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Effect of camonagrel, a selective thromboxane synthase inhibitor, on retinal vascularization in experimental diabetes

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Abstract

Platelet hyperactivity accompanied by an increased synthesis of thromboxane and/or a decreased prostacyclin production are important factors in ischemic diabetic retinopathy. We studied the effect of camonagrel and dazoxiben, two thromboxane synthase inhibitors, on retinal vascularization in a model of streptozotocin-induced diabetes in rats. Ten nondiabetic rats, 10 diabetic animals treated with saline (i.e., not treated), and 60 diabetic animals treated with dazoxiben or camonagrel (10, 50 or 100 mg kg^{-1} day^{-1} p.o.) were studied. All treatments lasted for 90 days. Dazoxiben and camonagrel produced a dose-dependent reduction in platelet aggregation and thromboxane synthesis. Dazoxiben increased prostacylin synthesis by 78% at 100 mg kg^{-1} day^{-1}, and camonagrel by 154%. Dazoxiben increased retinal vascularity by 74%, and by 183% after camonagrel treatment. Prostacyclin synthesis showed a direct linear correlation with the degree of retinal vascularization ($r^2 = 0.6733$, $P < 0.00001$). We conclude that an increased prostacyclin synthesis may have a greater influence than the inhibition of thromboxane synthesis in preventing ischemic diabetic retinopathy in experimental diabetes. Camonagrel may be an alternative treatment in the prevention of these lesions.

Keywords: Camonagrel; Dazoxiben; Diabetic retinopathy; Prostacyclin; Thromboxane synthase inhibitor

1. Introduction

Two of the mechanisms involved in the genesis and evolution of microangiopathic complications in patients with diabetes mellitus are increased platelet functioning (Dallinger et al., 1987; Ishii et al., 1992; De La Cruz et al., 1997b) along with increased thromboxane synthesis and decreased prostacyclin production (Hendra and Betteridge, 1989; Moreno et al., 1995a; De La Cruz et al., 1997b). Platelet alterations have been related with the course of microangiopathic changes in diabetes (Merimee, 1990; De La Cruz et al., 1997a) manifested most frequently as retinopathy and nephropathy. We previously showed that an imbalance in eicosanoid synthesis was directly related with the degree of retinal vascular alteration in an experimental model of diabetes in rats (Moreno et al., 1995a). In this model, some drugs such as acetylsalicylic acid (De La Cruz et al., 1990, 1997a), dipyridamole and mopidamole (De La Cruz et al., 1996), and ditazol (Moreno et al., 1995b) were able to prevent the genesis and evolution of retinal vascular alterations. One of the main conclusions drawn from these studies was that retinal vasculopathy could be optimally prevented by inhibiting thromboxane synthesis while not affecting, or increasing, the vascular production of prostacyclin. Dazoxiben and picotamide, selective inhibitors of thromboxane synthase, are able to bring about these two effects (Berretini et al., 1990; Gresele et al., 1991). In the present study, we investigated the effectiveness of camonagrel (Fig. 1), a new selective inhibitor of thromboxane synthase which in addition shows fibrinolytic action (Gryglewski et al., 1995), in preventing

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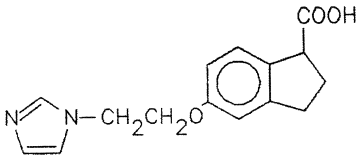

Fig. 1. Chemical structure of camonagrel. [+]-5-[2-imidazole-1-ethyloxy]-1-indan-carboxylic acid hydrochloride.

experimental diabetic retinopathy in rats, in comparison to dazoxiben, a standard thromboxane synthase inhibitor.

2. Materials and methods

2.1. 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 six experimental groups. In group I, 10 nondiabetic animals served as controls. In group II, 10 diabetic animals received 0.5 ml kg^{-1} day^{-1} of isotonic saline (p.o.) for 90 days. In group III, 30 diabetic animals received dazoxiben (10, 50 or 100 mg kg^{-1} day^{-1}, $n = 10$ rats per dose) (Grupo Ferrer Internacional, Barcelona, Spain) from day 1 after induction of diabetes. In group IV, 30 diabetic animals received 10, 50 or 100 mg kg^{-1} day^{-1} ($n = 10$ rats per dose) of camonagrel (Grupo Ferrer Internacional, Barcelona, Spain) from day 1 after induction of diabetes. All animals were treated orally every day through an endogastric catheter that was left in place between the administration of solutions during 90 days, in twice daily doses given between 09:00 and 10:00, and between 20:00 and 21:00. Drugs were diluted in isotonic saline at the final concentration used.

2.2. Experimental diabetes

Experimental diabetes was induced by a single dose (50 mg kg^{-1}) of streptozotocin (Sigma Chemical, St. Louis, MO) injected i.v. into the femoral vein. Nondiabetic animals received equivalent doses of normal saline solution. Blood glucose concentration was determined by a micromethod (Glucometer®, Menarini, Barcelona, Spain) in samples obtained via a small incision in the tail. Blood glucose 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 next day after diabetes had been induced (detection of glucose concentrations of 200 mg dl^{-1}). Animals in groups II and III were given intermediate-acting insulin, 3 IU day^{-1} s.c. (Insulatard HM®, Novo Nordisk, Bagsvaerd, Denmark) as an antidiabetic. Insulin was administered to support high glucose levels without mortality due to possible ketoacidosis.

2.3. Assessment of retinal vascularity

After completion of the protocol, animals were anesthetized with pentobarbital sodium (Nembutal®, Abbott), 40 mg kg^{-1} i.p., and 2 ml of blood was drawn from the left ventricle (1 ml was mixed with 3.8% trisodium citrate at a proportion of 1:10, and 1 ml without anticoagulant was placed in a glass tube). The descending carotid artery was tied and two segments of the abdominal aorta (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 horseradish peroxidase throughout the arterial territory of the internal carotid artery for some minutes, the eyeballs were enucleated and retinal tunics were processed histochemically with the Mesulam's technique (Mesulam, 1982). Retinal sections were incubated with a solution of tetramethylbenzidine and sodium nitroferrocyanide (Sigma) as chromogen substrates. Samples were subsequently dehydrated in a graded series of alcohol, incubated in xylene and mounted on slices for light microscopic examination. Retinal vessels labelled with horseradish peroxidase were examined at $40\times$. A computerized, digital image processing system (IBAS Kontron 2000) (Gonzalez and Wintz, 1987) was applied to microscopic photographs to evaluate the percentage of the retinal area occupied by horseradish peroxidase-labelled vessels. The retinal vascular pattern was also assessed qualitatively for the presence of arterial narrowing, tortuous vessels, dilations and images of fragmentation of the labelled substance.

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 10 μg ml^{-1} of collagen (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). The sample of whole blood without anticoagulant was placed 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. To assess the possible influence of platelet number on platelet

thromboxane B_2 production we used the formula described by Carter and Hanley (1985):

$$\text{Thromboxane } B_2\,(\text{nmol } 10^{-9}) = \frac{\text{Thromboxane } B_2\,(\text{nmol } l^{-1}) \times (1 - [\text{haematocrit}/100])}{\text{Platelet number } (\text{cells} \times 10^9 l^{-1})} \times 10^9$$

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^{-1}) 100 NaCl, 4 KCl, 25 $NaHCO_3$, 2.1 Na_2SO_4, 20 sodium citrate, 2.7 glucose and 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 International) and the mean value of the two aortic segments was calculated for each animal.

2.7. Blood cellular counts

We used a Baker-8000 automatic blood cell counter (Menarini).

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

2.8. Statistical analysis

All values in the text, tables and figures are presented as mean ± standard error of the mean (S.E.M.). The results were analysed with the Social Program for Statistical Sciences (SPSS, version 6.0 for Windows 95). One-way analysis of variance with Bonferroni's transformation was used to determine significant differences. The linear correlation test was used to determine significant correlations between variables. Differences were considered significant at $P < 0.05$.

3. Results

Blood glucose levels (expressed as the mean of all determinations throughout the 3-month study period) in the groups of diabetic animals were significantly higher than in the nondiabetic controls (Table 1). Camonagrel and dazoxiben had no significant effect on glycemia in diabetic animals at any dose (Table 1).

Neither of the drugs modified blood cell counts, body weight or the daily intake of food or fluids in diabetic animals (data not shown).

Maximum collagen-induced platelet aggregation in whole blood was significantly higher in untreated diabetic

Table 1
Blood glucose and maximum aggregation intensity in nondiabetic rats, untreated diabetic animals, and diabetic animals treated with dazoxiben or camonagrel

Group ($n = 10$)	Glycemia (mg dl^{-1})
Nondiabetic rats	95 ± 4.2[a]
Untreated diabetic rats	462 ± 10.5
Dazoxiben 10 mg kg^{-1} day^{-1} p.o.	486 ± 8.0
Dazoxiben 50 mg kg^{-1} day^{-1} p.o.	493 ± 6.5
Dazoxiben 100 mg kg^{-1} day^{-1} p.o.	427 ± 12.9
Camonagrel 10 mg kg^{-1} day^{-1} p.o.	488 ± 8.9
Camonagrel 50 mg kg^{-1} day^{-1} p.o.	475 ± 14.1
Camonagrel 100 mg kg^{-1} day^{-1} p.o.	471 ± 13.4

[a] $P < 0.05$ in comparison with all other groups.

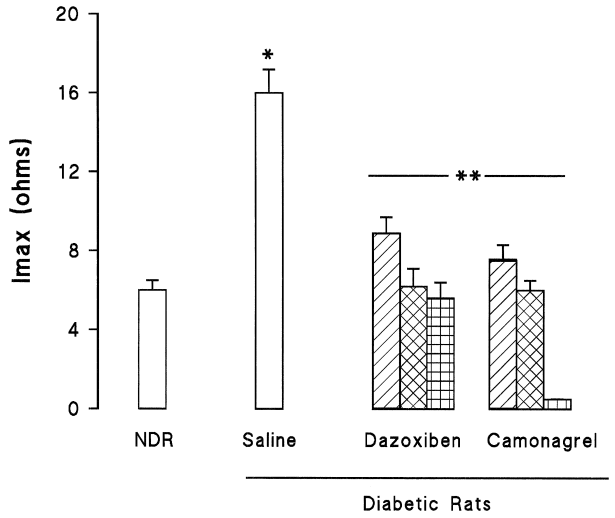

Fig. 2. Maximum platelet aggregation intensity (I_{max}) in whole blood, induced with 10 μg collagen ml^{-1}, in nondiabetic rats (NDR) and diabetic rats after treatment for 90 days with saline solution, dazoxiben or camonagrel at 10 (diagonal line in a square), 50 (x in a square) or 100 (cross in a square) mg kg^{-1} body weight per day. $*P < 0.05$ in comparison with NDR, $**P < 0.05$ in comparison with diabetic rats treated with saline.

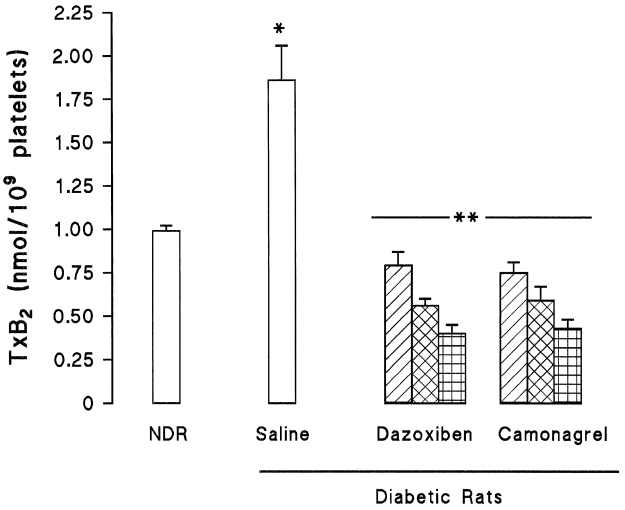

Fig. 3. Platelet thromboxane B_2 production in nondiabetic rats (NDR) and diabetic rats after treatment for 90 days with saline solution, dazoxiben or camonagrel at 10 (diagonal line in a square), 50 (x in a square) or 100 (cross in a square) mg kg^{-1} body weight per day. $*P < 0.05$ in comparison with NDR, $**P < 0.05$ in comparison with diabetic rats treated with saline.

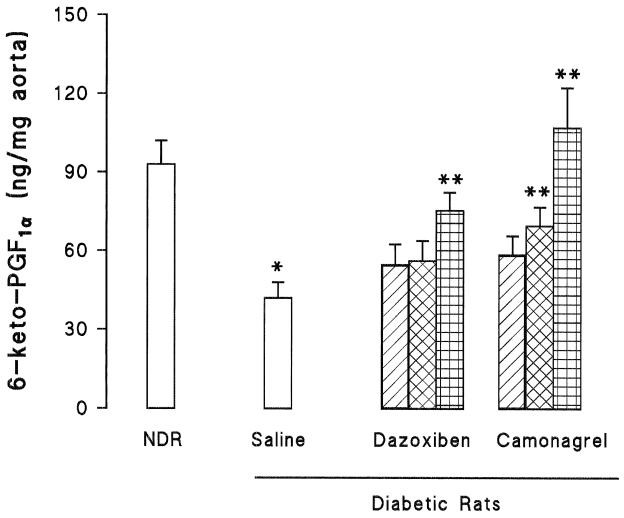

Fig. 4. Concentrations of 6-keto-prostaglandin $F_{1\alpha}$ in aortic segments from nondiabetic rats (NDR) and diabetic rats after treatment for 90 days with saline solution, dazoxiben or camonagrel at 10 (diagonal line in a square), 50 (x in a square) or 100 (cross in a square) mg kg^{-1} body weight per day. $*\ P < 0.05$ in comparison with NDR, $**\ P < 0.05$ in comparison with diabetic rats treated with saline.

rats (Fig. 2), and showed a dose-dependent reduction in groups treated with 10, 50 or 100 mg camonagrel (69%, 83% and 98% reduction, respectively) or dazoxiben (51%, 57% and 66% reduction, respectively).

Platelet production of thromboxane B_2 was significantly higher in diabetic animals than in nondiabetic controls (Fig. 3). Dazoxiben led to a dose-dependent reduction in thromboxane B_2 production (15% at 10 mg, 39 at 50 mg% and 54% at 100 mg), as did camonagrel (20% at 10 mg, 36% at 50 mg and 55% at 100 mg).

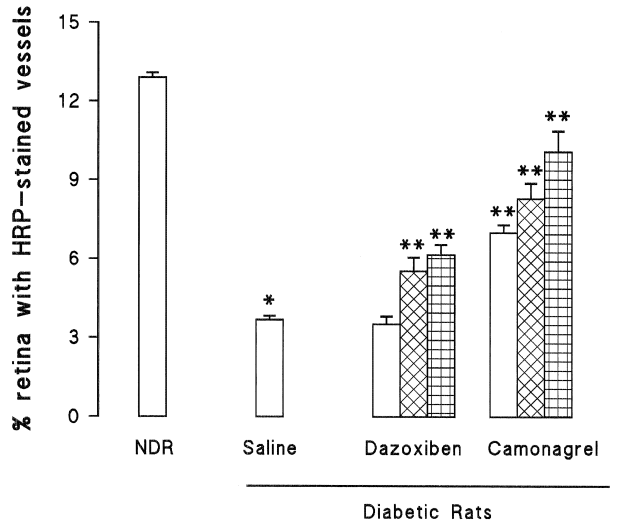

Fig. 5. Percentage area of the retina occupied by horse radish peroxidase-permeable vessels in nondiabetic rats (NDR) and diabetic rats after treatment for 90 days with saline solution, dazoxiben or camonagrel at 10 (diagonal line in a square), 50 (x in a square) or 100 (cross in a square) mg kg^{-1} body weight per day. $*\ P < 0.05$ in comparison with NDR, $**\ P < 0.05$ in comparison with diabetic rats treated with saline.

The production of 6-keto-prostaglandin $F_{1\alpha}$ was significantly lower in diabetic rats than in nondiabetic controls (Fig. 4). Dazoxiben increased 6-keto-prostaglandin $F_{1\alpha}$ production by 28% at a dose of 10 mg, by 36% at 50 mg, and by 78% at 100 mg. Camonagrel increased production by 38%, 60% and 154%, respectively.

The percentage of the retina occupied by horseradish peroxidase-permeable vessels was significantly lower in untreated diabetic animals than in nondiabetic controls (Fig. 5). Dazoxiben did not significantly increase the percentage area at 10 mg, but it did 55% at 50 mg and 74% at 100 mg. The increases in permeability obtained with camonagrel likewise failed to reach significance (101%, 132% and 183%, respectively).

We found a significant linear correlation ($y = 3.5x + 35.1$, $r^2 = 0.6733$, $P < 0.00001$) between 6-keto-prostaglandin $F_{1\alpha}$ values and percentage of the retinal area occupied by horseradish peroxidase-permeable vessels.

4. Discussion

Our results confirm the occurrence of platelet hyperactivity and imbalanced eicosanoid synthesis in the experimental model of diabetes mellitus we used. Earlier studies had reported similar findings in an experimental model (Moreno et al., 1995a) and in humans (Hendra and Betteridge, 1989). In addition, the present findings confirm the relationship between these alterations and ischemic retinal lesions in rats, as had previously been shown in another experimental model (Moreno et al., 1995a).

In streptozotocin-induced diabetes, high doses of aspirin, which inhibits both thromboxane and prostacyclin synthesis, increased the percentage area of the retina occupied by horseradish peroxidase-permeable vessels by 55% in comparison with untreated animals (De La Cruz et al., 1990). Together with physiopathological (Moreno et al., 1995a) and pharmacological evidence reported earlier (De La Cruz et al., 1990; Moreno et al., 1995b), this finding shows that prostacyclin synthesis was relatively more important than the inhibition of thromboxane synthesis in preventing ischemic vascular lesions in experimental diabetes.

Selective inhibitors of platelet thromboxane synthesis are a group of drugs that in theory satisfy one of the main requirements for platelet antiaggregant treatment: they inhibit the synthesis of an aggregating factor (thromboxane) while leaving the production of an endogenous antiaggregating factor (prostacyclin) unaffected (Gresele et al., 1991). Moreover, accumulated cyclic endoperoxides can be transferred to endothelial cells, where they act as a substrate for prostacyclin synthase. However, part of the endoperoxides binds to membrane receptors for thromboxane and endoperoxides on other platelets, which can thus become activated (Maguire and Wallis, 1983).

In the present study, both dazoxiben and camonagrel inhibited platelet aggregation and platelet thromboxane synthesis to a similar extent. Vascular prostacyclin production increased in a dose-dependent way in rats treated with camonagrel, the effect being especially evident at a dose of 100 mg kg^{-1} body weight per day.

If prostacyclin were important in improving retinal vascularization in experimental diabetes, camonagrel would be expected to improve vascularization. Our results showed this to be the case, as the percentage area of the retina occupied by horseradish peroxidase-permeable vessels increased more with camonagrel treatment than with equivalent doses of dazoxiben. At a dose of 100 mg camonagrel kg^{-1} body weight per day, percentage area approached that found in nondiabetic control rats.

The greater increase in prostacyclin production in animals given camonagrel may have benefit retinal vascularization via two mechanisms: (1) the vasodilating effect and (2) the increased endothelial fibrinolytic activity of prostacyclin (Gryglewski, 1995). If camonagrel increases prostacyclin production to a greater extent than does dazoxiben, it might also lead to a greater increase in fibrinolytic activity. In a study of patients with obliterating arteriopathy, Gryglewski et al. (1995) found that the oral administration of 800 mg camonagrel led to a decrease in thromboxane B_2, an increase in 6-keto-prostaglandin $F_{1\alpha}$, and an increase in tissue plasminogen activator activity; in addition, plasminogen activator inhibitor was inhibited, a finding that indicated stimulation of the fibrinolytic system. These actions may therefore enhance the beneficial effects of increased retinal vascularization seen in our experimental model.

The beneficial effects of selective inhibition of thromboxane synthesis on retinal vascularization can occur not only via inhibition of platelet aggregation and increased production of vasodilating eicosanoids, but also via thromboxane/endoperoxidase receptors, which have been described in several vascular and avascular structures in the human eye (Chen et al., 1994). Nonetheless, the actual functional significance of these receptors remains to be fully elucidated.

The selective inhibition of thromboxane synthase may constitute an alternative approach to the prevention of ischemic retinal lesions in diabetes. Further studies in humans will be necessary to confirm the therapeutic potential of these drugs.

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