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Effect of dipyridamole and aspirin on the platelet–neutrophil interaction via the nitric oxide pathway

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Abstract

This study was designed to determine the influence of the combination of aspirin and dipyridamole on the interaction in vitro between neutrophils and platelets through the nitric oxide (NO) pathway. Collagen-induced platelet aggregation (impedance method) was determined in platelet-rich plasma and in platelet-rich plasma + neutrophils, and cGMP (enzyme immunoanassay) and NO levels (electrochemical method, with a ISO-200 electrode) were also measured. The 50% inhibitory concentration (IC_{50}) of aspirin was 139 ± 11 μM in platelet-rich plasma, 367 ± 21 μM in platelet-rich plasma + L-N^G-nitro-arginine-methyl-ester (L-NAME), and 42 ± 3 μM in platelet-rich plasma + L-arginine. The IC_{50} for dipyridamole in platelet-rich plasma was not affected by L-NAME or L-arginine; the combination of aspirin with 20 μM dipyridamole (which has no effect per se) led to an IC_{50} of 51 ± 2 μM in platelet-rich plasma, 101 ± 7 μM in platelet-rich plasma + L-NAME, and 13 ± 2 μM in platelet-rich plasma + L-arginine. The cGMP levels showed the greatest increases in the aspirin plus dipyridamole group. Dipyridamole and aspirin increased the leukocyte production of NO: 50% increases were obtained at concentrations of 285 ± 31 μM aspirin, 110 ± 9 μM dipyridamole, and 16 ± 2 μM aspirin + dipyridamole. Dipyridamole alone at a concentration of 20 μM had no significant effect on NO levels. We conclude that the combination of aspirin and dipyridamole significantly increases the antiplatelet effect of leukocytes, through an increase of NO, and that this effect is further evidence of the therapeutic benefits of this combination of drugs.

Keywords: Aspirin; Dipyridamole; Nitric oxide (NO); Platelet; Polymorphonuclear leukocyte

1. Introduction

One pharmacological approach to preventing thrombotic accidents is to use dipyridamole in combination with acetylsalicylic acid (aspirin). In pharmacodynamic terms, this combination is based on the effect of these two drugs on the principal mechanisms of platelet function regulation: aspirin inhibits thromboxane synthesis (Roth and Majerus, 1975) and dipyridamole increases the intraplatelet concentration of cyclic nucleotides (cAMP and cGMP) (McElroy and Philp, 1975; Lugnier et al., 1986).

Dipyridamole increases cAMP by inhibiting the cellular uptake of adenosine, an endogenous antiplatelet substance (Roos and Pleger, 1972), and by inhibiting type III phosphodiesterase. However, its greatest effect is the inhibition of type V phosphodiesterase, the enzyme that degrades cGMP (Lugnier et al., 1986). Moreover, dipyridamole and nitric oxide (NO) have shown a synergistic effect in inhibiting platelet aggregation (Sakuma et al., 1990; Bult et al., 1991b).

The antiplatelet effect of aspirin is greater in whole blood than in platelet-rich plasma (De La Cruz et al., 1986a), and the effect is potentiated in the presence of leukocytes (De La Cruz et al., 1986b). It was recently shown that aspirin can increase leukocyte NO synthesis (Lopez-Farre et al., 1995).

The reasons for combining dipyridamole and aspirin are based on their intraplatelet effects, but their influence on leukocyte NO production and subsequent effect on platelets remain unknown. The present study was designed to determine the effect of the dipyridamole–aspirin combination on the leukocyte–platelet interaction in vitro, with particular attention being paid to the biochemical effects on the leukocyte NO-platelet cGMP axis.

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2. Material and methods

This study was done in samples of human blood obtained from healthy volunteers (all men, mean age 35.8 ± 2.5 years) who had not taken any oral medication during a period of at least 2 weeks before the study. Sodium citrate (3.8%) at a proportion of 1:10 (v/v) was used as an anticoagulant for the blood samples. All samples were collected between 0900 and 1000 h after an overnight fast. The study protocol was approved by the Research Ethics Committee of the School of Medicine of the University of Málaga.

2.1. Study samples

To separate the different fractions, part of each sample of whole blood was centrifuged at $180 \times g$ for 10 min at 20°C; platelet-rich plasma was obtained from the resulting supernatant. Part of the platelet-rich plasma was centrifuged at $2000 \times g$ for 15 min at 20°C to obtain platelet-poor plasma. Another part of the whole blood sample was centrifuged on a Ficoll gradient (densities of 1077 and 1119) at $1000 \times g$ for 20 min at 20°C to separate polymorphonuclear leukocytes.

The experiments were done in samples of platelet-rich plasma and platelet-poor plasma to which polymorphonuclear leukocytes were added in a number proportionate to that found in whole blood ($4.3 \pm 0.2 \times 10^9$ polymorphonuclear lekocytes/l) (Coulter MD 10, Coulter, Miami, FL, USA).

Each group of experiments was performed with 10 independent samples.

2.2. Drugs and reagents

Aspirin and dipyridamole were obtained from Boehringer Ingelheim, Barcelona, Spain. L-N^G-nitro-arginine-methyl-ester (L-NAME), an unspecific inhibitor of NO synthase, was used to inhibit NO synthesis. Hemoglobin was used as an extracellular scavenger for NO. Methylene blue was used as an unspecific inhibitor of guanyl cyclase. All reagents were from Sigma (St. Louis, MO, USA). Aspirin was diluted in ethanol to a concentration of 5 mM, then sodium carbonate (0.8%) was used as diluent to obtain the different concentrations in each type of experiment. Dipyridamole was diluted in a HCl–saline buffer (100 μl of HCl 0.01 N plus 1 ml saline), and then saline was used as diluent to obtain the different concentrations (all of them were buffered at pH 7.4). In each type of experiment, a group of samples was tested after incubation with the buffer in which the drug was diluted. Neither the buffer of aspirin nor the buffer for dipyridamole affected any technique.

2.3. Analytical techniques

2.3.1. Platelet aggregometry

Aggregation was quantified by optic transmission with an Aggrecorder 4210 aggregometer (Menarini Diagnóstica, Barcelona, Spain). We tested samples of platelet-rich plasma and platelet-rich plasma + polymorphonuclear leukocytes added in the same proportion as that found in whole blood. Samples of platelet-rich plasma (470 μl) were adjusted to 250×10^6 platelets/l, and polymorphonuclear leukocytes (10 μl) were tested at a concentration of 4.5×10^9 cells/l. Aggregation was induced with collagen (10 μl) (Aggrepack, Menarini Diagnóstica) at a final concentration of 2 μg/ml.

Drugs (10 μl) were incubated with the samples for 5 min at 37°C, then collagen was added. In experiments with platelet-rich plasma + polymorphonuclear leukocytes, the leukocytes were added to the platelet-rich plasma and the mixture was incubated for 5 min at 37°C before the drug or drugs were added. Percentage optic transmission was recorded continuously for 10 min, and maximum aggregation was determined as the maximum rate of optic transmission obtained in each sample. The results are given as percentages of aggregation determined in comparison with transmittance recorded in platelet-poor plasma.

The concentration of collagen was chosen on the basis of preliminary studies that showed that the 50% effective dose (EC_{50}) that stimulated platelet aggregation in platelet-rich plasma was 2.2 ± 0.3 μg/ml ($n = 10$).

2.3.2. Cyclic GMP levels

Platelets in platelet-rich plasma (1 ml) were diluted in 2 volumes of buffer consisting of NaCl (113 mM), NaH_2PO_4 (24 mM), KH_2PO_4 (4 mM), glucose (5 mM), apyrase (50 mg/l) and prostaglandin E_1 (5 nM). The samples were centrifuged at $1000 \times g$ for 15 min at 4°C, and the resulting pellet was resuspended in buffer (1 ml) consisting of NaCl (134 mM), NaH_2PO_4 (36 mM), $NaHCO_3$ (12 mM), $CaCl_2$ (0.1 mM), $MgCl_2$ (1 mM), HEPES (5 mM), glucose (5 mM), apyrase (50 mg/l) and bovine albumin (0.5 g/l).

The number of platelets and leukocytes was adjusted as in the aggregometry experiments. The samples were divided into aliquots (175 μl) to which we added 100 μM of zaprinast (type V phosphodiesterase inhibitor) (25 μl) and the drug (or drugs, 10 μl) under study or saline solution (10 μl) as a control. The samples were incubated for 5 min at 37°C, and the reaction was stopped by adding 28 μl of 1 N HCl. The samples were then centrifuged at $10\,000 \times g$ for 3 min, and 150 μl of the supernatant was neutralized with 15 μl of 1 N NaOH.

The cGMP levels were measured by enzyme immunoassay (Amersham International, Buckinghamshire, UK). Sensitivity of the method was 161 pg ml^{-1}, intra-assay variability was 7.5%, and inter-assay variability was 12.2%. Crossreactions with this method were: 100% cGMP,

$< 0.0005\%$ cAMP, $< 10^{-6}\%$ AMP, $< 10^{-6}\%$ ADP, $< 10^{-6}\%$ ATP, $< 0.0005\%$ GMP, $< 0.00025\%$ GDP, $< 0.00025\%$ GTP.

2.3.3. Leukocyte production of nitric oxide

Polymorphonuclear leukocytes were obtained as described above for aggregometry experiments. Nitric oxide production was measured with an electrochemical method (Shibuki, 1992), using a specific electrode coupled to an ISO-NO detector (World Precision Instruments, Aston, Stevenage, Hertsforshire, UK).

The experiments were designed to measure NO production through a calcium-dependent pathway. Samples of polymorphonuclear leukocytes (300 μl) were first incubated with the drug or with saline (10 μl) for 5 min at 37°C; then 10 μl of L-arginine was added at a final concentration of 100 μM, and the incubation was continued for 5 min at 37°C. After this time the calcium ionophore A 23187 (10 μl, final concentration 10 μM) was added. The NO electrode was left in the sample at all times, so that NO production was recorded constantly.

The mean number of polymorphonuclear leukocytes in each sample used for NO detection was $3.4 \pm 0.1 \times 10^9$ cells/l ($3.7 \pm 0.2 \times 10^9$ cells/l in whole blood).

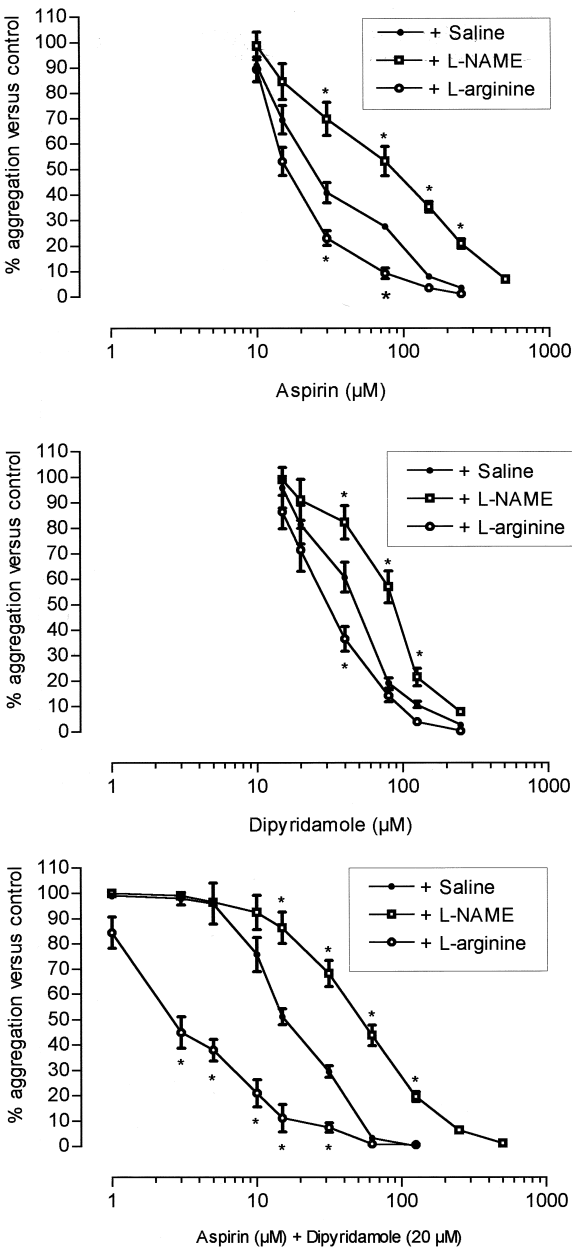

Fig. 1. Percent platelet aggregation in platelet-rich plasma plus polymorphonuclear leukocytes in samples incubated with different concentrations of aspirin, dipyridamole or aspirin plus 20 μM dipyridamole, in the presence of saline, L-arginine (100 μM) or L-N^G-nitro-arginine-methyl-ester (L-NAME, 300 μM). $^*P < 0.05$ versus saline. Each group of experiments was performed with 10 independent samples.

Table 1
Concentrations of aspirin and dipyridamole alone and in combination that produced 50% inhibition (IC_{50}) in collagen-induced platelet aggregation in platelet-rich plasma, without and with polymorphonuclear leukocytes
Each group of experiments was performed with 10 independent samples. L-Arginine: 100 μM; L-NAME: 300 μM. These isolated concentrations produced non-significant modifications in platelet aggregation.

	Aspirin	Dipyridamole	Aspirin + dipyridamole (20 μM)
Platelet-rich plasma			
Saline	139 ± 11	178 ± 16	51 ± 2
+ L-NAME	367 ± 21^a	183 ± 15	101 ± 7^a
+ L-arginine	42 ± 3^a	112 ± 11	13 ± 2^a
Platelet-rich plasma + polymorphonuclear leukocytes			
Saline	27 ± 2^b	54 ± 6^b	13 ± 1^b
+ L-NAME	89 ± 5^a	88 ± 7^a	65 ± 4^a
+ L-arginine	10 ± 1^a	26 ± 2^a	2.5 ± 0.3^a

[a] $P < 0.05$ in comparison to saline.
[b] $P < 0.05$ in comparison to saline-platelet-rich plasma.

2.4. Statistical analysis

All data given in the text, tables and figures are the means ± standard error of the mean (S.E.M.) of the values obtained in each experiment. We used analysis of variance with post-hoc Bonferroni tests. All statistical analyses were

Table 2
Mean values of cGMP (μmol/10^9 cells) in platelet-rich plasma and platelet-rich plasma plus polymorphonuclear leukocytes
Each group of experiments was performed with 10 independent samples.

	Platelet-rich plasma	Platelet-rich plasma + polymorphonuclear leukocytes
Saline	86 ± 7	138 ± 11^a
L-NAME (300 μM)	81 ± 7	68 ± 8^b
Hemoglobin (10 μM)	81 ± 9	76 ± 7^b
Methylene blue (50 μM)	41 ± 4^b	58 ± 7^b

[a] $P < 0.05$ in comparison to platelet-rich plasma.
[b] $P < 0.05$ in comparison to saline.

done with the SPSSx 8.0 program for Windows 95. A P value of < 0.5 was considered significant.

3. Results

3.1. Platelet aggregation

Incubation with aspirin and dipyridamole alone or in combination inhibited collagen-induced platelet aggregation in a concentration-dependent manner. Fig. 1 shows the results in platelet-rich plasma with polymorphonuclear leukocytes, in the presence of L-NAME, L-arginine or saline only. In all three treatments, the curves for L-NAME were shifted to the right, and those for L-arginine were shifted to the left, in comparison with the control curve.

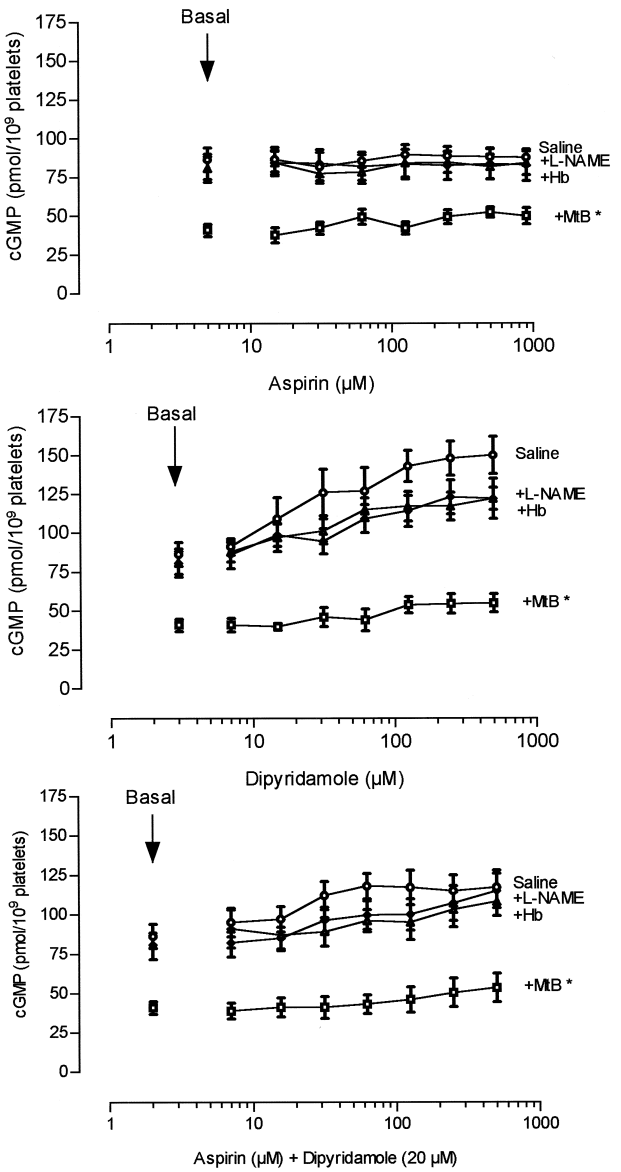

Fig. 2. Intracellular levels of cyclic GMP (cGMP) in washed platelets incubated without (basal) or with aspirin, dipyridamole or aspirin plus 20 μM dipyridamole, in the presence of saline or L-N^G-nitro-arginine-methyl-ester (L-NAME, 300 μM), hemoglobin (Hb, 10 μM) or methylene blue (MtB, 50 μM). $^*P < 0.05$ versus all other curves. Each group of experiments was performed with 10 independent samples.

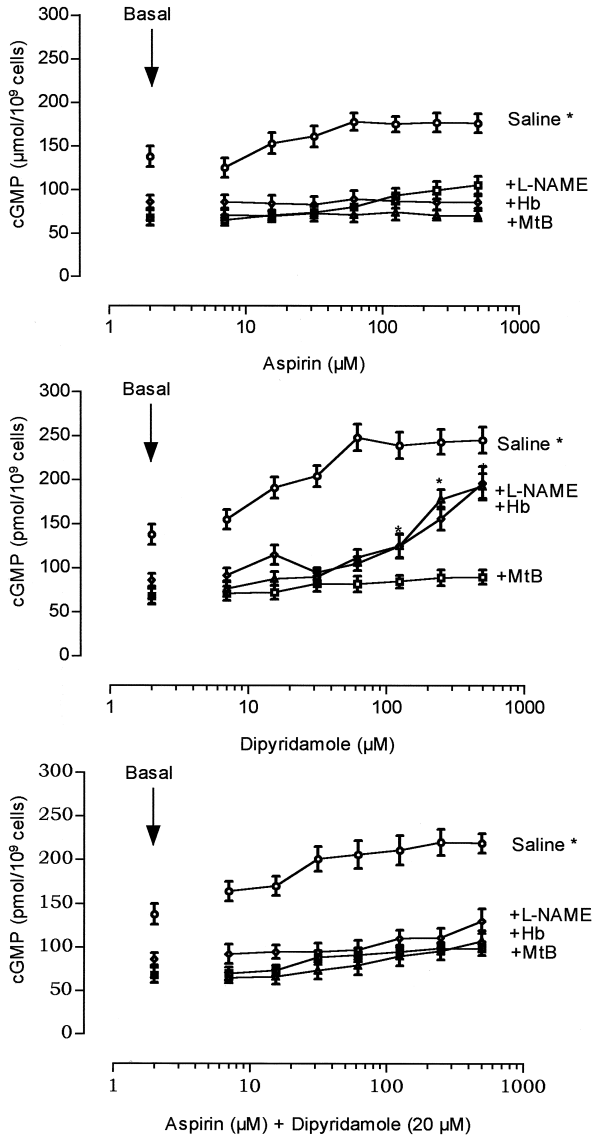

Fig. 3. Intracellular levels of cyclic GMP (cGMP) in washed platelets plus polymorphonuclear leukocytes, incubated without (basal) or with aspirin, dipyridamole or aspirin plus 20 μM dipyridamole, in the presence of saline or L-N^G-nitro-arginine-methyl-ester (L-NAME, 300 μM), hemoglobin (Hb, 10 μM) or methylene blue (MtB, 50 μM). $^*P < 0.05$ versus all other curves. Each group of experiments was performed with 10 independent samples.

Table 1 summarizes the concentrations of the drugs that led to 50% maximum inhibition (IC_{50}) of platelet aggregation obtained in control samples. Under basal conditions (no modification of NO synthesis), the IC_{50} of dipyridamole + aspirin was lower than that for either drug alone. The presence of polymorphonuclear leukocytes decreased

the IC_{50} of both drugs alone and in combination. Incubation with L-NAME increased the IC_{50} in samples containing either or both drugs, and L-arginine significantly decreased the values, especially in the presence of polymorphonuclear leukocytes.

3.2. Cellular cGMP concentration

In samples of platelet-rich plasma (Table 2), only methylene blue decreased cyclic GMP levels, whereas when polymorphonuclear leukocytes were present cyclic GMP concentration was reduced by L-NAME, hemoglobin, and methylene blue.

The amount of cyclic GMP in samples of platelet-rich plasma (Fig. 2) increased significantly after incubation with dipyridamole alone or with aspirin. Methylene blue significantly reduced this effect, whereas L-NAME and hemoglobin had no effect.

In samples of platelet-rich plasma with polymorphonuclear leukocytes (Fig. 3), all three treatments increased the cyclic GMP concentration in a dose-dependent way. This effect was significantly inhibited when methylene blue, L-NAME or hemoglobin was present.

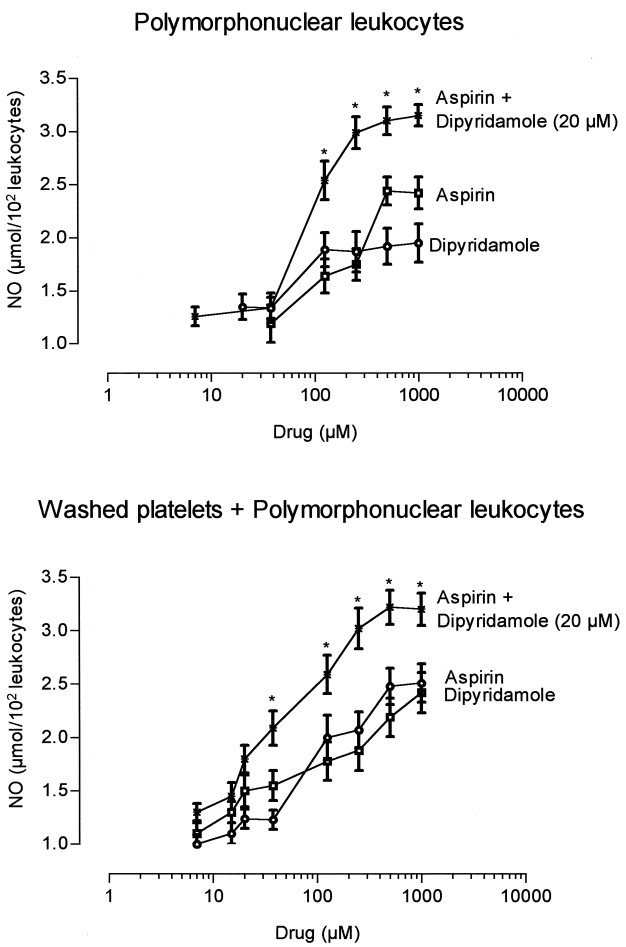

Fig. 4. Nitric oxide production in washed polymorphonuclear leukocytes or in polymorphonuclear leukocytes plus washed platelets, incubated with aspirin, dipyridamole or aspirin plus 20 µM dipyridamole. Each group of experiments was performed with 10 independent samples.

Table 3
Concentration of each drug (µM) that produced a 50% increase in nitric oxide levels in control samples
Each group of experiments was performed with 10 independent samples.

	Polymorphonuclear leukocytes	Platelet-rich plasma + polymorphonuclear leukocytes
Aspirin	285 ± 31	260 ± 29
Dipyridamole	110 ± 10[a]	30 ± 4[a,b]
Aspirin + dipyridamole (20 µM)	16 ± 2[a]	5.1 ± 0.2[a,b]

[a] $P < 0.05$ in comparison to aspirin.
[b] $P < 0.05$ in comparison to polymorphonuclear leukocytes.

3.3. Nitric oxide production

Aspirin, dipyridamole, and the combination of both all increased NO production in a dose-dependent way in samples of polymorphonuclear leukocytes and platelets + polymorphonuclear leukocytes (Fig. 4). In medium containing platelets alone, neither drug had a significant effect on NO production; the maximum increase obtained was 5.8 ± 0.7% in the presence of 1 mM aspirin + 20 µM dipyridamole.

Table 3 shows the concentrations of both drugs that led to a 50% increase in basal NO production (measured in the absence of drugs). In both types of samples the effect of the aspirin + dipyridamole combination was significantly greater than that of either drug alone.

Incubation in the presence of 300 µM L-NAME reduced basal NO production by 92.8 ± 2.8%. In the presence of this inhibitor of NO synthase, the greatest increase in NO production (10.9 ± 1.8% increase) was obtained with aspirin at a concentration of 1 mM in combination with dipyridamole.

4. Discussion

Our results show that, in general, both aspirin and dipyridamole favor the platelet–leukocyte interaction in vitro via the NO-cGMP biochemical pathway, and that combination of the drugs leads to an even greater enhancement of this biochemical interaction.

The aggregometric results show that leukocyte NO production, as a natural inhibitory mechanism of platelet aggregation, has a significant influence on the effects of aspirin and dipyridamole. When NO synthesis was blocked by L-NAME, the inhibitory effect on drug-induced platelet activation decreased (Table 1), and the values of IC_{50} doubled in medium poor in NO. This effect was much

clearer in the samples containing platelets + polymorphonuclear leukocytes than in samples of platelets alone, a logical finding given that polymorphonuclear leukocytes are the type of blood cell in which NO production is highest. In fact, the inhibitory effect of unstimulated polymorphonuclear leukocytes on platelet aggregation is the result of their high NO production (Nicolini and Mehta, 1990). This effect was not detected when dipyridamole was incubated with L-NAME in platelet rich plasma, without leukocytes; perhaps the NO production in platelets is not sufficient to demonstrate significant changes in the effect of dipyridamole, because when leukocytes are present this effect is significant.

The antiaggregant effect of aspirin was found to be greater in whole blood than in platelet-rich plasma (De La Cruz et al., 1986a); this difference was attributed mainly to the presence of leukocytes in the sample (De La Cruz et al., 1986b). More recently, aspirin was shown to enhance the inhibitory effect of polymorphonuclear leukocytes by increasing leukocyte NO production (Lopez-Farre et al., 1995). These results are consistent with our aggregometric findings and the changes in cGMP concentrations: aspirin did not significantly modify cGMP levels in isolated platelets, but led to a concentration-dependent increase when leukocytes were present. Thus, aspirin probably facilitates guanyl cyclase activation by leukocyte NO, as the presence of L-NAME (which inhibits NO synthesis) or hemoglobin (which scavenges NO) abolished this effect of aspirin. When we quantified NO production, we found that the effect of aspirin in samples containing polymorphonuclear leukocytes was similar regardless of whether platelets were present or not (Fig. 4). This indicates that aspirin affects mainly the production of NO by leukocytes.

Dipyridamole is a classic inhibitor of platelet function, which acts mainly in two ways: (1) by increasing cyclic nucleotides as a result of the inhibition of phophodiesterase (especially type V, which is cGMP-dependent) (Lugnier et al., 1986), and (2) by increasing extracellular levels of adenosine (Roos and Pleger, 1972), which leads to the activation of adenyl cyclase (Gresele et al., 1986). We have shown that the antiaggregant effect of dipyridamole is lower when leukocyte NO synthesis is reduced. The decrease in NO synthesis, caused by L-NAME or by the removal of NO from the intracellular milieu by hemoglobin, decreases the effect of dipyridamole on the cGMP content, but the decrease in this effect was smaller than the decrease in the effect of aspirin on cGMP levels under the same conditions. These findings are logical, as dipyridamole acts fundamentally to maintain intracellular levels of cGMP. Sakuma et al. (1990) and Bult et al. (1991a,b) showed that the antiaggregant effect of dipyridamole was potentiated in the presence of endogenous or exogenous NO. This was explained by the sum of the effect of NO on guanyl cyclase and the effect of dipyridamole on cGMP phosphodiesterase (not demonstrated in our study).

Iimura et al. (1996) showed that dipyrimadole increased NO production in cultures of vascular smooth muscle induced with interleukin 1β, i.e., by activation of inducible NO synthase. Our present findings complement these results by showing that dipyrimadole also stimulates leukocyte NO production through a calcium-dependent pathway, a phenomenon compatible with activation of constitutive NO synthase. Moreover, we show that this effect in vitro is quantitatively greater than the effect attained with aspirin.

Finally, it should be recalled that dipyrimadole has an antioxidant effect, decreasing the appearance of lipid peroxides in tissues (De La Cruz et al., 1992a), and reducing the formation of free radicals in neutrophils (Suzuki et al., 1992). This drug prevents superoxide anions from increasing platelet aggregation (De La Cruz et al., 1992b) or from shortening the half-life of vascular prostaglandin (De La Cruz et al., 1994). Because superoxide anions react with NO, by limiting the binding of NO to guanyl cyclase (McCall et al., 1989), dipyrimadole may reduce this effect of free radicals.

Aspirin is frequently combined with dipyrimadole for prophylactic use in secondary prevention of arterial thrombosis, mainly because the two drugs act through different mechanisms. However, in some tests of platelet function the final effect was greater than the sum of the effects of each drug separately (De La Cruz and Sanchez De La Cuesta, 1991), and this result has been confirmed in clinical assays on the prevention of secondary thrombotic events in patients with coronary bypass surgery (Sanz et al., 1990), diabetic retinopathy (DAMAD Study Group, 1989) or stroke (Diener et al., 1996). Our findings shed light on the influence of the aspirin + dipyridamole combination on the NO-cGMP axis: both drugs stimulate this biochemical pathway, hence, the combination of the two would be expected to have a greater effect on both leukocyte NO synthesis and on cGMP levels than either drug separately. The decrease in leukocyte NO synthesis clearly reduced the antiaggregant effect of this combination, while increasing cGMP levels. Nitric oxide synthesis was also increased when the two drugs were given together: the concentration that increased basal NO production by 50% was 85–95% lower than when either drug was used separately.

The concentration of dipyridamole of 20 μM used in the present experiments is the peak concentration reached in blood after chronic intake of the habitual dose (Bjornsson and Mahony, 1983); however, these concentrations are variable, because other authors have described plasma levels less than 10 μM (Bult et al., 1991a). When dipyrimadole was incubated alone, the effects of this concentration were weak and not statistically significant. This further supports the benefits of using the combination of both drugs in clinical practice.

In conclusion, the combination of aspirin and dipyridamole significantly increases the endogenous antiplatelet effect of polymorphonuclear leukocytes. This effect is

further evidence of the benefits of this combination of both drugs for patients who are at risk for thrombotic events.

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