THE PYRIMIDO-PYRIMIDINE DERIVATIVES, DIPYRIDAMOLE, MOPIDAMOL AND RA-642, PREVENT FROM RETINAL VASCULAR DEFECTS IN EXPERIMENTAL DIABETES MELLITUS

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Abstract We compared the effects of dipyridamole, RA-642, and mopidamol on platelet activity and thromboxane/prostacyclin balance in relation to the degree of retinal vascularization in a model of experimental streptozotocin-induced diabetes in rats. After 3 months, collagen-induced platelet aggregation in whole blood was 25% higher in diabetic animals than in nondiabetics. Dipyridamole inhibited 43% platelet aggregation, mopidamol 39%, and RA-642 36%. Platelet production of thromboxane B2 was 87% higher in untreated diabetic rats. Mopidamol and RA-642 produced a 46% and 41% inhibition of thromboxane B2. Dipyridamole did not inhibited thromboxane B2 synthesis. Aortic production of 6-keto-PGF1α was 43% lower in untreated diabetic animals and showed no change after treatment with either mopidamol or RA-642. In contrast, dipyridamole caused a 90% increase in aortic production of prostacyclin. Computerized analysis of retinal vascularization showed that untreated diabetic rats had a 81% decrease in the area occupied by peroxidase-labelled vessels as compared with nondiabetics. Treatment with dipyridamole, mopidamol, and RA-642 caused 2.5-fold, 2.8-fold and four-fold increases, respectively, in the percentage of retinal surface occupied by peroxidase-labelled vessels. Differences in retinal vascularization between diabetic animals given RA-642 and nondiabetic controls were negligible.

Key Words: Pyrimido-pyrimidine derivatives - Platelets - Diabetic retinopathy - Thromboxane - Prostacyclin.

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The majority of diabetic patients will eventually develop retinopathy after 10 to 12 years of disease. It has been estimated that 50% to 70% of patients with untreated retinopathy would present ophthalmologic signs of blindness in a 10-year period (1). However, despite advances in the treatment of this condition, diabetic retinopathy is the leading cause of blindness in the Western world (2).

Platelet hyperactivity accompanied by an imbalance of thromboxane/prostacyclin production (particularly due to a decrease in the vascular synthesis of prostacyclin) has been implicated in the etiopathogenesis of retinal alterations found in patients with diabetes mellitus (3-7). In accordance with this hypothesis, the development and progression of retinal vascular lesions could be modified by the administration of inhibitors of platelet aggregation; in fact, treatment with dipyridamole alone, dipyridamole plus acetylsalicylic acid (ASA), ticlopidine or triflusal has been shown to increase retinal vascularization (8-11).

The pyrimido-pyrimidine derivative, dipyridamole (RA-8), exhibits platelet antiaggregatory effects and causes an increase in prostacyclin synthesis. The congener, RA-642, has a similar antiplatelet action but shows a potent antiperoxidative effect. In contrast, platelet thromboxane synthesis is inhibited by mopidamol (RA-233) but unaffected by either dipyridamole or RA-642 (12-17). These prostanoid balance has been related with the vascular retinal changes in diabetes (1,5,7), and it is modified by these three compounds in different way; furthermore, RA 642 prevent from brain ischemia in a rat experimental model (16) by a mechanism independent to the prostanoid balance. For that reason, we have compared the effects of these three pyrimido-pyrimidine derivatives (dipyridamole, RA-642, mopidamol) on platelet activity and thromboxane/prostacyclin balance in relation to the degree of retinal vascularization in a model of experimental diabetes in rats, in order to evaluate if these biochemical changes alter or not the development of the retinal vascularization in this experimental diabetic model.

MATERIAL AND METHODS

Animals
A total of 100 male Wistar rats, aged between 3 and 4 months and weighing 200 to 350 g, were housed in plastic cages with unlimited access to food and water. Rats were divided at random into five experimental groups. In group I, 20 nondiabetic animals were not treated; in group II, 20 diabetic animals received 0.5 mL/kg per day of normal saline; in group III, 20 diabetic animals received 10 mg/kg per day of dipyridamole; in group IV, 20 diabetic animals received 8.34 mg/kg per day of mopidamol; and in group V, 20 diabetic animals received 10.08 mg/kg/day of RA-642. Doses of RA-642 and mopidamol were equimolar to that of dipyridamole. All study medications were administered orally (by a plastic endogastric cannula) and the daily doses were divided into two administration, one of them between 8:00 and 9:00 a.m., and other between 20:00 and 21:00 p.m.. The duration of treatment was 3 months.

This scheme of dosification were used according to two reason: first, the pharmacokinetic studies carried out in the Thomae Institute (confidential reports) show similar profiles and rates of intestinal absorption for the three compounds; second, in humans, the antiplatelet effects of RA-642 and dipyridamole are very similars, and the one of mopidamol are 2-3 times more potent. In order to investigate differences and similarities, we carried out a scheme of dosification based in the comparison of equimolar doses.
Experimental diabetes

Experimental diabetes was induced by a single dose of streptozotocin (50 mg/kg) intravenously injected into the left femoral vein. Nondiabetic animals received equivalent doses of normal saline. Blood glucose concentration was determined by a micromethod (Glumometer®, Menarini, Barcelona, Spain) following a small incision in the animal's tail. Glycemia was monitored daily for the first week and at 7-day intervals thereafter. Animals were divided at random into the five study groups on the next day after they had been considered to be diabetics (detection of glucose concentrations ≥ 200 mg/dL during two consecutive days). Animals in groups II, III, IV, and V were given monocomponent insulin, 2-4 IU/day subcutaneously (Monotard®, Novo España, S.A., Madrid) as antidiabetic. Animals in group I received normal saline.

Assessment of retinal vascularization

Retinal vascularization was assessed according to the method described by Wallow and Ingerman (18). Briefly, after completion of the protocol, one hour after the last dose of the drugs in all rats, animals were anesthetized with pentobarbital sodium, 40 mg/kg intraperitoneally, and 2 mL of blood was drawn from the inferior vena cava (1 mL was mixed with 3.8% trisodium citrate in a proportion 1:10, and 1 mL without anticoagulant was introduced in a bath at 37°C for 45 min). The internal carotid artery was cannulated and the descending carotid artery was tied at the level of its cardiac outlet. Then 180 mg/kg of horseradish peroxidase (HRP-type II, Sigma Chemical Co., St. Louis, MO) was injected into the carotid artery. A segment of the abdominal aorta was obtained and transferred to a conical tube, surrounded by crush ice, with 1 mL of buffer solution containing (in g/L) 6.21 NaCl, 0.29 KCl, 1.68 NaHCO₃, 0.28 Na₂SO₄, 5.58 trisodium citrate, 0.5 glucose, and 0.6 Tris, pH 8.5).

Ten minutes after HRP injection, eyeballs were enucleated and immersed in a standard fixative solution for 45 min. Lens were extracted and scleral and retinal tunics were processed histochemically by means of Mesulam’s technique. Specimens were fixed in 1.2% glutaraldehyde and 1% para-formaldehyde dissolved in 0.2 phosphate buffer (pH 7.3) for 48 hours and subsequently in 0.1 N phosphate buffer for 24 hours. A fine spatula was used to separate the retina from the sclera.

Retinal sections were incubated with a solution of 0.2% 3,3′,5,5′-tetramethylbenzidine (as chromogen of the reaction) and 100 mg sodium nitroferrocyanide (pH 3.3) for 20 min. Subsequently, 10 to 15 mL of 1% hydrogen peroxide was added and incubation was prolonged for 20 min. Sections were placed in the refrigerator in a wash solution of 10% glutaraldehyde and 1% para-formaldehyde dissolved in 0.2 phosphate buffer (pH 7.3) for 48 hours and subsequently in 0.1 N phosphate buffer for 24 hours. A fine spatula was used to separate the retina from the sclera.

Platelet aggregometry

Platelet aggregation was measured in whole blood samples by the electric impedance method described by Cardinal and Flower (20), as the maximum change in impedance (ohms) 10 min after the addition of the aggregating agent (10 µg/mL of collagen). One milliliter of anticoagulated blood with 3.8% trisodium citrate in a proportion 1:5 was used. Aggregometry
was performed at 37°C in a double-channel aggregometer (model 540, Chrono-Log Corp, Havertown, PA) with continuous stirring at 1,000 r.p.m.

**Platelet production of thromboxane B₂**
Thromboxane B₂ was measured by radioimmunoassay (³H-thromboxane B₂) (Amersham International plc, UK). A sample of whole blood was kept at 37°C during 45 min, and then we added 100 μmol/L of indomethacin, and was centrifuged at 10,000 x g for 3 min. The supernatant was removed and kept frozen at -80°C until analysis.

**Aortic production of 6-keto-PGF₁₀**
Aortic production of prostacyclin was determined by radioimmunoassay (³H-6-keto-PGF₁₀) (Amersham International plc, UK). A segment of abdominal aorta was incubated in cold buffer (whose composition has been mentioned above) at 37°C for 10 min. Subsequently, arterial sections were passed onto 1 mL of new buffer and 100 μmol/L of arachidonic acid was added. After 10 min of incubation at 37°C we added 100 μmol/L of indomethacin, and 500 μL samples of supernatant were extracted and frozen at -80°C before analysis. The cross-reactivity of both radioimmunoassay kits are less of 5% respect to other prostanoids (data from manufacturer).

All laboratory tests were carried out by researchers who were blind to the origin of samples and to the purpose of the study.

**Statistical analysis**
Statistical analysis of the results was carried out using the Statgraphics computer program (STSC Inc., MD). Differences of mean values between groups were analyzed by means of D'Agostino test to assess the normal distribution of data. The One-way analysis of variance (ANCOVA) was used, AND to determine significant differences the Student t test was carried out. Statistical significance was assumed at p < 0.05. All values in text, tables, and figures are presented as mean ± standard error of the mean (SEM).

**RESULTS**
Production of experimental diabetes was achieved in all animals within 5 days after the administration of streptozotocin. Blood glucose concentrations before injection of streptozotocin was lower statistically than the ones after this injection (p < 0.0001), and were also lower statistically when all diabetic animals were compared with all the diabetic groups (Table 1). Results of hematological parameters are shown in Table 2. Mean platelet count was significantly lower in animals in group II (diabetic controls) as compared with group I (nondiabetic controls) (p < 0.001), group IV (diabetes and mopidamol) (p < 0.03), and group V (diabetes and RA-642) (p < 0.01).

Maximum collagen-induced platelet aggregation was 25% higher in diabetic animals than in nondiabetics. Dipyridamole, mopidamol and RA-642 caused about 43%, 39% and 36% inhibition of platelet aggregation, respectively (Figure 1).
TABLE 1

Serum Glucose Levels (mg/dL) In The Study Groups

<table>
<thead>
<tr>
<th>Study group</th>
<th>Baseline</th>
<th>Streptozotocin-induced diabetes at 1 month</th>
<th>Streptozotocin-induced diabetes at 2 months</th>
<th>Streptozotocin-induced diabetes at 3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>70 ± 2'</td>
<td>79 ± 2'</td>
<td>81 ± 2'</td>
<td>82 ± 3'</td>
</tr>
<tr>
<td>II</td>
<td>71 ± 2'</td>
<td>446 ± 22</td>
<td>456 ± 20</td>
<td>442 ± 19</td>
</tr>
<tr>
<td>III</td>
<td>68 ± 1*</td>
<td>462 ± 16</td>
<td>478 ± 16</td>
<td>443 ± 18</td>
</tr>
<tr>
<td>IV</td>
<td>69 ± 2*</td>
<td>467 ± 26</td>
<td>441 ± 27</td>
<td>396 ± 22</td>
</tr>
<tr>
<td>V</td>
<td>70 ± 2*</td>
<td>458 ± 21</td>
<td>447 ± 20</td>
<td>397 ± 16</td>
</tr>
</tbody>
</table>

* I: nondiabetic controls; II: diabetic controls; III: diabetes and dipyridamole; IV: diabetes and mopidamol; V: diabetes and RA-642.
' p < 0.0001 vs all diabetic groups; * p < 0.0001 vs values of 1, 2 and 3 months evolution.

TABLE 2

Laboratory Data After 3 Months Of Treatment With Pyrimido-pyrimidine Derivatives

<table>
<thead>
<tr>
<th>Study groups</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leukocytes (x10^9/L)</td>
<td>3.5 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>2.9 ± 0.3</td>
<td>3.8 ± 0.1</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td>Erythrocytes (x10^12/L)</td>
<td>6.0 ± 0.2</td>
<td>6.1 ± 0.1</td>
<td>6.8 ± 0.3</td>
<td>7.4 ± 0.3</td>
<td>6.8 ± 0.3</td>
</tr>
<tr>
<td>Platelets (x10^12/L)</td>
<td>729 ± 38</td>
<td>506 ± 37</td>
<td>545 ± 31</td>
<td>690 ± 75</td>
<td>698 ± 59</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>32.2 ± 0.7</td>
<td>32.0 ± 0.9</td>
<td>36.5 ± 1.9</td>
<td>37.7 ± 1.1</td>
<td>37.8 ± 1</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>11.2 ± 0.2</td>
<td>10.5 ± 0.3</td>
<td>11.6 ± 0.5</td>
<td>12.5 ± 0.2</td>
<td>12.6 ± 0.2</td>
</tr>
<tr>
<td>Platelet volume (fL)</td>
<td>3.9 ± 0.03</td>
<td>4.1 ± 0.003</td>
<td>4.1 ± 0.004</td>
<td>4.3 ± 0.01</td>
<td>4.2 ± 0.008</td>
</tr>
</tbody>
</table>

* I: nondiabetic controls; II: diabetic controls; III: diabetes and dipyridamole; IV: diabetes and mopidamol; V: diabetes and RA-642.
' p < 0.001 vs group I, p < 0.03 vs group IV, and p < 0.01 vs group V.
Maximum intensity of platelet aggregation (Imax) induced by 10 μg/mL of collagen, in whole blood in non-diabetic rats (NDR) and diabetic rats treated with saline, dipyridamole (dip), mopidamol (Mop) and RA-642. *p < 0.01, **p < 0.05 vs diabetic rats treated with saline, and ***p < 0.05 vs nondiabetic rats.

Platelet production of thromboxane B₂ was 87% higher in untreated diabetic animals (group II) than in nondiabetic controls (Figure 2). Dipyridamole produced a nonsignificant decrease of this prostanoid, whereas mopidamol and RA-642 produced a statistically significant inhibition of platelet thromboxane B₂ production (46% and 41% decrease, respectively).

Aortic production of 6-keto-PGF₁₀, which was significantly lower (43% decrease) in untreated diabetic animals (group II), showed no change after treatment with either mopidamol or RA-642. In contrast, dipyridamole caused a 90% increase in aortic production of 6-keto-PGF₁₀, so that statistically significant differences between dipyridamole-treated rats and nondiabetic controls were not detected (Figure 3). The thromboxane/ prostacyclin index (thromboxane B₂/6-keto-PGF₁₀ ratio) was 5.8 ± 0.7 in nondiabetic controls, 19.2 ± 1.1 in untreated diabetic rats (p < 0.001), and 7.7 ± 0.5, 8.1 ± 0.7, and 8.9 ± 0.9 in diabetic animals treated with dipyridamole, mopidamol, and RA-642, respectively (p < 0.001 vs. untreated diabetic rats).

Animals in group II (untreated diabetes) showed a retinal pattern characterized by tortuous vessels, decreased vascular diameter, arterial narrowing, and multiple images of fragmentation of the labelled substance. Diabetic rats given dipyridamole also showed tortuous vessels and arterial narrowing, although the images of fragmentation of the labelled substance were less frequently observed. A less impaired retinal vascular pattern was found in diabetic animals treated with mopidamol. Differences in retinal vascularization between diabetic animals given RA-642 and nondiabetic controls were negligible.
**FIG 2**

Serum thromboxane B$_2$ (TxB$_2$) in non-diabetic rats (NDR) and diabetic rats treated with saline, dipyridamole (dip), mopidamol (Mop) and RA-642.

*p < 0.001 vs NDR; **p < 0.001 vs diabetic rats treated with saline.

**FIG 3**

Aortic 6-keto-PGF$_{1α}$ synthesis induced by 100 µmol/L of arachidonic acid, in non-diabetic rats (NDR) and diabetic rats treated with saline, dipyridamole (dip), mopidamol (Mop) and RA-642.

*p < 0.001 vs NDR; **p < 0.001 vs diabetic rats treated with saline.
Percentage of retinal surface covered by peroxidase-stained vessels, in non-diabetic rats (NDR) and diabetic rats treated with saline, dipyridamole (dip), mopidamol (Mop) and RA-642.

*p < 0.001 vs NDR; **p < 0.03, and ***p < 0.01, vs diabetic rats treated with saline.

With regard to quantitative assessment of retinal vascularization (Figure 4), untreated diabetic animals showed a significantly lower percentage of retinal area occupied by HRP-labelled vessels than nondiabetic controls (81% decrease). Treatment with dipyridamole, mopidamol, and RA-642 caused an increase in the percentage of retinal surface occupied by HRP-labelled vessels (2.5-fold, 2.8-fold and four-fold increases, respectively) as compared to values obtained in untreated diabetic animals. Statistically significant differences between animals given the pyrimido-pyrimidine derivative RA-642 and nondiabetic controls were not found.

DISCUSSION

Characteristic pharmacological properties of dipyridamole, mopidamol, and RA-642 in relation to their chemical structure may result in differential effects exerted by these compounds on retinal vascular pattern. Although there is a large number of studies dealing with morphological, biochemical, thrombocytic or endothelial alterations produced in experimental diabetes mellitus (21-24), there are a few reports studying all these parameters together. Given that in previous studies made by our group, platelet-vessel wall alterations and retinal vascular morphological changes were inconsistently documented within 2 months after induction of diabetes, data in the present study were assessed at 3 months.

Untreated diabetic animals showed increased platelet production of thromboxane, decreased vascular synthesis of prostacyclin, a statistically significant increase in collagen-induced platelet aggregation in whole blood as compared with nondiabetic rats. Moreover, marked morphological
abnormalities including reduction of the retinal area occupied by HRP-labelled vessels, tortuous vessels, arterial narrowing, and fragmentation of the labelled substance, were observed. These findings are in agreement with observations made by our group (25,26) and others (see reviews in refs. 27 and 28) who reported, in humans, an imbalance in thromboxane/prostacyclin production due to an increase in the former and/or a decrease in the latter.

A high thromboxane production, through increasing cytosolic Ca"++, plus the decrease of prostacyclin, through its platelet and vascular effects, can contribute to the appearance of areas of arterial narrowing and images of unlabelled retinal vessels. The presence of platelet microaggregates may be probably an aggravating circumstance.

With regard to collagen-induced platelet aggregation in whole blood, the present results are not in agreement with those observed in humans. According to studies of Rifkin et al. (29), Reuter (30), Eliasson and Bygdeman (31), and De La Cruz et al. (17), mopidamol is three to five times more potent than dipyridamole in inhibiting platelet aggregation; the effects of dipyridamole and RA-642 in whole blood are similar (15). Species-related differences may account for this lack of agreement (32).

The disrupted thromboxane/prostacyclin equilibrium in diabetic rats, is modified by the three pyrimido-pyrimidinic compounds: dipyridamole mainly by an increase in prostacyclin production, and mopidamol and RA-642 by an inhibition of thromboxane synthesis. These biochemical effects were reported previously by several groups (15,17,33,34).

If we accept the hypothesis that thromboxane/prostacyclin balance is essential for a correct maintenance of tissue perfusion, then, a modification of such balance caused by pyrimido-pyrimidinic compounds should be reflected by qualitative and quantitative changes of retinal vascular pattern. In fact, all three pyrimido-pyrimidinic derivatives caused an increase in the retinal surface occupied by HRP-labelled vessels as compared with animals in the group of untreated diabetes. Differences in retinal vascularization between diabetic animals given RA-642 and nondiabetic controls were negligible. Therefore, although all three pyrimido-pyrimidinic derivatives produced an improvement in retinal vascular pattern, the greatest quantitative effect was obtained after the administration of RA-642. In a previous study using this experimental model, we reported that dipyridamole and RA-642 caused a delay in the formation of sugar cataracts, while mopidamol had no effect (35). However, the increase of prostacyclin synthesis or the inhibition of thromboxane production alone, is not enough to improve a beneficial effect on the retinal vascular lesions, perhaps is necessary the addition of the two effects to observe a preventive effect (25).

According to the present results, improvement in retinal vascular pattern observed in animals treated with pyrimido-pyrimidinic derivatives may be explained by a combination of the effects on these compounds on platelet and vascular parameters or by other mechanisms not investigated in our study. It may therefore be postulated that the effect of dipyridamole, mopidamol, and RA-642 on retinal vascularization does not exclusively depend upon a single factor. In this case, an altered retinal vascular pattern may be the result of two fundamental factors: a re-equilibrium of thromboxane/prostacyclin balance and a vascular enhancing effect on blood flow, particularly for RA-642, as has been shown in the cerebral circulation in rats (16) and in the cardiac vascular territory in dogs (36).
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REFERENCES


