifi-

cation.[10] Within 10-20 seconds of stimulation there is a first, very brief acidification phase. This is followed after about 3-5 seconds by the alkalinisation phase, which continues stably for some minutes (at least 5) and is far more marked when thrombin is used. In a number of experiments, we studied the effects of amiloride (200 µg/ml) *in vitro* on the Na^+/H^+ pump stimulated by thrombin (1 UI/l) as well as on platelet adhesion induced by ADP 10 µM and by thrombin (0.01-0.1 U/ml). The interassay variability and intra-assay reproducibility for pHi determination were respectively 4% and 3.2%.

Cytosolic calcium concentration

Cytosolic calcium concentration was measured in platelets with the fluorescent indicator Fura 2, as previously described by Grynkiewicz *et al.*[11] After loading with 2 µM Fura 2 AM in platelet rich plasma (PRP), the platelets were collected by centrifugation and suspended (75×10^6 cell/ml) in the same buffer as for adhesion. The external calcium and magnesium concentration was restored to 1 mM. Fluorescent measurements were recorded at 37°C by F-2000 fluorescence spectrophotometer, using excitation wavelengths 340/380 nm and emission wavelengths 500 nm. Basal and ADP (10 µM)-stimulated calcium, before and after ticlopidine, were then measured recording the platelet suspension fluorescence.

Statistical analysis

Data are expressed as means±SEM. Results were analysed statistically, using Student's "t"-test for paired data (pre- *versus* post-therapy for each patient). Results with $p<0.05$ were regarded as significant.

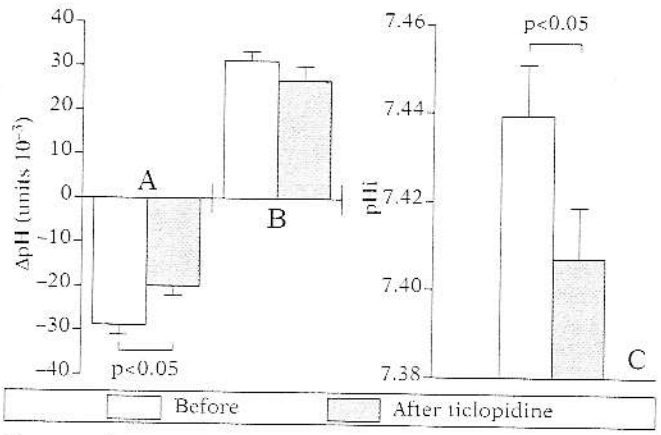

Fig. 1.—Thrombin-induced variations of intracellular pH (Δ pHi), measured as acidification (A) and alkalinisation (B), and basal pHi (C) before and after treatment with ticlopidine.

Results

Figure 1 shows the changes of the platelet Na^+/H^+ pump following one month of treatment with ticlopidine.

The treatment significantly reduced basal pHi and the thrombin-induced acidification phase (1 U/ml). For the alkalinisation phase, mean alkalinisation values were lower after treatment (pH variations 33.3±2.4 mU before *vs* 26.1±3.4 mU after), though this decrease was not statistically significant. Ticlopidine did not affect basal

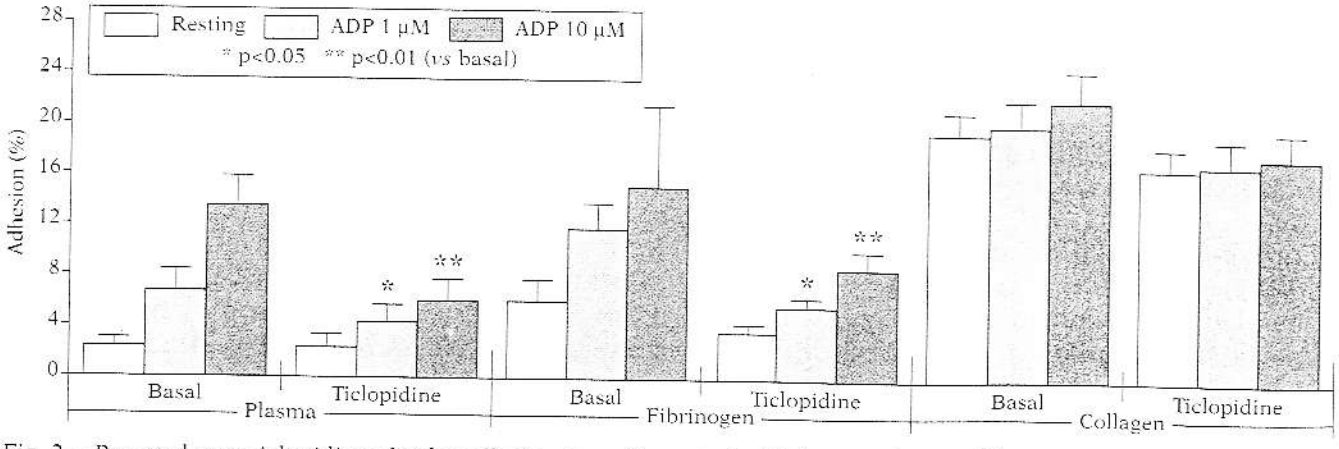

Fig. 2.—Pre- and post-ticlopidine platelet adhesion to wells coated with human plasma, fibrinogen and collagen, in resting conditions and after ADP stimulation (1 µM and 10 µM), (mean±SEM).

and post-ADP stimulation intracellular calcium, measured in the presence of extracellular calcium 1 mM: 41±3 nM and 304±38 nM before ticlopidine *vs* 48±6 nM and 351±27 nM after ticlopidine, respectively.

Figure 2 shows platelet adhesion basally, and after stimulation with ADP 1 and 10 μM. ADP at these doses activated adhesion to the human plasma and fibrinogen substrates. When collagen was used as a coating, adhesion was almost maximal even without ADP, which increased platelet adhesion only slightly.

Treatment with ticlodipine caused a marked (about 50%), statistically significant reduction in ADP-dependent adhesion to human plasma and fibrinogen, but did not affect adhesion to collagen. Addition of amiloride *in vitro* significantly reduces both the Na^+/H^+ pump and adhesion; in both cases an inhibitory effect was observed, about 40% for the Na^+/H^+ pump and 30-40% for adhesion (data not shown).

Discussion and conclusions

The inhibitory effect of ticlopidine on platelet function is a matter of active investigation, but the underlying mechanisms are still unclear.[1,3,12-14] It is unlikely that inhibition of fibrinogen binding induced by ticlopidine can be attributed to direct modification of the GPIIb/IIIa complex.[15] In fact, recent studies showed that it is quantitatively unaffected and different monoclonal antibodies recognize GPIIb/IIIa in the same way before and after treatment.[1,3,15] Ticlopidine-induced inhibition of some GPIIb/IIIa-independent platelet responses to ADP also suggests that the drug can block some earlier stages in the transduction signal between the ADP receptor and activation of the fibrinogen binding sites.[12] More recently, the possible action of an analogue of ticlopidine on the ADP receptor [13] and the interaction of this receptor with its G protein [14] have also been examined.

In this study, ADP-induced adhesion, measured on microplates coated with both human plasma and fibrinogen, was significantly reduced after one month of ticlopidine. When the well was coated with collagen, marked adhesion occurred even without the agonist, and there was no change after treatment. This finding is consistent not only with the constitutional expression of collagen receptors on the platelet membrane but also with the twin role of the platelet receptor for collagen (VLA-2), as both receptor for adhesive proteins and receptor-agonist.[16,17] Basally and with any type of coating, adhesion of non-stimulated platelets is not affected by treatment with ticlopidine. The explanation for this could be that the action of ticlopidine is not exerted directly on the adhesion protein, but only on one of the steps leading to its enhanced ADP-dependent function.

Therapy with ticlopidine affected neither basal nor ADP-stimulated $[Ca^{2+}]_i$. Our data are thus consistent with those of Hardisty [3] who, using Quin 2, detected no effect of ticlodipine on human platelets in terms of either calcium influx or mobilisation in response to ADP, thrombin and ionomycin. With regard to the Na^+/H^+ exchanger, this is the first study directly concerning the activity of this pump in treatment with ticlopidine. Ticlopidine significantly reduces basal pHi, i.e. when measured *in vivo* under static conditions, suggesting a decrease in basal exchanger activity *in vivo*. However, agonist-induced alkalinization, although reduced in many cases, is not significantly decreased by ticlopidine. This alkalinization is a dynamic event studied *in vitro*, under stress conditions which might explain the discrepancy between the two phenomena. The only finding in the literature which indirectly supports inhibition of Na^+/H^+ exchange by ticlopidine is that the drug blocks the potentiation of ADP-induced aggregation by epinephrine, which can stimulate Na^+/H^+ exchange through a direct effect on the α_2 adrenergic receptors and proteinkinase-C.[6,18] The observed partial *in vitro* inhibition of both the Na^+/H^+ exchanger and adhesion induced by amiloride suggests that the two phenomena might be related.

As far as the reduced initial acidification by the agonist is concerned, the early acidification is a phenomenon independent from Na^+/H^+ exchanger activity, be it an agonist-induced increase in proton production, an increase in Ca-ATPase activity or an artefact related to shape change.[19] It seems, however, to be linked to the first stage in cellular activation and, in this respect, its modification after ticlopidine therapy is of interest.

In conclusion, consistent with previous reports in the literature, biological response in terms of ADP-induced platelet adhesion to plasma and fibrinogen is significantly decreased by ticlopidine. However, this reduction does not seem to be related to a parallel reduction of intracellular calcium. There is thus a discrepancy between biological response and one of the steps in transduction, i.e. intracellular calcium influx. Though *in vitro* alkalinisation after thrombin does not seem to be significantly reduced, there is a reduction of pIIi measured *in vitro*, which might in some way be related to a decrease in GPIIb/IIIa function. The pHi reduction may give some contribution to the interpretation of mechanisms by which ticlopidine inhibits platelet activation. In terms of Na^-/H^- exchange, however, it is more difficult to base any definitive conclusions on our data and new studies are necessary to clarify this issue.

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