

Effect of thalidomide and rosiglitazone on the prevention of diabetic retinopathy in streptozotocin-induced diabetic rats

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Abstract

Aims/hypothesis. Our aim was to compare the therapeutic effect of thalidomide and rosiglitazone on the prevention of diabetic retinopathy in streptozotocin-induced diabetic rats.

Methods. Male Holtzman rats of 6 to 8 weeks of age and weighing 170 ± 30 g were randomly divided into four groups: control ($n=13$), untreated diabetic ($n=17$) and diabetic rats treated with thalidomide ($200 \text{ mg kg}^{-1} \text{ day}^{-1}$) ($n=8$) or rosiglitazone ($1 \text{ mg kg}^{-1} \text{ day}^{-1}$) ($n=22$) for 3 months. Diabetes was induced by streptozotocin with the rats having a body weight of 70 mg/kg. After treatment, vascular endothelial growth factor (VEGF) concentrations in ocular fluid were compared between the different groups, and retinal capillary basement membrane thickness was measured by electron microscopy.

Results. Higher VEGF concentrations in ocular fluid and thicker basement membranes were observed in

untreated diabetic rats compared to the control rats. Similar VEGF concentrations and basement membrane thickness were observed for the thalidomide-treated group compared with the control group, whereas no difference in these parameters was observed between the rosiglitazone-treated rats and the control or untreated diabetic rats.

Conclusions/interpretation. Our findings confirm the association between VEGF concentrations and diabetic retinopathy as suggested by other investigators. Thalidomide, but not rosiglitazone, was associated with the inhibition of basement membrane thickening and the blockade of the increase of VEGF in ocular fluid, thus representing a potential therapeutic drug for the prevention of diabetic retinopathy. [Diabetologia (2003) 46:1669–1675]

Keywords Diabetes, diabetic retinopathy, thalidomide, rosiglitazone.

Diabetes mellitus, one of the major public health problems, is a highly prevalent disease and an important cause of mortality and morbidity because of the chronic complications frequently associated with it

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Abbreviations: T, Thalidomide; R, rosiglitazone; VEGF, vascular endothelial growth factor; BM, basement membrane; BMT, basement membrane thickness.

[1]. Diabetic retinopathy is the most common and specific vascular complication of both Type 1 and Type 2 diabetes, with 70 to 100% of patients with Type 1 diabetes being estimated to develop retinal alterations that may result in blindness [2, 3]. Although strict glycaemic control has been shown to considerably reduce the incidence of retinopathy, it is not easily achieved and maintained. Its efficacy has not been completely confirmed being that 12% of diabetic patients under intensive treatment develop retinopathy [3, 4].

Proliferative diabetic retinopathy is characterised by the extensive formation of new blood vessels in the retina (angiogenesis) and invasion towards the vitreous body, followed by haemorrhage and the traditional

detachment of the retina, which lead to visual alterations and blindness [5, 6]. The presence of ischaemic areas in the retina is fundamental for angiogenic stimulation, which is mediated by growth factors, including VEGF [7, 8, 9, 10, 11, 12, 13, 14, 15]. It is currently believed that retinopathy is an inflammatory process, since various studies have shown that the circulating leukocytes activated in diabetic patients play a crucial role in the increased adhesion of leukocytes, macrophages and vascular adhesion molecules and in the process of vascular occlusion and retinal ischaemia [16, 17, 18, 19]. Several alterations in the retinal capillaries of diabetic patients are observed with electron microscopy: numerous capillaries with open inter-endothelial junctions, increased cytoplasmic vacuolization (enhanced vesicular transport), pericytes presenting degenerative alterations, and capillary BM (lamina) thickening. These can be reproduced in experimental studies [5, 6, 20]. The cause of this thickening, is still unknown [20, 21]. It alters cellular function and/or oxygen diffusion and reduces the contact between pericytes and endothelial cells, contributing to the vascular alterations observed in diabetic patients [20, 22].

Some drugs such as thalidomide and rosiglitazone have shown an inhibitory action on angiogenesis in vivo and in vitro [23, 24, 25, 26], which might represent a potential preventive therapeutic measure for proliferative retinopathy. Thalidomide has been used successfully for various autoimmune and inflammatory diseases [27, 28, 29] characterised by increased concentrations of macrophage-produced TNF- α and VEGF, because it interferes with cellular adhesion molecules, reducing the expression especially of β 2-integrin produced by leukocytes, thus inhibiting cell-matrix interactions [23, 30]. The action of Rosiglitazone (R), a peroxisome proliferator-activated receptor γ (PPAR- γ) ligand, a member of a new class of anti-diabetic drugs, has also been related to the inhibition of macrophages and monocytes, as well as to the inhibition of tumour cell proliferation [31, 32]. A recent study [25] showed that PPAR- γ ligands inhibit angiogenesis in vitro and in vivo, with VEGF inhibition being one of the mechanisms proposed. In a more recent study [24] in vitro inhibition of VEGF-induced chorioidal angiogenesis by PPAR- γ ligands.

Our objective was to evaluate the therapeutic action potential of T and R as antiangiogenic agents to prevent the development of diabetic retinopathy in rats as evaluated by BM thickening and VEGF concentration in ocular fluid.

Material and methods

A total of 120 male Holtzman rats of 6 to 8 weeks of age and weighing 170 ± 30 g were randomly divided into two groups: a normal control group and a streptozotocin-induced diabetic

group (70 mg/kg by a single intraperitoneal injection). The diabetic rats (glycaemia >250 mg/dl) were subdivided into three groups: untreated diabetic rats, diabetic rats treated with T and diabetic rats treated with R. All experimental procedures and animal care followed the ethical guidelines of the Brazilian College of Animal Experimentation.

Procedures. All rats were maintained in individual cages under adequate conditions at an ambient temperature of $21 \pm 2^\circ\text{C}$ on a 12-h light/dark cycle, with standard diet and water available ad libitum. Treatment of diabetic rats was initiated after confirmation of the diabetic state (3 days after induction) with either T tablets (100 mg) provided by the Health Secretariat of the Brazilian State of Minas Gerais, or R tablets (4 mg) (GlaxoSmith-Kline, Rio de Janeiro, Brazil). The tablets were pulverised and the individual dose, 200 and 1 mg/kg for T [23] and R respectively [33], was calculated. The drugs were mixed daily with a pelleted vehicle consisting of casein and starch prepared by the Animal house staff, and the standard diet (commercial ration for small rodents, NUVILAB CR1, Nuvital Nutrientes, Belo Horizonte, Minas Gerais, Brazil) was given only after the vehicle was completely ingested by the animals. The control animals received the same vehicle diet but without the drug. Weight and fasting glycaemia (capillary blood glucose) were measured every 2 weeks throughout the experiment to adjust the dose of the drug according to weight and to evaluate the diabetic state of the rats.

At the end of the 12-week treatment period, the animals were killed by ethyl ether inhalation, and material was collected immediately for biochemical tests (glucose, insulin and VEGF) and morphological analysis of the retina.

Biochemical tests. Capillary blood samples were collected from the caudal vein after a daytime physiological fast to measure glycaemia, using commercial reagent strips (Advantage, Hofman La Roche, Indianapolis, Ind., USA) based on an enzymatic method (glucose dehydrogenase). Insulin was measured in duplicate with a commercial liquid phase radioimmunoassay kit (Linco Research, Saint Louis, Mo., USA) using ^{125}I -labelled insulin and an antibody specific for rat insulin.

The VEGF was measured in ocular fluid, a mixture of the aqueous and vitreous humour, and immediately after the rats were killed it was removed by puncturing the ocular globe with disposable 1-ml syringes connected to a 13×3.8 mm needle. The material was stored at -70°C until the time for use. This peptide was measured on microplates coated with rat and mouse VEGF-specific polyclonal antibody by an enzyme immunoassay [11, 12] using a commercial kit (Quantikine M. Murine VEGF Immunoassay, R&D System, Minneapolis, Minn, USA). The detection limit of the method was 7.8 pg/ml, with the standard curve being linear up to 500 pg/ml. All measurements were carried out in duplicate. Analysis of intra-assay variability showed a coefficient of variation (CV) of 8.2% (37.7 ± 3.1 pg/ml) for sample A (40 pg/ml) and of 4.7% (144 ± 6.2 pg/ml) for sample B (150 pg/ml). Inter-assay variations showed a CV of 8.4% for sample A (38.3 ± 3.2 pg/ml) and of 6.7% for sample B (138 ± 7.8 pg/ml). Samples were quantified by colorimetric measurement in a microplate reader equipped with a computerised program, considering the standard curve obtained in the same assay.

Morphological analysis of the retina. Retinal capillary BMT was measured by electron microscopic analysis. After puncture, the retina was enucleated and isolated. The ocular globe was removed surgically and sectioned at the level of the equator of the eyeball to isolate the posterior segment where the retina is located. This tissue sample was cut in a trapezoid

shape, with the smaller side, i.e. the portion proximal to the optic nerve, consisting of sclera, choroid and retina, which is easily detached from the remaining tissue.

The material was fixed in 3% glutaraldehyde for 24 h, washed in 0.1 mol/l phosphate buffer, pH 7.2, and postfixed in 1% osmium tetroxide. After washing and dehydration in alcohol, the specimens were immersed in Epon resin and acetone and embedded in Epon DMP-30, with the smaller side facing the surface. After polymerization, the blocks were cut into semi-thin sections and the sections stained with 0.5% toluidine blue and sodium borate. Ultrathin sections were obtained and counter-stained with uranyl acetate and lead citrate. All material was prepared at the Electron Microscopy Center of the Federal University of Minas Gerais (CEMEL) and analysed ultrastructurally with a Zeiss EM 10 electron microscope.

About 20 panoramic photomicrographs of the retinal capillaries were randomly selected from each group. Poor quality images were discarded: control group (16 photomicrograph), untreated diabetic rats ($n=14$), diabetic rats treated with T ($n=8$), and diabetic rats treated with R ($n=20$). Each negative was corrected using the respective magnification factor in such a way that the final magnification for analysis was $\times 30\,000$.

The morphometric method used implies the three-dimensional interpretation of the capillary which depends on its section plane; thus, analysis of the capillary lumen area might be important for the interpretation of BMT determined for each capillary. We therefore considered the ratio between BMT and capillary lumen area (BM:lumen) as the comparative parameter between groups.

The basement membrane thickness was measured with a pachymeter at 1-cm intervals around the vessel perimeter and is reported in mm with a total of ten measurements for each vessel. The results were converted into nm and corrected for the vessel lumen area. The capillary lumen area was estimated by multiplying the values obtained for the cross-sectional and longitudinal measurements of the extreme points. In the case of vessels with a quite irregular lumen, two measurements were made at points with a larger and smaller diameter, and the means were then multiplied. Values in cm^2 were converted to nm^2 and corrected for the magnification of the image ($\times 30\,000$). The BM:lumen ratio (nm^{-1}) was used as the final measurement.

Statistical analyses. Parametric data obtained in both the longitudinal and cross-sectional study were compared by the paired Student's *t* test and, when more than two time points were analysed, by repeated measures of ANOVA, followed by the Student-Newman-Keuls test. Non-parametric data were compared by the Wilcoxon test in the longitudinal study and by the Mann-Whitney U test in the cross-sectional study when no more than two time points were analysed. Otherwise, the Friedman test was applied, followed by the Müller-Dunn test

as the post test. An alpha risk value equal to or lower than 5% ($p<0.05$) of committing a type I or 1st species error, and a beta risk equal to or lower than 20% of committing a type II or 2nd species error were considered for analysis. A *p* value of less than 0.05 was considered statistically significant.

Results

We started the study with 15 rats in the control group and 35 in each diabetic group; i.e. the untreated group, treated with R and treated with T. At the end we had 13, 17, 22 and 15 respectively. However, it was particularly difficult to get sufficient ocular fluid from the T group to measure the VEGF because those animals were extremely dehydrated. This did not occur with the R group, probably because of its liquid retention effect. At the end it was possible to collect enough material from only eight animals.

Animal weight. No significant difference in body weight between control and diabetic rats was observed at the beginning of the experiment (170 ± 17 vs 159 ± 25 g, mean \pm SE, $p>0.05$). At the end of the 12 weeks, weight gain was higher in the control animals ($p<0.001$). The final body weight was 268 ± 8 g for the control group, 161.6 ± 3 g for the untreated diabetic group, 152.5 ± 4 g for the T-treated group and 176.6 ± 6 g for the R-treated group. Statistical significance was found just between the final weight in the R-treated group and to the T-treated group ($p<0.05$, Table 1).

Glucose. Initial glycaemia of the diabetic group was measured 3 days after streptozotocin was injected. The diabetes developed was moderate to severe. Mean glycaemia was higher in the diabetic group than in the control group ($p<0.001$) (Table 1).

At the end of treatment, no significant difference in glycaemia was observed between untreated, T-treated and R-treated diabetic rats ($p>0.05$). In the control group, the same concentrations of glycaemia were observed at the beginning and at the end of the 12 weeks.

Insulin. Plasma insulin concentrations were measured at the beginning of the experiment after hyperglycae-

Table 1. Weight and glycaemia at the end of the study

	Control ($n=13$)	Group D ($n=17$)	Group T ($n=8$)	Group R ($n=22$)
Final glycaemia (mg/dl); (mmol/L)	76 ± 2 ; 4.22 ± 0.39	454 ± 22^a ; 25.2 ± 5.0	388 ± 20^a ; 21.7 ± 3.1	432 ± 18.6^a ; 23.9 ± 4.8
Final weight (g)	268.5 ± 8	161.6 ± 3^a	152.5 ± 4^a	$176.5\pm 6^{a,b}$

Untreated diabetic rats (group D), thalidomide-treated diabetic rats (group T), rosiglitazone-treated diabetic rats (group R)
Data are reported as means \pm standard error for *n* rats in each group

Glycaemia: ^a $p<0.001$ vs control
Weight: ^a $p<0.001$ vs control; ^b $p<0.05$ vs group T

Table 2. VEGF concentration in ocular fluid, retinal capillary basement membrane thickness, capillary lumen area (CLA) and the ratio between basement membrane thickness and retinal capillary lumen area (BM/LA)

	Control	Group D	Group T	Group R
VEGF (pg/ml)	(n=12) 1454±199	(n=17) 2327±197 ^a	(n=8) 838±207 ^b	(n=22) 1982±222 ^c
BMT (nm)	(n=16) 38±2	(n=14) 106±4 ^a	(n=18) 58±2 ^{b,c}	(n=20) 98±2 ^a
CLA (×10 ⁶ nm ²)	21±4	21±4	19±2	29±4
BM/LA (×10 ⁶ nm ⁻¹)	3.7±1	6.9±1 ^a	3.5±0.3 ^b	5.0±0.7

Untreated diabetic rats (group D), thalidomide-treated diabetic rats (group T) and rosiglitazone-treated diabetic rats (group R) Data are reported as means ± standard error for *n* capillaries analysed

VEGF: ^a $p < 0.05$ vs control; ^b $p < 0.01$ vs group D; ^c $p < 0.05$ vs group T

BMT: ^a $p < 0.001$ vs control; ^b $p < 0.001$ vs group D; ^c $p < 0.01$ vs group R

LA: not significant

BM/LA: ^a $p < 0.01$ vs control; ^b $p < 0.01$ vs group D

mia was detected to validate the diabetic model. Control rats showed higher mean insulin concentrations than the diabetic groups ($p < 0.001$).

VEGF in ocular fluid. The VEGF concentration in ocular fluid was higher in untreated diabetic rats than in the control rats ($p < 0.05$) and T-treated diabetic ($p < 0.01$) rats, whereas no significant difference was observed between the untreated diabetic and R-treated rats ($p > 0.05$). There was also no significant difference between the two treated diabetic groups and the control group ($p > 0.05$). On the other hand, VEGF concentrations were lower in the T-treated group compared to the R-treated group ($p < 0.05$, Table 2).

Morphometric study of the basement membrane. Capillary BMT is reported as the mean±SE of individual measurements in each group.

Untreated diabetic rats ($n=14$) had a thicker BM than the control ($n=16$) and T-treated rats ($n=18$) ($p < 0.001$ for both groups), whereas BMT did not differ between untreated and R-treated animals ($n=20$) ($p > 0.05$). A thicker BM was observed for the R-treated group compared to the control ($p < 0.001$) and T-treated ($p < 0.01$) groups, while there was no difference between control and T-treated rats ($p > 0.05$) (Table 2). Analysis of capillary lumen area showed no significant difference between the four groups ($p > 0.05$, Table 2).

To exclude possible influences of section plane and vessel-diameter, correction of BMT for capillary lumen area (BM/lumen) was done. The results were similar to those obtained before correction, except for the difference observed for the R-treated group, showing a clearly thickened BM compared to the control and T-treated groups, which was no longer significant after correction. These results indicate the absence of any correlation between BMT and capillary lumen in the different groups. Therefore, absolute values of BMT without lumen correction were considered for analysis.

Discussion

This study shows an increase in VEGF concentration in the ocular fluid of untreated diabetic rats compared to control rats, and this increase is associated with retinal capillary BM thickening. These data agree with several studies showing an increase in VEGF in the ocular fluid and serum of diabetic patients before they developed complications of the disease [12, 13, 14, 34].

For long-term studies, the most widely accepted model for the evaluation of retinal complications is the streptozotocin-induced diabetic rat. The retinal lesions observed in rats resemble the initial process of diabetic retinopathy that occurs in humans [20]. Thickening of the BM is always observed in a generalised manner in all species with increased glucose concentrations and represents the first lesion during the retinopathic process which culminates in the formation of new vessels [6, 21]. The mechanisms underlying this thickening are still not well understood, but at least two stages are known to be of fundamental importance in this: (i) increased synthesis of extracellular matrix through hyperglycaemia-stimulated biochemical reactions [5, 17, 35], and (ii) reduced proteolysis of laminin and fibronectin (glycoprotein components of the capillary BM) due to an increase in tissue plasminogen activator inhibitor 1 (PAI-1) [35, 36]. The increase in VEGF concentrations has been related to active retinal neovascularization as observed in ischaemic diseases of the retina [11, 14]. Two studies [14, 15] have shown increased VEGF mRNA expression in retinal vessels of diabetic rats, and blockade of the VEGF receptors suppressed capillary BM thickening and retinal neovascularization in other species.

Thus, our data showed that T-treated diabetic rats did not develop the capillary lesions observed in untreated diabetic animals with the same disease duration. The simultaneous presence of normal VEGF

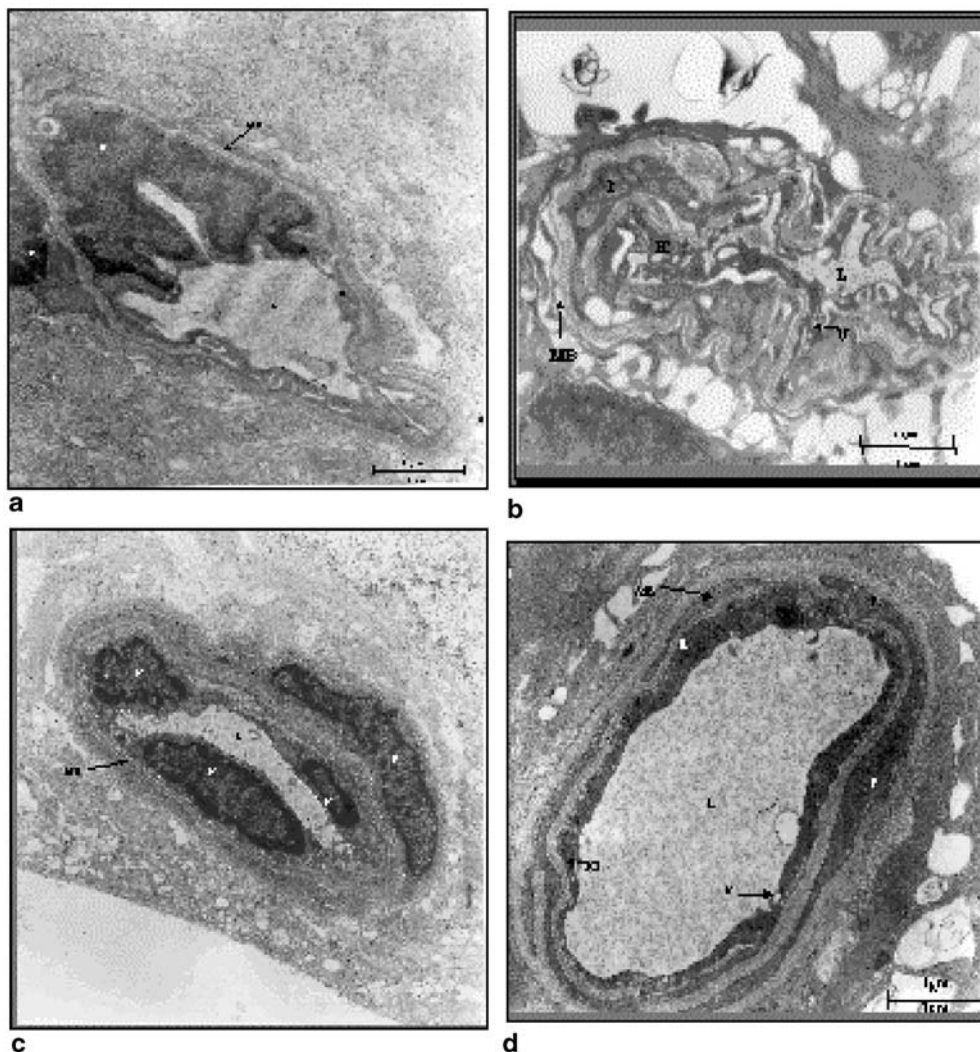


Fig. 1a–d. Retinal capillary sections from (a) non-diabetic rats, (b) untreated diabetic rats, (c) diabetic rats treated with thalidomide and (d) diabetic rats treated with rosiglitazone (MB, basement membrane; L, vessel lumen; V, vacuolization; P, pericyte; E, endothelium)

concentrations in ocular fluid and the absence of alterations in the capillary BM might suggest an association between them. The VEGF response to the hyperglycaemic stimulus, which is known to stimulate VEGF production in the retina [10, 11, 12, 13], was somehow blocked in T-treated rats. Little is known about the anti-angiogenic properties of T, and no studies have reported that this drug has the ability to inhibit the retinal-capillary BM thickening provoked by diabetic hyperglycaemia. In humans, T is transformed into various metabolites through hepatic metabolization, one of them being responsible for its antiangiogenic as well as teratogenic effects. However, in rats T is not metabolised by the liver and does not show any teratogenic activity [37]. These differences in T metabolism between rats and humans are important when

considering that another action of this drug might be responsible for its inhibitory effect of angiogenesis in retinopathy. In contrast, it is known that tissue hypoxia resulting from ischaemic processes is an important stimulus for the production of VEGF [10, 12] and retinal tissue ischaemia caused by capillary occlusion is a physiopathological characteristic of diabetic retinopathy.

Based on the therapeutic efficacy of T in leprosy, AIDS, auto-immune disease and multiple myeloma [28, 38], and based on previous findings on the inhibition of IGF-1-mediated angiogenesis by T in non-diabetic rabbit cornea [23] and on our results, thalidomide could be used for the prevention of diabetic retinopathy. The side effects of this drug do not exclude its therapeutic effects as long as treatment is done properly.

In contrast to the response obtained with the use of T, diabetic rats treated with R showed VEGF concentrations in ocular fluid similar to those observed for untreated diabetic and normal rats but higher than in T-treated diabetic rats. Thus, R was not effective in inhibiting VEGF secretion in hyperglycaemic diabetic rats. In addition, retinal capillary BMT in R-treated

rats was similar to that observed for the untreated diabetic group and was significantly greater than in the control and T-treated groups, again indicating an association between VEGF concentrations and BMT. However, when BMT was adjusted for vessel lumen area, which did not differ between groups, no significant difference in the BM:lumen ratio was observed between the R-treated group and the untreated diabetic, T-treated and control groups.

Some authors [39] did not consider this adjustment. This procedure can be important in the assessment of BM thickening if variable dimensions of the capillary BM are observed in the same rat or in the same group of rats due to differences in section planes or vessel size. This situation was not found in the four groups studied, as BMT showed little variation within the same group considering the variations in lumen area of the same capillaries analysed.

Based on the data above, we conclude that R was not effective in preventing the development of capillary lesions in diabetic rats.

The antiangiogenic effect of R was initially shown by [25], who induced angiogenesis in rat cornea with VEGF and observed inhibition of neovascularization in animals treated with PPAR- γ ligands. Another important study [24] also showed the inhibition of choroidal angiogenesis induced by photocoagulation in rats after intravitreal injection of troglitazone. In a more recent study [26] retinal ischaemia was induced in newborn mice by oxygen hyperperfusion and intravitreal injections of R maleate (100 $\mu\text{mol/l}$) were applied, resulting in inhibition of retinal neovascularization irrespective of VEGF over-expression in the ganglion-cell layer of the retina. This suggests that R inhibited retinal neovascularization by blocking the angiogenic action of VEGF rather than by direct inhibition of this growth factor. Another relevant observation [26] was the inhibition of protein kinase, an enzyme recognised to be important in the development of diabetic retinopathy, in endothelial retinal cells. The same results have been confirmed in *in vitro* studies [25], differing only in the effect of the dose.

Various studies have shown that the activation of PPAR- γ is associated with endothelial protection [40, 41] and even with the inhibition of monocytes and macrophages [31]. Furthermore, both *in vitro* and *in vivo* studies in experimental animals agree that PPAR- γ ligands are inhibitors of angiogenesis, but the exact mechanism remains controversial. Evidence indicates that the effect occurs in the post-VEGF production phase probably by blocking the synthesis of its receptors. These findings are useful for interpreting previous results showing that troglitazone induces an increase in the plasma of VEGF in Type 2 diabetic patients treated with this drug [42].

Our results show that R does not prevent the development of diabetic retinopathy in rats. This is probably related to the inability of R to block the action of

VEGF at its receptor level rather than to its inability to reduce the increased VEGF concentrations triggered by the diabetic state. Since the antiangiogenic effect of R is dose-dependent, it is possible that the dose, time and especially the route of administration used in our study were not adequate for this animal model. Therefore oral R at the dose of 1 mg kg⁻¹ day⁻¹ administered for a period of 3 months is probably not effective in preventing retinopathy in streptozotocin-induced diabetic rats.

In conclusion, our data shows increased VEGF concentrations in ocular fluid and retinal capillary-basement-membrane thickening in diabetic rats, compared to normal rats, 3 months after the onset of the disease. Although no differences in the parameters studied were observed after the use of R, daily administration of T to diabetic rats for the same period of 3 months was associated with the blockade of the increase of VEGF in ocular fluid and the inhibition of BM thickening, thus indicating the potential action of this drug in preventing retinal neovascularization in humans (Fig. 1).

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