Effect of Aspirin Plus Dipyridamole on the Retinal Vascular Pattern in Experimental Diabetes Mellitus

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ABSTRACT

Platelet hyperactivity has been one of the mechanisms implicated in the pathogenesis of diabetic retinopathy. Antiplatelet agents have been shown, in experimental models, to prevent the development of retinal vascular abnormalities when given from the first day after the onset of diabetes. We assessed the effect of aspirin plus dipyridamole (6 + 12 mg/kg daily) on the retinal vascular pattern in experimental streptozotocin-induced diabetes in rats, when the treatment was given at different intervals after the induction of diabetes, over a 3-month study period. Saline-pretreated diabetic rats showed a time-dependent increase in the platelet production of thromboxane B_{2} (r = 0.981, P < .0001) and a decrease in the aortic production of 6-keto-PGF_{1α}. The percentage of retinal area occupied by horseradish peroxidase-labeled vessels decreased progressively in relation to the length of time of the evolution of diabetes (r = 0.983, P < .00001) and the thromboxane/prostacyclin ratio. Treatment with aspirin plus dipyridamole caused an inhibition of the platelet production of thromboxane B_{2} and a decrease in the vascular synthesis of prostacyclin. Treatment with antiplatelet agents slowed down the decrease in the percentage of retinal area occupied by horseradish peroxidase-labeled vessels. These data provide further evidence to support the results of previous clinical trials in which antiplatelet agents had a beneficial effect on the evolution of retinal lesions in early diabetic retinopathy.

Diabetic retinopathy is the earliest and most important microangiopathic complication of diabetes mellitus, especially of type I (insulin-dependent) (Kalter-Leibovici et al., 1991). Although the pathogenetic mechanism responsible for retinopathy is imperfectly understood, considerable evidence implicates biochemical reactions (e.g., excessive polyol production and nonenzymatic glycosylation) and hemorheologic abnormalities (e.g., reduced deformability of erythrocytes, increase in blood viscosity) in the genesis of diabetic microangiopathy (Merimee, 1990). All these processes lead to capillary occlusion and retinal ischemia, in which platelet hyperactivity seems to play a predominant role (Osternmann and Van de Loo, 1986). Various platelet abnormalities have been demonstrated in diabetic patients, particularly in those with rapidly deteriorating retinopathy (Ishii et al., 1992; Dallinger et al., 1987; Moreno et al., 1995a). Accordingly, it seems that the development and progression of retinal vascular lesions could be modified by antiplatelet drug therapy. In fact, we have shown that different inhibitors of platelet aggregation, administered from the first day after the induction of diabetes, prevented or delayed the development of retinal vascular lesions in rats (De La Cruz et al., 1990; 1994; Moreno et al., 1995b). The purpose of the present study was to determine the efficacy of antiplatelet agents when they are introduced at increasingly later times after the development of diabetes in the experimental model.

We assessed the effect of aspirin plus dipyridamole on the retinal vascular pattern in a model of experimental streptozotocin-induced diabetes in rats. The choice of this treatment was based on previous studies made by our group with this experimental model (De La Cruz et al., 1990, 1994; Moreno et al., 1995c), in which aspirin plus dipyridamole showed the greatest beneficial effect as compared with other antiplatelet drugs or aspirin alone. In the present investigation, aspirin combined with dipyridamole was given at different intervals after the onset of diabetes over a 3-month study period.

Materials and Methods

Animals. A total of 120 male Wistar rats weighing 200 to 250 g were housed in plastic cages with unlimited access to food and water. Rats were divided at random into 12 experimental groups. In group I, 10 nondiabetic animals served as controls. In group II, 60 diabetic animals received 0.5 ml/kg/day of isotonic saline (p.o.) for 15 (group IIa, 10 animals), 30 (group IIb, 10 animals), 45 (group IIc, 10 animals), 60 (group IId, 10 animals), 75 (group IIe, 10 animals) and 90 days (group IIf, 10 animals). In group III, 50 diabetic animals received 6 mg/kg/day of aspirin (Sigma Chemical Co., St. Louis, MO) plus 12 mg/kg per day of dipyridamole (Boehringer Ingelheim).
vascularity in nondiabetic and saline-pretreated diabetic rats.

Thus all animals were treated every day p.o. for 90 days, and the differences between groups were in the period with saline vs. the period with antiplatelet treatment. All study medications were administered p.o. (through an endogastric catheter that was left in place between administrations of solutions) as single daily doses given between 9:00 A.M. and 10:00 A.M. Drugs were diluted in isotonic saline to the final concentration used. The duration of treatment was 3 months.

**Experimental diabetes.** Experimental diabetes was induced by a single dose (50 mg/kg) of streptozocin (Sigma) injected i.v. into the femoral vein. Nondiabetic animals received equivalent doses of normal saline. Blood glucose concentration was determined by a micro-method (Glucometer, Menarini, Barcelona, Spain) after a small incision was made 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 aforementioned experimental groups on the day after they were determined to be diabetics (detection of glucose concentrations of 200 mg/dl). Animals in groups II and III were given intermediate-acting insulin, 3 IU/day s.c. (Insulatard HM, Novo Nordisk A.S., Bagsvaerd, Denmark) as antidiabetic. Insulin was administered in order to support high glucose levels without mortality due to a possible ketoacidotic situation.

**Assessment of retinal vascularity.** After completion of the protocol, animals were anesthetized with pentobarbital sodium (Nembutal, Abbott S. A., Madrid, Spain), 40 mg/kg, i.p. and 2 mL of blood was drawn from the left ventricle (1 mL was mixed with 3.8% trisodium citrate in the proportion 1:10, and 1 mL without anticoagulant was introduced in a glass tube). The descending carotid artery was tied and two segments of abdominal aorta of 52.1 ± 2.8 mg were excised. Then 180 mg/kg of HRP type II, (Sigma) was injected into the internal carotid artery.

After the heart had pumped HRP throughout the arterial territory of the internal carotid artery for several minutes, eyeballs were enucleated and retinal tunics were processed histochemically by means of Mesulam’s technique (Mesulam, 1982). Retinal sections were incubated with a solution of tetramethylbenzidine and sodium nitroferrocyanide (Sigma) as chromogen substrates. Samples were weighted and the supernatant was frozen at −80°C until analysis. Aortic production of PGI2 was determined by measuring its stable metabolite 6-keto-PGF1α, via radioimmunoassay (H-6-keto-PGF1α) (Amersham), and the mean value of the two aortic segments was calculated for each animal.

**Platelet volume and cellular counts.** Platelet volume and cellular counts were carried out by an automatic blood cell counter Baker-8000 (Menarini Diagnostica, Barcelona, Spain).

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

**Statistical analysis.** All values in text, tables and figures are presented as mean ± S.E.M. Statistical analysis of the results was carried out using the Epistat computer program. Student’s t test for unpaired data was used to determine significant differences. Statistical significance was set at P < .05.

### Results

Mean values of serum glucose levels, maximum collagen-induced platelet aggregation, platelet production of TxB2α, aortic production of 6-keto-PGF1α, platelet volume and percentage of retinal area occupied by HRP-labeled vessels in nondiabetic controls and untreated diabetic animals are shown in table 1. When these two groups of animals were compared, there were statistically significant differences in all parameters during the 3 months of the study except for maximum collagen-induced platelet aggregation at 90 days.

**Table 1**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Glycemia (mg/dl)</th>
<th>PA (ohms)</th>
<th>TxB2α (nmol/109 plate)</th>
<th>6-keto-PGF1α (pg/mg tissue)</th>
<th>Platelet Voluem (fl)</th>
<th>% Retinal Area with Vascularity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>96.6 ± 3.1</td>
<td>5.7 ± 0.3</td>
<td>0.38 ± 0.01</td>
<td>283.0 ± 9.1</td>
<td>4.17 ± 0.03</td>
<td>21.6 ± 0.9</td>
<td></td>
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<tr>
<td><strong>Untreated diabetics</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>15 days</td>
<td>423 ± 27**</td>
<td>7.1 ± 0.3</td>
<td>0.71 ± 0.05**</td>
<td>108.0 ± 10.0**</td>
<td>4.36 ± 0.08**</td>
<td>17.3 ± 0.3**</td>
</tr>
<tr>
<td>30 days</td>
<td>451 ± 10**</td>
<td>6.8 ± 0.9</td>
<td>0.82 ± 0.07**</td>
<td>126.0 ± 9.4**</td>
<td>4.53 ± 0.05**</td>
<td>15.6 ± 0.3**</td>
</tr>
<tr>
<td>45 days</td>
<td>418 ± 20**</td>
<td>8.1 ± 0.6</td>
<td>1.19 ± 0.09**</td>
<td>97.6 ± 9.2**</td>
<td>4.47 ± 0.12**</td>
<td>13.1 ± 0.4**</td>
</tr>
<tr>
<td>60 days</td>
<td>448 ± 19**</td>
<td>8.2 ± 0.2</td>
<td>1.30 ± 0.11**</td>
<td>108.0 ± 9.0**</td>
<td>4.30 ± 0.06**</td>
<td>9.2 ± 0.2**</td>
</tr>
<tr>
<td>75 days</td>
<td>437 ± 21**</td>
<td>7.6 ± 0.5</td>
<td>1.59 ± 0.09**</td>
<td>106.0 ± 11.1**</td>
<td>4.50 ± 0.10**</td>
<td>5.0 ± 0.3**</td>
</tr>
<tr>
<td>90 days</td>
<td>387 ± 19**</td>
<td>6.4 ± 0.9</td>
<td>1.72 ± 0.08**</td>
<td>94.1 ± 8.06**</td>
<td>4.45 ± 0.04**</td>
<td>2.9 ± 0.4**</td>
</tr>
</tbody>
</table>

* P < .05, ** P < .001 vs. nondiabetic controls.
Saline-pretreated diabetic animals showed a time-dependent increase in the platelet production of TxB₂ \((r = 0.981, P < .0001)\), a non-time-dependent decrease in aortic production of 6-keto-PGF₁α \((r = 0.236, P < .08)\), and a time-dependent decrease in the percentage of retinal area occupied by HRP-labeled vessels. There was a statistically significant correlation between the TxB₂/6-keto-PGF₁α ratio and retinal vascularity (fig. 1). We measured aortic 6-keto-PGF₁α as an indication of systemic PGI₂ production, including retinal vascularity, because endothelial production of PGI₂ follows the same mechanisms in several types of arterial vessels.

The administration of aspirin plus dipyridamole caused a 100% inhibition of maximum collagen-induced platelet aggregation in all groups of animals (independently of the interval at which treatment was begun) and between 93% and 97% inhibition of platelet production of TxB₂ at the end of the study (fig. 2). Intragroup differences were not statistically significant. Aortic production of 6-keto-PGF₁α decreased significantly, although the percentage of inhibition was inversely proportional to the duration of antiplatelet treatment (fig. 3).

At 90 days, the percentage of retinal area occupied by HRP-labeled vessels was significantly higher in animals treated with aspirin and dipyridamole than in saline-pretreated diabetic rats (fig. 4). The earlier treatment began, the greater the differences were.

In nondiabetic rats, retinal vascular morphological changes were not documented (fig. 5). By contrast, diabetic rats showed tortuous vessels, arterial narrowing and multiple images of fragmentation of the labeled substance (figs. 6A and 7A). These alterations in the retinal vascularity were less frequently observed in diabetic rats that were given aspirin plus dipyridamole. Moreover, animals treated early showed a more preserved retinal vascular pattern than animals in which antiplatelet therapy was delayed for up to 75 days after the induction of diabetes (figs. 6B and 7B).

**Discussion**

Untreated diabetic animals, in general terms, showed a state of platelet hyperactivity characterized by an imbalance of TxB₂/PGL₂ production due to an increase in the former and a decrease in the latter. These findings are in agreement with observations made by our group in rats with long-standing diabetes (Moreno et al., 1995c; De La Cruz et al., 1990, 1994) and by other authors (Ostermann and Van de Loo, 1986; Ishii et al., 1992; Dallinger et al., 1987) who reported, in humans, an imbalance in TxB₂/PGL₂ production, in which decrease in prostacyclin levels was not correlated with the time of induction of disease.

As compared with nondiabetic controls, saline-pretreated diabetic rats showed a marked increase in collagen-induced platelet aggregation in whole blood and a significant increase in platelet aggregation in response to collagen in the citrated plasma. Moreover, animals treated early showed a more preserved retinal vascular pattern than animals in which antiplatelet therapy was delayed for up to 75 days after the induction of diabetes (figs. 6B and 7B).

**Fig. 1.** Linear correlation between TxB₂/6-keto-PGF₁α and the percentage of retinal area occupied by HRP-labeled vessels.

**Fig. 2.** Time course of platelet production of TxB₂ in untreated diabetic animals (□) and animals given aspirin plus dipyridamole from the indicated times after the induction of diabetes (abscissa) to the end of treatment (90 days) (■). * P < .0001 as compared with saline-pretreated diabetic rats at 90 days.
in platelet volume, which is an indirect sign of platelet hyperaggregation (D’Erasmo and Acca, 1992). The greater production of TxB₂ by platelets from diabetic animals than by those from nondiabetic controls is consistent with previous investigations (Moreno et al., 1995c; De La Cruz et al., 1990, 1994). Therefore, an increase in platelet cyclooxygenase activity seems to occur at early stages of the diabetic state and to progress time-dependently during the course of the disease.

Tx, through its interaction with its specific membrane receptor, causes vasoconstriction and plays an important role in the Ca²⁺ mobilization process, increasing the rate of cytosolic Ca²⁺ and thus stimulating platelet aggregation (Samuelsson et al., 1978). These actions favor the development of diabetic retinal vascular lesions, because vasoconstriction reduces blood flow, and platelet hyperactivity may cause microaggregates that, in turn, would enhance the ischemic state (Kohner et al., 1995).

In a result consistent with previous studies (Moreno et al., 1995c; De La Cruz et al., 1990, 1994), untreated diabetic animals showed a decrease in the vascular synthesis of PGI₂ that was not dependent on the duration of diabetes. The active mechanism based on the formation of PGI₂ that defends normal vessels against platelet deposition (Moncada et al., 1977) appears to be absent in our experimental model, so, the aggregating and vasoconstriction activity of Tx is not prevented by PGI₂ produced by vessel walls. Therefore, a decrease in the vascular synthesis of PGI₂ associated with an increase in the platelet production of Tx, i.e., an imbalance of...
Tx/PGL₂ production (Moreno et al., 1995c; Engerman, 1989), contributes to the appearance of areas of arterial narrowing and images of unlabeled retinal vessels. The statistically significant correlation between retinal vascularity and the TxB₂/6-keto-PGF₁α ratio, a thrombogenic index (Moncada and Amezgua, 1979), confirms the important role that these prostanoids could have played in the development of retinal vascular lesions in this model of experimental diabetes. According to the results of the present study, TxB₂ seems to contribute in a higher proportion to the TxB₂/6-keto-PGF₁α ratio; however, modifications in PGL₂ production are important to the pharmacological prevention of retinopathy in our experimental model in rats. In saline-treated diabetic rats, TX synthesis seems to be mathematically more important than prostacyclin production, but the minor alteration in vascular synthesis is sufficient to explain the mechanism of pharmacological protection.

The results obtained in diabetic animals treated with aspirin plus dipyridamole confirm the well-known properties of these aggregation inhibitors. Because sensitivity to the pharmacological effects of aspirin is almost 50% lower in rats than in humans (De La Cruz et al., 1990), the dose of 6 mg/kg/day used in this study may be considered to reflect the “high-dose” range in humans. With regard to the effects of antiplatelet drug therapy on Tx/PGL₂ balance, and in agreement with previous observations (Moreno et al., 1995b; De La Cruz et al., 1990, 1994), there was a rapid and nearly complete (93%–97%) inhibition of the platelet production of TxB₂ regardless of when treatment with aspirin plus dipyridamole was begun. In accordance with the “high doses” of aspirin tested, inhibition of vascular synthesis of PGL₂ varied between 30% and 66% in the different groups of diabetic animals. However, the amount of PGL₂ inhibition was significantly lower in the group of rats given aspirin plus dipyridamole after the onset of diabetes than in the remaining groups.

Because Tx/PGL₂ balance is essential for the correct maintenance of tissue perfusion, modification of such balance by antiplatelet drug therapy should be reflected in qualitative and quantitative changes in retinal vascular pattern. In fact, aspirin plus dipyridamole given at different intervals after the induction of diabetes interrupted the progression of retinal vascular lesions as compared with untreated diabetic animals. It should be noted that in this experimental model, inhibitors of platelet aggregation not only prevented a decrease in retinal vascularity from the onset of diabetes but also slowed down the progression of altered retinal pattern from the moment when they were given. This latter effect was independent of time since the induction of diabetes. The favorable effects of aspirin plus dipyridamole on retinal vascularity may be explained by their action on platelet aggregation as well as by the vasodilator properties of dipyridamole (Kinsella et al., 1962).

Some clinical studies using the association of dipyridamole and aspirin (ESPS, 1990, in cerebrovascular events; DAMAD, 1989, in evolution of diabetic retinopathy; PARIS-II, 1986, in coronary ischemia) have revealed no differences in percentages of prevention of the respective events between women and men. However, we used only male rats because some studies, mainly in prevention of ischemic cerebrovascular events, have demonstrated that aspirin alone shows a lesser protective effect in women than in men. We also demonstrated [De La Cruz et al., 1986] that this difference is observed in vitro and that it is influenced by the hormonal levels of estrogens in women. Because these factors could introduce new variables that were not related to our objectives, we used only male rats to eliminate the gender factor.

In addition to suggesting the importance of intensive therapy to delay the onset and slow the progression of retinopathy [DCCTRG, 1993], the present findings provide further evidence in support of previous clinical trials [Pagani et al., 1989; DAMAD, 1989; Esmatjes et al., 1989; TIMAD, 1990] in which antplatelet agents had a beneficial effect on the evolution of retinal lesions in early diabetic retinopathy.

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References


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