Influence of nitric oxide on the in vitro antiaggregant effect of ticlopidine

J.P. De La Cruz*, M.M. Arrebola, A. Guerrero, F. Sánchez de la Cuesta

Department of Pharmacology and Therapeutics, School of Medicine, University of Málaga, Campus de Teatinos s/n, Málaga 29071, Spain

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Abstract

The aim of this study was to evaluate the influence of leukocyte nitric oxide (NO) production on the antiplatelet aggregant effect of aspirin and ticlopidine. This in vitro study was done with platelets (platelet-rich plasma, PRP) and polymorphonuclear leukocytes (PMNLs) separated from samples of human blood. Collagen-induced platelet aggregation and calcium-dependent NO production by PMNL were quantified. The inhibition of NO production in PRP significantly reduced the antiaggregant effect of aspirin (IC50 2.64-fold greater), whereas it had no significant effect on the effect of ticlopidine (IC50 1.03-fold greater). Incubating PMNL in PRP increased the antiaggregant effect of both aspirin (IC50 5.09-fold lower) and ticlopidine (IC50 10.16-fold lower). The inhibition of NO production in PMNL significantly reduced the antiaggregant effect of both aspirin (IC50 2.21-fold greater) and ticlopidine (IC50 3.26-fold greater). Both drugs increased leukocyte NO production. The concentration of aspirin that raised NO production by 50% was greater than 1000 μM, whereas the concentration of ticlopidine that led to this effect was 9.14±0.87 μM. We conclude that the effect of ticlopidine on leukocyte NO production may constitute an addition mechanism to the IIb/IIIa glycoprotein complex inactivation in the inhibition of platelet activation. © 2002 Elsevier Science Inc. All rights reserved.

1. Introduction

Aspirin and ticlopidine are platelet antiaggregant drugs that have proved useful in the secondary prevention of thrombotic events such as cerebrovascular accidents, coronary ischemic accidents and peripheral artery disease (Antiplatelet Trialist Collaboration, 1994a,b), and in the prevention of diabetic retinopathy (TIMAD Investigators, 1990; ETDRS Study Group, 1991). These two drugs work through different mechanisms of action: aspirin blocks the enzyme cyclooxygenase, whereas ticlopidine prevents the activation of glycoprotein IIb/IIIa complex by platelet ADP (Vane, 1975; Geiger et al., 1998).

An analysis of earlier studies on the prevention of cerebrovascular accidents has shown the preventive effect of aspirin to be significantly lower in patients with diabetes mellitus, whereas the effect of ticlopidine in these patients is not diminished (Grotta et al., 1992). In diabetes mellitus, nitric oxide (NO) production is reportedly reduced (Rabini et al., 1998). In an experimental model of cerebral thrombosis in rabbits, aspirin (Thomas et al., 1995) but not ticlopidine (Bednar et al., 1996) antagonized the thrombolytic effects of recombinant tissue-type plasminogen activator (rtPA), an effect that is prevented by the administration of NO donors (Bendar et al., 1997).

The aim of this study was to determine whether decreased leukocyte NO production modified the antiaggregant effect of aspirin and ticlopidine. It was hoped that the findings would help explain some of the differences in the effects of these two drugs in diabetes mellitus and stroke. An additional aim was to determine whether ticlopidine affects leukocyte NO production, as aspirin is known to do (Lopez Farre et al., 1995; De La Cruz et al., 1987, 2000). In an experimental model of cerebral thrombosis in rabbits, aspirin (Thomas et al., 1995) but not ticlopidine (Bednar et al., 1996) antagonized the thrombolytic effects of recombinant tissue-type plasminogen activator (rtPA), an effect that is prevented by the administration of NO donors (Bendar et al., 1997).

2. Materials and methods

This in vitro study was done in samples of human blood obtained from healthy volunteer donors (men, mean age 37.9±1.6 years) who had not taken any drugs for at least 2 weeks prior to the time blood was withdrawn. One sample was obtained from each donor, and all samples...
were collected with 3.8% sodium citrate at a proportion of 1:10 (vol/vol) as an anticoagulant. All blood samples were obtained between 0900 and 1000 h before the donor had had anything to eat.

2.1. Study samples

To separate the blood fractions, part of the sample of whole blood was centrifuged at 1800 × g for 10 min at 20 °C to yield platelet-rich plasma (PRP). Part of the PRP was centrifuged at 2000 × g for 15 min at 20 °C to obtain platelet-depleted plasma (PDP). Another portion of whole blood was centrifuged with Ficoll gradients (densities of 1077 and 1119) at 1000 × g for 20 min at 20 °C to separate the fraction of polymorphonuclear leukocytes (PMNLs).

All experiments were done with samples of PRP alone and PRP to which PMNLs were added in proportion to the number of cells found in counts of whole blood (4.1 ± 0.3 × 10^9 l^-1) (Coulter MD 10; Coulter, Miami, FL).

2.2. Drugs and reagents used

The drugs investigated were aspirin (Sigma, St. Louis, MO) and ticlopidine (Sanofi-Winthrop, Barcelona, Spain). L-N^G-nitro-arginine-methyl-ester (L-NAME; Sigma), a nonspecific inhibitor of NO synthase, was used to inhibit NO synthesis.

2.3. Analytical techniques

2.3.1. Platelet aggregometry

Platelet aggregation was determined by optical transmission with an Aggrecorder 4210 aggregometer (Menarini Diagnóstica, Barcelona, Spain). Samples of PRP alone and PRP with PMNLs added at the same proportion as in whole blood were used. Samples of PRP were adjusted to 250 × 10^6 platelets/l, and PMNLs were incubated at a concentration of 4.5 × 10^9 cells/l. Collagen (Aggrepack; Menarini Diagnóstica), at a final concentration of 2 μg/ml, was used to induce platelet aggregation.

Drugs were incubated with the samples for 5 min at 37 °C, then collagen was added. In samples of PRP with PMNL, the leukocytes were added to PRP and the mixture was incubated for 5 min at 37 °C, then either aspirin or ticlopidine was added and the mixture incubated again. Percent optical transmittance was recorded continuously for 10 min, and maximum intensity of aggregation was recorded as the maximum rate of optical transmittance in each sample. The results were expressed as percent aggregation referred to transmittance measured in a sample of platelet-poor plasma (PPP).

The concentration of collagen used to stimulate aggregation in samples of PRP was determined in a series of preliminary experiments. The 50% effective concentration (EC_{50}) of collagen was found to be 2.2 ± 0.3 μg/ml (n=10).

2.3.2. Leukocyte production of NO

Cellular NO production was studied in PMNLs obtained in the same way as for the aggregometry experiments. The amount of NO was quantified with an electrochemical method (Shibuki, 1992) that used a specific electrode coupled to an ISO-NO detector (World Precision Instruments, Aston, Stevenage, Hertfordshire, UK).

The experiments were designed to determine NO production initially with the drug under study or with saline solution (control sample) for 5 min at 37 °C. Then L-NAME was added to the sample at a final concentration of 300 μM and the mixture was incubated for 5 min at 37 °C. After this step, calcium A 23187 ionophore was added. The NO electrode was kept immersed in the sample throughout the procedure, so that NO production was recorded constantly. The mean number of PMNLs in the samples used for NO detection was 3.3 ± 0.2 × 10^9 cells/l.

2.4. Statistical analysis

All data given in the text, tables and figures are the mean ± standard error of the mean (S.E.M.) of the values obtained in each type of experiment. The results were subjected to analysis of variance (ANOVA) with the Bonferroni posthoc test. All data were processed with the Statistical Package for the Social Sciences, v. 10.0 for Windows (SPSS, Chicago, IL). The differences were considered statistically significant at P<.05.

3. Results

The addition of L-NAME to samples of PRP significantly reduced the antiplatelet aggregant effect of aspirin, whereas no effect was seen in cultures of PRP with ticlopidine (Figs. 1 and 2). Table 1 summarizes the values of the 50%
inhibitory concentration (IC$_{50}$) for the two drugs. These data show that the IC$_{50}$ for aspirin was 2.64-fold higher after incubation with L-NAME, whereas incubation with ticlopidine led to an increase of only 1.03-fold.

The addition of PMNL to PRP significantly increased the antiaggregant effect of both aspirin and ticlopidine (Figs. 1 and 2, Table 1). The values of IC$_{50}$ were 5.09-fold lower for aspirin and 10.16-fold lower for ticlopidine.

The inhibition of NO synthase activity in cultures of PMNL significantly reduced the antiaggregant effect of both drugs (Figs. 1 and 2, Table 1). The IC$_{50}$ for aspirin was 2.21-fold greater and that for ticlopidine 3.26-fold greater, in comparison with the values measured before L-NAME was added.

Both drugs significantly increased the leukocyte production of calcium-dependent NO (Table 2). The increase was statistically significant for aspirin at a dose of 500 μM or higher, whereas the increase became significant for ticlopidine at a dose of 1 μM. The concentration of aspirin that led to a 50% increase in NO production was greater than 1000 μM, whereas the equivalent concentration for ticlopidine was 9.14±0.87 μM.

### Table 1
Mean values of 50% inhibitory concentration (IC$_{50}$) for aspirin and ticlopidine for collagen (2 μg/ml)-induced platelet aggregation in PRP with or without PMNLs and with or without L-NAME (300 μM) (n=10 samples per group).

<table>
<thead>
<tr>
<th></th>
<th>Aspirin (μM)</th>
<th>Ticlopidine (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without L-NAME</td>
<td>139±11.26</td>
<td>513±38.47</td>
</tr>
<tr>
<td>With L-NAME</td>
<td>367±21.08*</td>
<td>532±39.41</td>
</tr>
<tr>
<td>PRP+PMNL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without L-NAME</td>
<td>27.26±1.93</td>
<td>50.49±4.37</td>
</tr>
<tr>
<td>With L-NAME</td>
<td>60.00±4.88*</td>
<td>165±15.53*</td>
</tr>
</tbody>
</table>

*P<.05 in comparison to the value obtained without L-NAME.

### Table 2
Calcium-dependent NO production in PMNLs after incubation with aspirin or ticlopidine (n=10 samples per group).

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>Aspirin (μmol/10$^6$ cells)</th>
<th>Ticlopidine (μmol/10$^6$ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.80±0.12</td>
<td>1.89±0.14</td>
</tr>
<tr>
<td>1</td>
<td>1.79±0.15</td>
<td>2.54±0.31*</td>
</tr>
<tr>
<td>10</td>
<td>1.88±0.14</td>
<td>2.87±0.29*</td>
</tr>
<tr>
<td>100</td>
<td>1.95±0.19</td>
<td>4.21±0.35**</td>
</tr>
<tr>
<td>500</td>
<td>2.57±0.22*</td>
<td>5.74±0.49**</td>
</tr>
<tr>
<td>1000</td>
<td>2.57±0.24*</td>
<td>5.52±0.58**</td>
</tr>
</tbody>
</table>

**P<.001, in comparison to the control sample (without drug).

*P<.05, in comparison to the control sample (without drug).

### 4. Discussion

Our study shows that PMNLs condition the antiaggregant effect of ticlopidine, possibly by affecting NO production. The effect seen in this drug is proportionally higher than that seen in aspirin.

The antiaggregant effect of ticlopidine was more potent when aggregation was induced with ADP (Geiger et al., 1998). However, in our study, we chose collagen to induce platelet aggregation because this inducer most frequently stimulates platelets in the early phases of activation, as in the subendothelial stimulation by collagen in blood vessels affected in lesions to the vascular wall (Badimon and Badimon, 1996). The relation between PMNL and platelets is connected with (among other factors) NO production by PMNL, which stimulates platelet guanyl cyclase activity and thus inhibits PMNL activation (Radomski and Moncada, 1993). This mechanism operates in phases of the platelet activation process previous to the activation of the glycoprotein IIb/IIa complex, when ticlopidine acts (Geiger et al., 1998). A novel contribution of this study is therefore the finding that ticlopidine can act in phases earlier than platelet aggregation, which is when the IIb/IIa complex is activated.

In our study, the relation between ticlopidine and NO appears to occur in the presence of the PMNL. This was suggested by our finding that in PRP, blocking NO production (by incubation with L-NAME) did not modify the antiplatelet aggregant effect of ticlopidine, whereas L-NAME did have this effect when PMNLs were present in the medium (Fig. 2, Table 1). The effect became evident when we quantified NO production by PMNL and found that ticlopidine stimulated production. An earlier study by De Logeril et al. (1998) showed that ticlopidine did not modify prostacyclin production but did increase systemic NO production in heart transplant recipients. Our results confirm this finding, and we therefore propose that the effect occurs in neutrophils (among, possibly, other types of PMNLs).
of cell. Bednar et al. (1995) showed that ticlopidine non-
specifically increased PMNL functioning; in this con-
nection, it can be assumed that ticlopidine stimulates the
capacity of PMNL to inhibit platelet aggregation.

Earlier studies have also reported that PMNL increased the
antiaggregent effect of aspirin (De La Cruz et al., 1987), and
that aspirin stimulated NO production by PMNL (Lopez
Farre et al., 1995; De La Cruz et al., 2000). In addition to
the fact that aspirin is the antiplatelet aggregator used most
widely in the secondary prevention of thrombotic events,
these effects led us to chose aspirin as the control drug in
this study. Experiments done in vitro have shown that PMNLs
seem to influence the antiplatelet effects of ticlopidine to a
greater extent that they do the effect of aspirin. This effect
would, in principle, be expected to reduce the effect of
ticlopidine in situations in which NO production is low, such
as in patients with diabetes (Rabin et al., 1998). However,
ticlopidine can also be postulated to stimulate NO production
to a greater extent under these circumstances, thus offsetting
the NO deficit typical of the underlying disease.

In conclusion, the effect of ticlopidine on leukocyte
NO production may constitute an additional mechanism
of inhibition of the IIb/IIIa complex activation. This
would imply that ticlopidine exerts its antiplatelet effect
not only during the final phase of platelet activation, but
may also influence mechanisms, such as leukocyte NO
production, that modulate platelet function in earlier stages
of this process.

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References

Antiplatelet Trialist Collaboration, 1994a. Collaborative overview trials of
antiplatelet therapy: I. Prevention of death, myocardial infarction, and
stroke by prolonged antiplatelet therapy in various categories of pa-

Antiplatelet Trialist Collaboration, 1994b. Collaborative overview trials of
antiplatelet therapy: II. Maintenance of vascular graft or arterial patency

Badimon, L., Badimon, J.J., 1996. Interaction of platelet activation and
coaulation. In: Fuster, V., Ross, R. (Eds.), Thrombosis in Cardiovascular

Ticlopidine augments luminol-dependent chemiluminescence in human

Combination tissue plasmienogen activator and ticlopidine therapy in

1997. Nitric oxide reverses aspirin antagonism of t-PA thrombolysis in

De La Cruz, J.P., Camara, S., Bellido, I., Carrasco, T., Sánchez de la Cuesta,
F., 1987. Platelet aggregation in human whole blood after chronic ad-

De La Cruz, J.P., Blanco, E., Sanchez De La Cuesta, F., 2000. Effect of
dipyridamole and aspirin on the platelet–neutrophil interaction via the

De Loberl, M., Bordet, J.C., Salen, P., Durbin, S., Defrey, G., Delaye, J.,
Botsonat, P., 1998. Ticlopidine increases nitric oxide generation in
heart-transplant recipients: a possible novel property of ticlopidine.

ETDRS Study Group, 1991. Effects of aspirin treatment on diabetic reti-
opathy. ETDRS report number 8. Early Treatment Diabetic Retinopathy

specificity and ticlopidine effects distinguish three human platelet ADP

ticlopidine: who benefits most? TASS baseline and angiographic data

Lopez Farre, A., Caramelo, C., Esteban, A., Alberola, M.L., Millas, I.,
Monton, M., Casado, S., 1995. Effect of aspirin on platelet–neutrophil
interaction. Role of nitric oxide and endothelin-1. Circulation 91,
2080–2088.

Rabin, R.A., Staffolani, R., Funelli, P., Mutus, B., Curatola, G., Mazzanti,
IDDM and NIDDM. Diabetologia 41, 101–104.

Radomski, M.W., Moncada, S., 1993. The biological and pharmacological
251–264.

Shibuki, K., 1992. Detection of nitric oxide by an electrochemical micro-
probe. A companion to methods in neuroscience. Neuroprotocols 1,
151–157.

Thomas, G.R., Thibodeaux, H., Errett, C.J., Bendor, M.M., Gross, C.E.,
Bennett, W.F., 1995. Intravenous aspirin causes a paradoxical attenua-

TIMAD Investigators, 1990. Ticlopidine treatment reduces the progres-
sion of nonproliferative diabetic retinopathy. Arch. Ophthalmol. 108,
1577–1583.

Vane, J.R., 1975. Inhibition of prostaglandin synthesis as a mechanism of