Dipyridamole Inhibits Platelet Aggregation Induced by Oxygen-derived Free Radicals

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ABSTRACT
Pyrogallol (a generator of superoxide anions) caused 50% increase in platelet aggregation induced by 400 μM of arachidonic acid. Dipyridamole did not produce a statistically significant inhibition of arachidonic-acid induced platelet aggregation, but it caused 100% inhibition of pyrogallol-stimulated platelet aggregation. Ferrous salts (Fe^{2+}) induced 34% platelet aggregation which was inhibited (79.6%) by a concentration of dipyridamole of 10 μM. Dipyridamole inhibited ferrous-induced lipid peroxidation with IC-50 values of 17.5 μM. When arachidonic acid was used as aggregating agent, the corresponding IC-50 value was 140.5 μM. These results indicate that dipyridamole prevented platelet activation induced by oxygen-derived free radicals.

INTRODUCTION
Endogenous enzymatic mechanisms for the detoxification of oxygen-derived free radicals include superoxide dismutase which catalyzes the dismutation of the superoxide anion, and catalases and glutathione peroxidases which catalyze the reduction of hydrogen peroxide (1,2). An important source of hydroxyl radicals is the metal-dependent breakdown of hydrogen peroxide, where metal ions, such as iron and copper, and superoxide anions participate in hydroxyl radical formation (3). Oxygen radicals are involved in the genesis of lipid membrane peroxidation (3), breakdown of endothelium-derived vascular relaxing factor (EDRF) (4), and inhibition of prostacyclin synthetase to a greater extent than inhibition of thromboxane synthetase which results in a disequilibrium in favour of thromboxane A_2 formation.

We have shown that ferrous salts enhanced arachidonic acid-induced platelet aggregation, lipid peroxidation, and morphologi-

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cal changes in the platelet surface (5). It has been also shown that superoxide anions increase thrombin-induced platelet adhesion and aggregation (6). On the other hand, there is evidence that dipyridamole inhibits the production of superoxide anions and ferrous-induced lipid peroxidation in cell membranes (7-11). In this study we evaluate the effect of dipyridamole on the enhancement of platelet aggregation induced by oxygen-derived free radicals.

**MATERIAL AND METHODS**

**Materials**

Dipyridamole was obtained from Boehringer Ingelheim, S.A. (Barcelona, Spain), arachidonic acid from Biodata Corp. (Hatboro, PA, U.S.A.), and malondialdehyde-bis-diethyl-acetal from Aldrich-Chemie (Steinheim/Albuch, Germany). The rest of the reagents were obtained from Sigma Chemical Co., (St. Louis, MO, U.S.A.).

**Isolation of human platelets**

Venous blood was obtained from healthy male volunteers, aged (mean ± SD) 27.3 ± 1.1 years, who had received no medication which was known to modify platelet response for 15 days prior to blood collection. Blood was anticoagulated with a solution of 3.8% trisodium citrate in a proportion 1:10. Platelet-rich plasma (PRP) was prepared by centrifugation (180 g x 10 min) and diluted up to 300,000 platelets per μL with autologous platelet-poor plasma (1,800 g x 15 min). PRP samples with erythrocyte and/or leukocyte counts over 100,000/μL and 100/μL, respectively, were discarded.

**Platelet aggregation**

Diluted PRP (physiological saline pH 7.4, 37°C, 1:1 v/v) was incubated at 37°C for 2-3 min in a double channel Chrono-Log aggregometer (model 540, Chrono-Log Corp., Haverton, PA, U.S.A.) with continuous stirring at 1,000 r.p.m. Platelet aggregation was measured by the electric impedance method described by Cardinal & Flower (12) as the maximum change in impedance (ohms) 10 min after the addition of the aggregating agent. Platelet aggregation was also measured by the method of single platelet counting described by Saniabadi et al. (13) in which 40 μL samples PRP were taken at 5, 10, 15, 20, 25, and 30 min after the addition of the aggregating agent. For cell fixation glutaraldehyde was diluted to 0.5% (1:10 v/v) with phosphate buffer saline (pH 7.4) 0.1 M. Single platelets were counted with a cell counter System 8000 (Baker Instruments Co., Allentown, PA, U.S.A.). Controls for these experiments were prepared by stirring samples of PRP without each of the aggregating agents.

Arachidonic acid (400 μM) was used as inducer of platelet aggregation. Superoxide anion production was stimulated with pyrogallol (14). Increasing equimolecular concentrations of ferrous sulfate and ascorbic acid (FeAs) were used to induce lipid peroxidation via the formation of hydroxyl anions (15). Stock solutions of pyrogallol and FeAs were prepared in helium-deoxygenated water and incubated at 37°C for 1 min before the
addition of arachidonic acid. Dipyridamole was incubated for 5 min before the addition of oxygen radical inducers.

Lipid peroxidation

The products resulting from the reaction with thiobarbituric acid, of which the most significant is malondialdehyde (MDA), were taken to be indicators of lipid peroxidation (16). Arachidonic acid and/or pyrogallol or FeAs-induced PRP samples to which 500 μL of 20% trichloroacetic acid have been added, were centrifuged at 12,000 g for 3 min. Subsequently, 300 μL of 0.5% thiobarbituric acid (diluted in phosphate buffer saline 0.1 M, pH 7.4) were added to 700 μL of the supernatant. Samples were incubated at 100°C for 15 min and then the amount of MDA produced was measured by the spectrophotometric absorbance of the supernatant at 532 nm using a Lambda 1 (Perkin-Elmer, Illinois, U.S.A.) spectrophotometer. The absorbances obtained were compared to that of a standard curve using malondialdehyde-bis-diethyl-acetal.

Statistics

Results are expressed as mean ± standard error of the mean (SEM) for (n) experiments. The Student’s t test for unpaired data was applied to determine the significance of differences between means. P < 0.05 was taken as significant.

RESULTS

In the experiments in which platelet aggregation was measured by electric impedance, the addition of pyrogallol (4 μM) produced 50% increase in arachidonic acid-induced platelet aggregation (Table 1). The incubation of pyrogallol alone resulted in platelet aggregation of 3.5 ± 0.06 ohms. The addition of FeAs (10 μM) did not cause a significant increase in arachidonic acid-induced platelet aggregation (17% increment). The incubation of FeAs alone resulted in platelet aggregation of 0.5 ± 0.009 ohms.

Dipyridamole did not produce statistically significant inhibitions of platelet aggregation induced by both arachidonic acid (P > 0.6) and arachidonic acid plus FeAs (P > 0.7). However, dipyridamole at any concentration caused a statistically significant inhibition of platelet aggregation induced by arachidonic acid plus pyrogallol (Table 1).

In the experiments in which platelet aggregation was measured by single platelet counting, arachidonic acid (400 μM) produced 88.9 ± 4.03% of platelet aggregation after 5 min of incubation (Figure 1) which persisted up to 30 min (86.01 ± 7.68%). No concentration of dipyridamole caused inhibition of arachidonic-acid induced platelet aggregation (P > 0.8). FeAs (10 μM) was able to induce statistically significant platelet aggregation starting from 15 min of incubation (34.0 ± 2.8% at 25 min, P = 0.0047 as compared to PRP samples without the aggregating agent) (Figure 2).
Table 1

Effects of arachidonic acid, pyrogallol and ferrous sulfate/ascorbic acid (Fe/As) on platelet aggregation measured by electric impedance

<table>
<thead>
<tr>
<th>Inductor</th>
<th>Dipyridamole (µM)</th>
<th>Maximum platelet aggregation (ohms)</th>
<th>%</th>
</tr>
</thead>
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<tr>
<td>Arachidonic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(400 µM)</td>
<td>0</td>
<td>10.6 ± 1.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.8 ± 0.7</td>
<td>92.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>8.9 ± 1.1</td>
<td>83.9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>10.1 ± 0.9</td>
<td>95.3</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(400 µM) plus pyrogallol (4 µM)</td>
<td>0</td>
<td>15.9 ± 1.1b</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11.3 ± 1.1c</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>9.5 ± 0.8d</td>
<td>89.6</td>
</tr>
<tr>
<td></td>
<td>100</td>
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<td>86.8</td>
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<tr>
<td>Arachidonic acid</td>
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<tr>
<td>(400 µM) plus FeAs (10 µM)</td>
<td>0</td>
<td>12.5 ± 1.2</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12.6 ± 1.4</td>
<td>118</td>
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<tr>
<td></td>
<td>100</td>
<td>10.9 ± 0.7</td>
<td>103</td>
</tr>
</tbody>
</table>

Each value is the mean ± SEM of six independent experiments.
* vs b P = 0.023; vs c P = 0.013; vs d P = 0.0072; e vs b P = 0.0016

Dipyridamole did not cause statistically significant inhibition of FeAs-induced platelet aggregation until 15 min of incubation. Maximum percentages of inhibition after concentrations dipyridamole of 10 µM and 100 µM were 79.6 ± 5.8% and 97.6 ± 5.7%, respectively. The maximum inhibitory effect of dipyridamole 100 µM was not different (P > 0.8) from that obtained in PRP samples without the aggregating agent. When PRP samples were incubated with pyrogallol alone, statistically significant platelet aggregation was not induced (data not shown).

Dipyridamole showed a dose-related inhibitory effect on both arachidonic acid-induced (400 µM) and FeAs-induced (10 µM) MDA production (Figure 3). IC-50's values were 140.5 ± 6.3 µM for dipyridamole when MDA production was induced by arachidonic acid and 17.5 ± 1.8 µM when MDA production was induced by FeAs (P < 0.00001) (baseline values: 1.17 ± 0.18 nmol/10⁶ platelets for arachidonic acid-induced MDA production and 1.15 ± 0.09 nmol/10⁶ platelets for FeAs-induced MDA production, P > 0.8).
Platelet aggregation measured by the single platelet counting method after induction with arachidonic acid (400 μM) alone (solid triangles) or dipyridamole (10 μM, open triangles; 100 μM, open squares) as compared with PRP samples without aggregating agent (solid squares). Each value is the mean ± SEM of six independent experiments.

Platelet aggregation measured by the single platelet counting method after induction with ferrous sulfate and ascorbic acid (10 μM) alone (solid triangles) or dipyridamole (10 μM, open triangles; 100 μM, open squares) as compared with PRP samples without aggregating agent (solid squares). Each value is the mean ± SEM of six independent experiments.
Dose-response curves of the inhibitory effect of increasing concentrations of dipyridamole on malondialdehyde (MDA) production induced by arachidonic acid (400 μM, open circles) or ferrous sulfate and ascorbic acid (10 μM, solid circles)

DISCUSSION

In the present study, dipyridamole prevented the effect of pyrogallol on arachidonic acid-induced platelet aggregation and caused an inhibition of FeAs-induced aggregation. Given that superoxide anion radicals are formed during the autoxidation of pyrogallol (14) and that pyrogallol enhances platelet aggregation induced by thrombin (6), it can be stated that dipyridamole prevents the enhancement of arachidonic acid pathway activation by superoxide anions.

The generation of superoxide radicals and its appearance in the surrounding medium of platelets are by-products of oxidative metabolism in platelets that escaped the dismutating capacity of platelet superoxide dismutase (17). It is possible that superoxide radicals detected in the surrounding medium of platelets represent the resulting toxic oxygen products generated by stimulated leucocytes (18), in which a scavenging property of dipyridamole has been demonstrated (19). Since neutrophils were not a source for superoxide anions in our experiments (leucocyte count < 100/μL), the mechanism of action of dipyridamole was exerted on platelets.

Dipyridamole scavenges oxygen radicals and protects from free radical-induced peroxidative damage in different tissues (8,10,11,19) and may therefore exert a similar action in platelets. However, it should be taken into consideration the inhibitory effect of superoxide radicals on guanylate cyclase (20) and that dipyridamole inhibits cGMP-dependent phosphodiesterase (21) and potentiates the stimulating effect on cGMP production by platelet of endothelium-derived relaxing factor (22,23).
On the other hand, activated platelets produce hydroxyl radicals, which in turn could be a further platelet stimulus \( (24) \). We have shown that \text{FeAs} (1 mM) (an agent that can speed up the Fenton reaction) enhanced platelet lipid peroxidation and morphological changes in the platelet surface induced by arachidonic acid \( (5) \). Such effect was not apparent in the present study in which lower concentrations of \text{FeAs} were used, although \text{FeAs}-induced platelet aggregation was demonstrated by the single platelet counting method which is able to detect the occurrence of aggregates at earlier stages than electric impedance \( (13) \).

The inhibitory effect of dipyridamole on platelet aggregation indicates that the mechanism of action of this drug is related to the oxidative stimulating pathway used by \text{FeAs}. It has been shown that dipyridamole inhibited ferrous-induced lipid peroxidation in several tissues \( (7-11) \). In our study, dipyridamole inhibited \text{FeAs}-induced MDA production at eight-fold lower IC-50's values than did when arachidonic acid was used as aggregating agent.

This study provides further evidence of the protective properties of dipyridamole on platelet damage induced by oxygen-derived free radicals. The inhibition of platelet aggregation may be explained by a scavenging action of superoxide radicals and/or the oxidative effect of ferrous salts.

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