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Effect of WEB 2086-BS, an antagonist of platelet-activating factor receptors, on retinal vascularity in diabetic rats

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Abstract

Specific antagonists of platelet-activating factor (PAF) receptors inhibit platelet aggregation and thromboxane synthesis. These two processes have been implicated in the course of diabetic retinopathy. We assessed the effect of a specific PAF receptor antagonist, WEB 2086-BS (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2-f) (1,2,4 triazolo-(4,3-a(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone) on retinal vascularity in a model of experimental streptozocin-induced diabetes in rats. Rats were divided into five experimental groups (10 animals/group): group I, non-diabetic group II, untreated diabetic group III, diabetic given 1 mg/kg per day of WEB 2086-BS (p.o.) group IV, diabetic given 5 mg/kg per day (p.o.) and group V, diabetic given 10 mg/kg per day (p.o.). After 3-month treatment, platelet aggregometry, platelet synthesis of thromboxane B_2, aortic production of 6-keto-prostaglandin $F_{1\alpha}$, platelet and vascular lipid peroxidation, and percentage of the retinal area occupied by horseradish peroxidase-labeled vessels were measured. Untreated diabetic rats showed an increase in platelet reactivity, reduced 6-keto-prostaglandin $F_{1\alpha}$ production, increased thromboxane B_2 and lipid peroxides, and a decrease in the percentage of retinal area occupied by horseradish peroxidase-labeled vessels. WEB 2086-BS produced a decrease in platelet aggregation induced by collagen in whole blood, in thromboxane B_2 synthesis and lipid peroxide production, and an increase in the percentage of retinal area occupied by horseradish peroxidase-labeled vessels ($13.9 \pm 1.1\%$ in group II and $9.9 \pm 0.8\%$ in group V). There was a statistically significant linear correlation ($Y = -0.72 + 137X$, $r^2 = 0.7247$, $P < 0.0007$) between thromboxane B_2 values and the percentages of retinal area occupied by horseradish peroxidase-labeled vessels in the groups of animals treated with WEB 2086-BS.

Keywords: Diabetic retinopathy; Platelet; PAF (platelet-activating factor); Thromboxane lipid peroxidation

1. Introduction

Specific antagonists of membrane receptors for platelet-activating factor (PAF), such as WEB 2086-BS (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno(3,2-f) (1,2,4 triazolo-(4,3-a(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone),have shown a potential therapeutic effect on a series of PAF-mediated processes including bronchoconstriction (Casals-Stenzel et al., 1987; Stevenson et al., 1987), release of histamine and thromboxane mediators (Chung et al., 1986), infiltration of eosinophils (Lellouch-Tubiana et al., 1985), gastric hypersecretion (Brambilla et al., 1987), microvascular leakage of pulmonary vessels (Casals-Stenzel et al., 1987), and platelet accumulation in lung tissue (Casals-Stenzel et al., 1987). Moreover, PAF receptor antagonists inhibit platelet aggregation (O'Donnell and Barnett, 1981). Platelet hyperactivity and increased thromboxane synthesis have been implicated in the pathophysiology and evolution of diabetic microangiopathy (Hendra and Betteridge, 1989; Ishii et al., 1992; de la Cruz et al., 1997b). In a model of experimental streptozocin-induced diabetes in rats, these alterations were directly related to the development of retinal ischemic lesions (Moreno et al., 1995a) and control of platelet hyperactivity correlated with a quantitative improvement of the retinal vascular pattern (de la Cruz et al., 1990; Moreno et al., 1995b; de la Cruz et al., 1996, 1997a, 1998). This study was, therefore, conducted to assess the

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effect of a specific PAF receptor antagonist, WEB 2086-BS, on retinal vascularity in the experimental streptozocin-induced diabetes model in relation to platelet aggregation and synthesis of eicosanoids.

2. Materials and methods

2.1. Animals

A total of 50 male wistar rats weighing 200 to 250 g were housed in plastic cages with unlimited access to food and water. The rats were divided at random into five groups. In group I, 10 non-diabetic animals served as controls. In group II, 10 diabetic animals received 0.5 ml/kg/day of isotonic saline (p.o.) for 90 days. In group iii, 10 diabetic animals received 1 mg/kg per day of WEB 2086-BS (Dr. Karl Thomae Institute, Biberach an der Riss, Germany) from day 1 after induction of diabetes. In groups IV and V, 10 diabetic animals in each group received 5 and 10 mg/kg per day of WEB 2086-BS, respectively. All animals were treated every day orally (through an endogastric catheter that was left in place between administrations of solutions) for 90 days, in twice daily doses given between 0900 and 1000 and between 2000 and 2100. Drugs were diluted in isotonic saline to the final concentration used. The duration of treatment was 3 months.

2.2. Experimental diabetes

Experimental diabetes was induced by a single dose (50 mg/kg) of streptozocin (Sigma Chemical, St. Louis, MO) injected intravenously into the femoral vein. Non-diabetic animals received equivalent doses of normal saline. Blood glucose concentration was determined by a micromethod (Glucometer®, Menarini Diagnóstica, 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. The animals were divided at random into the aforementioned experimental groups on the day after they had been considered to be diabetic (detection of glucose concentrations of 200 mg/dl). Animals in groups II, III, IV and V were given intermediate-acting insulin, 3 IU/day subcutaneously (Insulatard HM®, Novo Nordisk, Bagsvaerd, Denmark) as antidiabetic. Insulin was administered in order to support high glucose levels without mortality due to a possible ketoacidotic situation.

2.3. Assessment of retinal vascularity

After completion of the protocol, the animals were anesthetized with pentobarbital sodium (Nembutal®, Abbott, 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 1:10, and 1 ml without anticoagulant was put 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 horseradish peroxidase (horseradish peroxidase-type II, Sigma) was injected into the internal carotid artery.

After the heart was left to pump horseradish peroxidase throughout the arterial territory of the internal carotid artery for some minutes, eyeballs were enucleated and retinal tunics were processed histochemically by means of Mesulam (1982) technique. Retinal sections were incubated with a solution of tetramethylbencidine and sodium nitroferrocyanide (Sigma) as chromogen substrates. The samples were subsequently dehydrated in a graded series of alcohol, incubated in xylene and mounted on slides for light-microscopic examination. Retinal vessels labeled with horseradish peroxidase were examined at $40\times$ magnification. A computerized, digital image processing system (IBAS Kontron 2000) (Gonzalez and Wintz, 1987) was applied to photomicrographs in order to evaluate the percentage of the retinal area occupied by horseradish peroxidase-labeled vessels. The retinal vascular pattern was also assessed qualitatively for the presence of arterial narrowing, tortuous vessels, dilatations and images of fragmentation of the labeled agent.

2.4. Platelet aggregometry

Platelet aggregation was measured in whole blood samples by the electric impedance method described by Cardinal and Flower (1980), as the maximum change in impedance (Ω) 10 min after the addition of collagen (final concentration 10 μg/ml, Menarini). Aggregometry was performed at 37°C in a double-channel aggregometer (model 540, Chrono-Log, Havertown, PA) with continuous stirring at 1000 rpm.

2.5. Platelet production of thromboxane B_2

Thromboxane B_2 (stable metabolite of thromboxane A_2) was measured by radioimmunoassay ([^{3}H]thromboxane B_2) (Amersham International, UK). The sample of whole blood without anticoagulant was introduced in a bath at 37°C for 45 min (platelet stimulation by formed thrombin) and then centrifuged at $2500\times g$ at 4°C for 15 min. The serum was removed and kept frozen at -80°C until analysis. In order to assess a possible influence of platelet number on platelet thromboxane B_2 production, the formula described by Carter and Hanley (1985) was applied as follows:

$$\text{Thromboxane } B_2\ (\text{nmol}/10^9\ \text{platelets}) = \frac{\text{Thromboxane } B_2\ (\text{nmol/l}) \times (1 - (\text{Haematocrit}/100))}{\text{Platelet number}\ (\text{cells} \times 10^9/\text{l})}.$$

2.6. Aortic production of 6-keto-prostaglandin $F_{1\alpha}$

Aortic segments were incubated in 1 ml of a buffer solution containing (in mmol/l): 100 NaCl, 4 KCl, 25

Table 1
Serum glucose levels and maximal platelet aggregation in the various groups of rats

Group	Glycemia (mg/dl)	Platelet aggregation (Ω)
Non diabetic ($n=10$)	87 ± 9.4[a]	5.5 ± 0.3
Untreated diabetic ($n=10$)	475 ± 10.2	15.9 ± 1.3[b]
Treated with WEB 2086-BS (p.o.)		
1 mg/kg per day ($n=10$)	468 ± 7.0	6.1 ± 0.5[c]
5 mg/kg per day ($n=10$)	476 ± 5.5	5.2 ± 0.8[c]
10 mg /kg per day ($n=10$)	461 ± 6.8	3.2 ± 0.4[c]

[b] $P < 0.05$ as compared with diabetic rats.
[b] $P < 0.05$ as compared with non-diabetic rats.
[c] $P < 0.05$ as compared with untreated diabetic rats.

$NaHCO_3$, 2.1 Na_2SO_4, 20 sodium citrate, 2.7 glucose, 50 Tris (pH 8.3). After 5 min 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 $F_{1\alpha}$, by radioimmunoassay ([^{3}H]6-keto-prostaglandin $F_{1\alpha}$) (Amersham); the mean value for the two aortic segments was calculated for each animal.

2.7. Platelet and aortic lipid peroxidation

An aliquot of the serum sample for thromboxane B_2 determination was incubated with thiobarbituric acid (0.5% in 20% trichloroacetic acid) at 100°C for 15 min. Absorbance was calculated spectrophotometrically at 520 nm. The absorbances obtained were compared to that from a standard curve using malondialdehyde-bis-diethyl-acetol and lipid peroxidation was quantified based on the products of the reaction with thiobarbituric acid (thiobarbituric acid reactive substances), the most significant of which is malondialdehyde. Aliquots of aortic tissue samples were treated with 20% trichloroacetic acid, homogenized in the same incubation buffer, centrifuged at $2500 \times g$ for 20 min at 4°C and the supernatant was processed for detection of thiobarbituric acid reactive substances production as described above.

2.8. Blood cell counts

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

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

2.9. Statistical analysis

All values in text, tables and figures are expressed as means ± standard error of the mean (S.E.M.). Statistical analysis 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 (ANOVA) with Bonferroni's correction was used to determine the significance of differences. Pearson's product-moment correlation coefficient was used to assess the association between two continuous variables. Statistical significance was set at $P < 0.05$.

3. 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 those in the group of non-diabetic controls (Table 1). The administration of WEB 2086-BS did not have a significant effect on serum glucose levels in diabetic animals at any of the doses used (Table 1), nor did it modify blood cell counts, body weight or the amount of food and water ingested daily by the diabetic rats (data not shown).

The maximal intensity of collagen-induced platelet aggregation in whole blood increased significantly in untreated diabetic animals (Table 1), whereas WEB 2086-BS caused a significant inhibition of collagen-induced maximal platelet aggregation in whole blood (62.1 ± 6.3% inhibition with 1 mg/kg per day, 68.4 ± 5.6% inhibition with 5 mg/kg per day and 80.0 ± 6.6% inhibition with 10 mg/kg per day).

Platelet production of thromboxane B_2 showed a significant increase in diabetic animals as compared with that in non-diabetic rats (Fig. 1). Treatment with WEB 2086-BS produced a dose-dependent decrease in thromboxane B_2 platelet production, although statistically significant differences were only obtained with the 10 mg/kg per day dose (0.5 ± 0.1% of inhibition with 1 mg/kg per day, 16.2 ± 0.9% inhibition with 5 mg/kg per day and 48.9 ± 29% inhibition with 10 mg/kg per day) (Fig. 1). Vascular

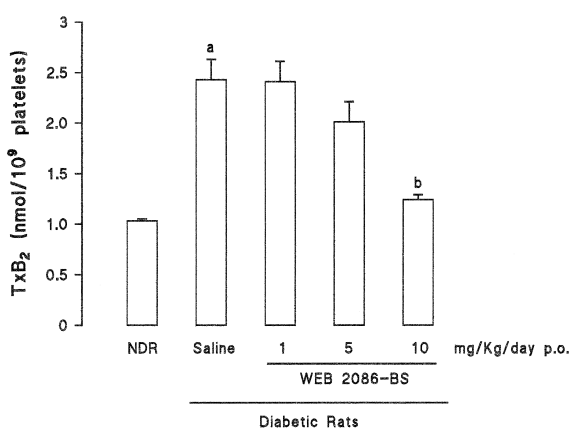

Fig. 1. Platelet production of thromboxane B_2 in non-diabetic rats (NDR) and in diabetic animals treated with isotonic saline or WEB 2086-BS ([a] $P < 0.05$ as compared with NDR; [b] $P < 0.05$ as compared with diabetic animals treated with saline).

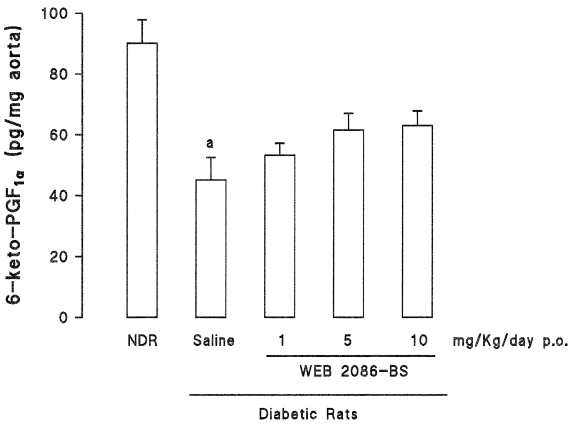

Fig. 2. Aortic production of 6-keto-prostaglandin $F_{1\alpha}$ in non-diabetic rats (NDR) and in diabetic animals treated with isotonic saline or WEB 2086-BS ($^{a}P < 0.05$ as compared with NDR; $^{b}P < 0.05$ as compared with diabetic animals treated with saline).

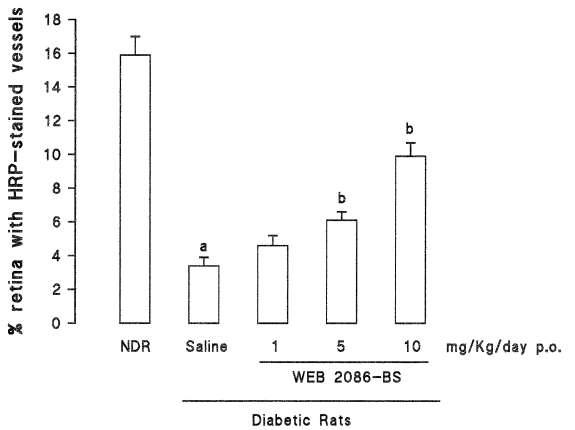

Fig. 4. Percentage of retinal area occupied by horseradish (horseradish peroxidase)-labeled vessels in non-diabetic rats (NDR) and in diabetic animals treated with isotonic saline or WEB 2086-BS ($^{a}P < 0.05$ as compared with NDR; $^{b}P < 0.05$ as compared with diabetic animals treated with saline).

production of 6-keto-prostaglandin $F_{1\alpha}$ was significantly decreased in diabetic animals with respect to that in non-diabetic rats (Fig. 2). None of the doses of WEB 2086-BS had any effect on this parameter.

Lipid peroxidation, measured as malondialdehyde production both in platelets and in aortic segments, increased significantly in untreated diabetic animals (Fig. 3). Treatment with WEB 2086-BS was followed by a statistically significant reduction of platelet lipid peroxidation ($70.2 \pm 5.8\%$ inhibition with 1 mg/kg per day, $74.3 \pm 5.9\%$ inhibition with 5 mg/kg per day and $81.2 \pm 7.7\%$ inhibition with 10 mg/kg per day). In contrast, the administration of WEB 2086-BS had no significant effect on lipid peroxidation in aortic tissue samples (Fig. 3).

The percentage of retinal area occupied by horseradish peroxidase-labeled vessels was a significantly reduced in untreated diabetic animals as compared with non-diabetic controls (Fig. 4). Treatment with WEB 2086-BS produced an increase in the percentage of retinal area occupied by horseradish peroxidase-labeled vessels. The increase was $31.3 \pm 1.8\%$ after treatment with 1 mg/kg per day, $78.9 \pm 6.5\%$ with 5 mg/kg per day and $190 \pm 10.8\%$ with 10 mg/kg per day (Fig. 4).

There was a statistically significant correlation between thromboxane B_2 values and the percentages of retinal area occupied by horseradish peroxidase-labeled vessels in the groups of animals treated with WEB 2086-BS ($Y = -072 + 137X$, $r^2 = 0.7247$, $P < 0.0007$).

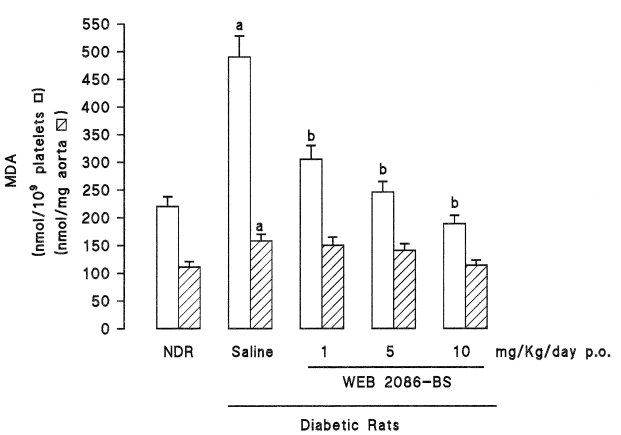

Fig. 3. Malondialdehyde production of platelets (□) and aortic segments (□) in non-diabetic rats (NDR) and in diabetic animals treated with isotonic saline or WEB 2086-BS ($^{a}P < 0.05$ as compared with NDR; $^{b}P < 0.05$ as compared with diabetic animals treated with saline).

4. Discussion

The present results confirm that there is a clear platelet hyperactivity and an unbalanced synthesis of eicosanoids in diabetes mellitus and particularly in the experimental model used in this study. Such findings have been reported by various groups, either for experimental models (Moreno et al., 1995a) or from studies with humans (Hendra and Betteridge, 1989). Our results confirm that there is a relationship between these abnormalities and the ischemic-type lesions that appear in the retina of the animals, effects previously shown by our group, using the same experimental model (Moreno et al., 1995a).

The administration of WEB 2086-BS to diabetic animals was followed by a reduction in the retinal ischemic areas. This effect may have resulted from a reduction of platelet activity, given that, in this experimental model, platelet activity is related to the development of these lesions. The effect may also have resulted from prevention of fluid leakage in the retinal vessels, given that PAF exerts this effect on the microvasculature level (Evans et al., 1987).

PAF may be involved in the course of retinal lesions through various mechanisms, so that antagonists of PAF receptors may decrease the activity of such mechanisms.

Firstly, PAF can produce platelet activation through stimulation of the phospho-inositol pathway (O'Rourke et al., 1985). Moreover, platelets from diabetic patients are more sensitive to PAF-related activation than are those from non-diabetic subjects (Shukla et al., 1992). Excessive platelet stimulation has been related to a greater area of retinal ischemic lesions in the experimental model used in this study (Moreno et al., 1995a) and those agents causing a reduction of platelet activation also lead to improvement of retinal ischemic lesions (de la Cruz et al., 1990; Moreno et al., 1995b; de la Cruz et al., 1996, 1997a, 1998).

Secondly, PAF induces the formation of thromboxane A_2 (Chung et al., 1986) and blockage of PAF receptors decreases thromboxane A_2 release; in addition, specific antagonists of thromboxane A_2 synthesis also produce a reduction of some PAF effects (Kato et al., 1993). The present study provided evidence that in diabetic animals, WEB 2086-BS led to a statistically significant reduction of thromboxane B_2 production, probably from synthesis in both platelets and leukocytes. Accordingly, the platelet activation effect of this prostanoid would be reduced, resulting in smaller retinal ischemic areas. The linear correlation between thromboxane synthesis and the percentage of retinal area occupied by horseradish peroxidase-labeled peroxidase would support this possible effect of PAF receptor antagonists on retinal ischemic lesions in our model of experimental diabetes.

Thirdly, PAF enhances the formation of superoxide anions (Kato et al., 1993). These oxygen-derived free radicals enhance the peroxidation of structurally important polyunsaturated fatty acids within the phospholipid structure of the cell membrane—an effect already documented for the crystalline lens in diabetic rats (de la Cruz et al., 1994). On the other hand, platelet thromboxane synthesis is stimulated by lipid peroxides (Warso and Lands, 1983) and for this reason, PAF receptor antagonists may modulate thromboxane synthesis negatively by decreasing the production of oxygen radicals, which may contribute to the beneficial effect of these substances on retinal vascularity in experimental diabetes.

Finally, PAF receptor antagonists prevent microvascular exudation induced by PAF (Casals-Stenzel et al., 1987; Evans et al., 1987). In this respect, PAF receptor antagonists may be useful in diabetic patients with exudative retinopathy in whom alterations of platelets and thromboxane synthesis are greater than in other types of diabetic retinopathy (de la Cruz et al., 1997b). In the present study, it was not possible to measure extravasation of the dye due to the molecular weight of peroxidase, since there should be an actual vascular rupture to document the extracapillary presence of peroxidase.

The dose-relationship for the drug in this model, differs somewhat from that for its PAF-receptor antagonist potency (Casals-Stenzel et al., 1987). It seems to show antiperoxidative and antiaggregatory effects in whole blood at doses lower than the PAF-receptor antagonistic ones. It is possible that WER-2086-BS could exerts these effects by a mechanism different from that for antagonism of PAF receptors.

In summary, WEB 2086-BS may exert its beneficial effect on ischemic retinal lesions in experimental diabetes by decreasing the effect of PAF at this site. Accordingly, blockage of PAF receptors may constitute an alternative approach to the reachof diabetic ischemic retinal lesions, although further studies in humans are needed to establish a definitive conclusion.

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