REGULAR ARTICLE

Effect of DT-TX 30, a Combined Thromboxane Synthase Inhibitor and Thromboxane Receptor Antagonist, on Retinal Vascularity in Experimental Diabetes Mellitus

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Abstract

Combined thromboxane synthase inhibitors and thromboxane receptors antagonists have been shown to have a beneficial effect on different models of thrombosis in vivo. We studied the action of one of these compounds (DT-TX 30) compared with dazoxiben (a thromboxane synthase inhibitor) on retinal vascularity in streptozotocin-diabetic rats. Ten nondiabetic animals were treated with isotonic saline, 30 (10 animals per group) were given 0.4, 4, and 8 mg kg⁻¹ per day of DT-TX 30 (p.o.) and 30 (10 animals per group) were given 10, 50, and 100 mg kg⁻¹ per day of dazoxiben (p.o.) over a 90-day study period. DT-TX 30 caused a dose-dependent decrease of platelet aggregation and thromboxane B₂ synthesis. There was an increase of 9, 65, and 166% in the synthesis of prostacyclin after treatment with 0.4, 4, and 8 mg kg⁻¹ per day, respectively. Retinal vascularity increased in 51, 72, and 182% of animals in response to the three doses used. Synthesis of prostacyclin and the degree of retinal vascularity showed a linear correlation (r² = 0.6528, p < 0.00001). Dazoxiben, at doses that inhibited thromboxane synthesis as much as DT-TX 30, increased prostacyclin production and retinal vascularity with less potency than DT-TX 30. In conclusion, the antagonism of thromboxane receptors may be an important additional effect to the inhibition of thromboxane synthase in the prevention of ischemic retinal lesions in experimental diabetes. © 2000 Elsevier Science Ltd. All rights reserved.

Key Words: Thromboxane synthase; Thromboxane receptors; Platelets; Prostacyclin; Diabetic retinopathy


Abbreviations: TxB₂, thromboxane B₂; HRP, horseradish peroxidase; PGF₁α, prostaglandin F₁α.

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tor (PAF)-receptors [12] among other drugs) have shown a prophylactic effect on the development and evolution of these retinal vascular lesions in this experimental model. One of the main conclusion drawn from these studies was the need to inhibit platelet production of thromboxane, together with protecting or even increasing vascular production of prostacyclin to obtain an optimal prevention of retinal lesions in diabetic retinopathy. In this respect, dual specific thromboxane synthase inhibitors and blockers of thromboxane-endoperoxidase receptors have been shown to meet this premise [13,14]. Therefore, this study was conducted to assess the effect of one of these compounds, DT-TX 30 (E-6-(2-(4-chloro-benzene- sulfonyl - amino)ergyl)phenyl) - 6 - (3-pyridyl) - 5 - hexenoic acid) [15], in comparison with the selective inhibitor of thromboxane synthase dazoxiben (4-[2-(1H-imidazol-1-yl)ethoxy]-benzoic acid) [16,17] (Figure 1) on the prevention of experimental diabetic retinopathy in rats.

1. Methods

1.1. Animals

A total of 80 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 eight experimental groups. In group I, 10 non-diabetic animals served as controls. In group II, 10 diabetic animals received 0.5 mL kg$^{-1}$ per day of isotonic saline (p.o.) for 90 days. In groups III, IV, and V, 10 diabetic animals in each group received 0.4, 4, and 8 mg kg$^{-1}$ per day of DT-TX 30 (Dr. Karl Thomae Institute, Biberach an der Riss, Germany), respectively, from day 1 after induction of diabetes. In groups VI, VII, and VIII, 10 diabetic animals in each group received 10, 50, and 100 mg kg$^{-1}$ per day of dazoxiben (Grupo Ferrer Internacional S.A., Barcelona, Spain), respectively, from day 1 after induction of diabetes. All animals were treated every day orally (through an endogastric catheter that was left in place between administrations of solutions) for 90 days, in doses given twice daily between 9:00 a.m. and 10:00 a.m. and between 8:00 and 9:00 p.m. Drugs were diluted in isotonic saline to the final concentrations used.

Doses of drugs were decided according to previous experiments in our laboratory (data not shown) in which we measured platelet thromboxane $B_2$ (TxB$_2$) synthesis after the administration of several doses of both drugs in nondiabetic rats. We chose these doses because they inhibited TxB$_2$ production in similar percentages.

The experimental protocol was approved by the Animal Ethical Committee in the School of Medicine of the University of Málaga.

1.2. Experimental Diabetes

Experimental diabetes was induced by a single dose (50 mg kg$^{-1}$) of streptozocin (Sigma, St. Louis, MO, USA) injected intravenously into the femoral vein. Nondiabetic animals received equivalent doses of normal saline. Blood glucose concentration was determined by a micromethod (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 previously mentioned experimental groups on the next day, after they had been considered to be diabetic (detection of glucose concentrations of 200 mg dL$^{-1}$). Animals in groups II, III, IV, V, and VI were given intermediate-acting insulin, 3 IU per day subcutaneously (Insulatard HM®, Novo Nordisk A.S., Bagsvaerd, Denmark) as antidiabetic
treatment. Insulin was administered to support high glucose levels without mortality due to a possible ketoacidotic situation.

1.3. Assessment of Retinal Vascularity

After completion of the protocol, animals were anesthetized with pentobarbital sodium (Nembutal®, Abbott S.A., Madrid, Spain), 40 mg kg$^{-1}$ intraperitoneally, and 2 mL of blood was drawn from the left ventricle (1 mL was mixed with 3.8% trisodium citrate in a 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$^{-1}$ of horseradish peroxidase (HRP, type II, Sigma) was injected into the internal carotid artery.

After the heart was left to pump 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 [18]. Retinal sections were incubated with a solution of tetramethylbencidine and sodium nitroferrocyanide (Sigma) as chromogenic substrates. Samples were subsequently dehydrated in a graded series of alcohol, incubated in xylene, and mounted on slices for light-microscopic examination. Retinal vessels labeled with HRP were examined at 40× magnification. A computerized, digital image processing system (IBAS Kontron 2000, Kontron Bi analyse, Basel, Switzerland) [19] was applied to microscopic photographs to evaluate the percentage of the retinal area occupied by HRP-labeled vessels. Retinal vascular pattern was also assessed qualitatively for the presence of arterial narrowing, tortuous vessels, dilations, and images of fragmentation of the labeled agent.

1.4. 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 minutes after the addition of 10 µg mL$^{-1}$ of collagen (Menarini Diagnostica, Barcelona, Spain). Aggregometry was performed at 37°C in a double-channel aggregometer (model 540, Chrono-Log Corp., Havertown, PA, USA) with continuous stirring at 1000 rpm.

1.5. Platelet Production of TxB$_2$

TxB$_2$ (stable metabolite of thromboxane A$_2$) was measured by radioimmunoassay ($^3$H-TxB$_2$) (Amer sham International plc, Little Chalfont, Buckinghamshire, UK). The sample of whole blood without anticoagulant was introduced in a bath at 37°C for 45 minutes (platelet stimulation by formed thrombin) and then centrifuged at 2500×g at 4°C for 15 minutes. The serum was removed and kept frozen at −80°C until analysis. To assess a possible influence of platelet number in platelet TxB$_2$ production, the formula described by Carter and Hanley [21] was applied as shown in Eq. (1):

$$TxB_2 \text{ (nmol/10}^9 \text{ platelets)} = \frac{TxB_2 \text{ (nM)} \times (1 - \text{[hematocrit/100]}) \times 10^9}{\text{platelet number (cells} \times 10^9 \text{ L}^{-1})}$$

1.6. Aortic Production of 6-keto-prostaglandin F$_{1\alpha}$

Aortic segments were incubated in 1 mL of a buffer solution containing 100 mM NaCl, 4 mM KCl, 25 mM NaHCO$_3$, 2.1 mM Na$_2$SO$_4$, 20 mM sodium citrate, 2.7 mM glucose, and 50 mM Tris (pH 8.3). After 5 minutes of incubation at 37°C, tissue samples were weighed and the supernatant was frozen at −70°C until assay. Aortic production of prostacyclin was determined by measuring its stable metabolite 6-keto-prostaglandin F$_{1\alpha}$ (6-keto-PGF$_{1\alpha}$) by radioimmunoassay ($^3$H-6-keto-PGF$_{1\alpha}$) (Amer sham); the mean value of the two aortic segments was calculated for each animal.

1.7. Blood Cell Counts

Cellular counts were carried out by an automatic blood cell counter Baker-8000 (Menarini).

1.8. Statistical Analysis

All tests were carried out by researchers who were blind to the origin of the samples and to the purpose of the study. All values in text, table, and figures are expressed as mean±SEM. Statistical analysis
Table 1. Serum glucose levels and maximal platelet aggregation in the different groups of rats

<table>
<thead>
<tr>
<th>Group (ohms)</th>
<th>Glycemia (mg dL(^{-1}))</th>
<th>Maximal platelet aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nondiabetic ((n=10))</td>
<td>97±4.2*</td>
<td>5.8±0.6*</td>
</tr>
<tr>
<td>Untreated diabetic ((n=10))</td>
<td>478±11.2</td>
<td>16.1±1.1*</td>
</tr>
<tr>
<td>Treated with DT-TX 30 (p.o.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 mg kg(^{-1}) per day ((n=10))</td>
<td>494±9.8</td>
<td>8.9±0.8**</td>
</tr>
<tr>
<td>4 mg kg(^{-1}) per day ((n=10))</td>
<td>471±12</td>
<td>6.2±0.9**</td>
</tr>
<tr>
<td>8 mg kg(^{-1}) per day ((n=10))</td>
<td>483±22</td>
<td>1.4±0.09**</td>
</tr>
<tr>
<td>Treated with dazoxiben (p.o.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mg kg(^{-1}) per day ((n=10))</td>
<td>483±10</td>
<td>7.2±0.9**</td>
</tr>
<tr>
<td>50 mg kg(^{-1}) per day ((n=10))</td>
<td>481±11</td>
<td>6.8±0.7**</td>
</tr>
<tr>
<td>100 mg kg(^{-1}) per day ((n=10))</td>
<td>479±16</td>
<td>5.5±0.6**</td>
</tr>
</tbody>
</table>

\(*p<0.05\) compared with nondiabetic rats, \(**p<0.05\) compared with untreated diabetic rats.

of the results was carried out using the Statistical Package for the Social Sciences (SPSS, version 6.0 for Windows 95). The one-way analysis of variance with Bonferroni’s correction was used to determine significant differences. The Pearson’s product-moment correlation coefficient was used to assess the association between two continuous variables. Statistical significance was set at \(p<0.05\).

2. Results

Serum glucose levels (mean value of all measurements carried out during the 3 months of the study) in the groups of diabetic animals were significantly higher than in the group of nondiabetic controls (Table 1). The administration of DT-TX 30 or dazoxiben did not show a significant effect on serum glucose levels in diabetic animals at any of the doses used (Table 1). Blood cell counts, body weight, or the amount of food and water daily ingested by diabetic rats were not modified either (data not shown).

Maximal intensity of collagen-induced platelet aggregation in whole blood showed a significant increase in untreated diabetic animals (Table 1), and a dose-dependent inhibition in rats treated with DT-TX 30 (45% inhibition with 0.4 mg kg\(^{-1}\) per day, 61% with 4 mg kg\(^{-1}\) per day, and 91% with 8 mg kg\(^{-1}\) per day). Dazoxiben inhibited platelet aggregation by 54% with 10 mg kg\(^{-1}\) per day, 57% with 50 mg kg\(^{-1}\) per day, and 67% with 100 mg kg\(^{-1}\) per day.

Platelet production of TxB\(_2\) (Figure 2) showed a significant increase in diabetic animals compared to nondiabetic rats (Figure 2). Treatment with 0.4, 4, and 8 mg kg\(^{-1}\) per day of DT-TX 30 was followed by 18, 47, and 72% decreases in the platelet production of TxB\(_2\), respectively. Dazoxiben reduced TxB\(_2\) production by 16% with 10 mg kg\(^{-1}\) per day, 42% with 50 mg kg\(^{-1}\) per day, and 70% with 100 mg kg\(^{-1}\) per day.

Vascular production of 6-keto-PGF\(_{1\alpha}\) (Figure 3) showed a significant decrease in diabetic animals with respect to nondiabetic rats (Figure 3). The production of 6-keto-PGF\(_{1\alpha}\) showed a 16% in-
48% in the retinal area occupied by HRP-labeled vessels after administration of 0.4 mg kg$^{-1}$ per day, 68% after 4 mg/kg/day, and 168% after 8 mg kg$^{-1}$ per day. The administration of 10, 50, and 100 mg kg$^{-1}$ per day of dazoxiben was followed by 1, 52, and 64% increases in retinal vascularity, respectively.

There was a statistically significant correlation between 6-keto-PGF$_{1\alpha}$ values and the percentages of retinal area occupied by HRP-labeled vessels ($Y=3.9+38.4X$, $r^2=0.6528$, $p<0.00001$), considering all the animals in the study (nondiabetic rats, diabetics without treatment, diabetics treated with dazoxiben, and diabetics treated with DT-TX 30). There was a nonsignificant correlation between platelet thromboxane production and retinal vascularity ($r^2=0.1223$, $p<0.1$).

### 3. Discussion

The present results confirm the presence of a marked platelet hyperactivity and imbalance of the synthesis of eicosanoids in diabetes mellitus and, especially, in the experimental model used in this study. These findings have been previously demonstrated by different groups, either in experimental models [5] or in studies with humans [4]. In addition, our results confirm that there is a relationship between these abnormalities and the ischemic-type lesions that appear in the retina of the animals, data previously shown by our group in the same experimental model [5].

In previous findings made by our group in the same experimental model, physiopathological and pharmacological evidence [5,7,10–12] was provided in relation to the fact that preservation of prostacyclin synthesis was proportionally more important than inhibition of thromboxane synthesis to obtain a prophylactic effect of ischemic vascular lesions in experimental diabetes.

Specific inhibitors of platelet thromboxane synthesis are compounds that theoretically fulfill a main requirement of antiplatelet drugs, that is, to inhibit the synthesis of an inducer of platelet aggregation (thromboxane) and to preserve the production of an endogenous factor that prevents platelet aggregation (prostacyclin) [14]. Moreover, cyclic endoperoxides that are accumulated may be transferred to the endothelial cells and serve as a sub-
strate for prostacyclin-synthase, although a part of them are bound to membrane receptors for thromboxane/endoperoxides of other platelets promoting platelet activation [22]. This could be the reason for the lesser effect of dazoxiben than DT-TX 30, although both of them produced a similar inhibition in TxB₂ production. For this reason, dual specific thromboxane synthase inhibitors and blockers of thromboxane-endoperoxide receptors may provide an important differential aspect with regard to drugs with no action on these receptors [14]. This is the case for DT-TX 30, a compound that has already shown a specific inhibitor effect on platelet thromboxane synthase (IC₅₀ 4 nM) and an antagonist action on thromboxane/endoperoxide receptors (IC₅₀ 19 nM) [15,23].

Our results show that DT-TX 30 produced a dose-dependent inhibition of platelet aggregation, with an effect similar to that of aspirin when the highest dose was tested (8 mg kg⁻¹ per day). In addition, the specific inhibition of platelet thromboxane synthesis was demonstrated by dose-dependent decreases of TxB₂ as well as by dose-dependent increases of endothelial production of 6-keto-PGF₁α. That is, DT-TX 30 shows a pharmacological profile different from that of aspirin.

The hypothesis on the important role of prostacyclin in the better retinal vascularity in experimental diabetes was confirmed by the finding of a dose-dependent increase in the retinal area occupied by HRP-labeled vessels in the animals treated with DT-TX 30. The values obtained in the group given 8 mg kg⁻¹ per day were even close to results found in the nondiabetic group. The presence of linear correlation between 6-keto-PGF₁α and the percentage of retinal area occupied by HRP-labeled vessels confirms this assumption.

Finally, specific inhibition of thromboxane synthase combined with blockage of thromboxane/endoperoxide receptors not only may exert its beneficial effect on retinal vascularity through an inhibition of platelet aggregation and an increase of a vasodilator eicosanoid, but also on thromboxane/endoperoxide receptors, which have been reported in different vascular and nonvascular structures of the human eye [24], although the definitive functional significance of this finding is unknown.

In summary, specific inhibition of thromboxane synthase and blockage of thromboxane/endoperoxide receptors may constitute an alternative in the prophylaxis of ischemic retinal lesions in diabetes, although further studies in humans are needed to draw definitive conclusions.

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