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Inhibition of choroidal angiogenesis by calcium dobesilate in normal Wistar and diabetic GK rats

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Abstract

Calcium dobesilate reduces vascular endothelial growth factor (VEGF) over-expression in diabetic rat retina, but its effect on intraocular angiogenesis is unknown. Therefore, we tested calcium dobesilate for its in vitro and ex vivo effects on choroidal explant angiogenesis in spontaneously diabetic Goto–Kakizaki (GK) rats. Choroidal explants were cultured in gels of collagen. Budded microvessels numbers and VEGF formation were taken as markers of angiogenesis. Ex vivo studies were performed in GK rats orally given 100 mg/kg/day calcium dobesilate for 10 days. In vitro, calcium dobesilate dose- and time-dependently inhibited both microvessel formation and VEGF production, at concentrations ≥25 μg/ml (i.e. ≥60 μM), with complete inhibition at 100 μg/ml. Oral treatment of diabetic GK rats with calcium dobesilate induced a significant reduction of choroidal angiogenesis ex vivo (38.8% after 3 days of culture). In conclusion, calcium dobesilate inhibited choroidal explant angiogenesis both in vitro and ex vivo. This effect may be due, at least in part, to inhibition of VEGF production. Antiangiogenesis by calcium dobesilate can be involved in its therapeutic benefit in diabetic retinopathy.

Keywords: Diabetes; VEGF (vascular endothelial cell growth factor); Angiogenesis; Calcium dobesilate; (Rat)

1. Introduction

Calcium dobesilate is a potent antioxidant compound (Brunet et al., 1998a,b; Szabo et al., 2001), which stabilizes blood–retinal barrier in patients with diabetic retinopathy (Nemeth et al., 1975; Grignolo et al., 1979; Leite et al., 1990; Vojnikovic, 1991; for review, see Berthet et al., 1999). Recent animal studies showed that calcium dobesilate inhibits advanced glycation end product formation, which in turn decreases retinal over-expression of vascular endothelial growth factor (VEGF) and vascular leakage (Rota et al., 2004).

VEGF is a growth factor, which promotes neovascularization (or angiogenesis) by increasing vascular permeability and other mechanisms (for review, see Ferrara, 2000). In diabetic retinopathy, VEGF plays a major role in mediating intraocular angiogenesis (Aiello et al., 1994; Ozaki et al., 2000), and angiogenesis is a main contributor to the pathogenesis of diabetic retinopathy (Aiello, 2003; Campochiaro, 2000; Campochiaro and Hackett, 2003).

The above arguments pushed us to test calcium dobesilate for its effects on vascular proliferation. Kobayashi et al. (1998) developed a quantitative assay for angiogenesis, by using cultured choroidal tissues from diabetic Goto–Kakizaki (GK) rats (see also Nicosia and Ottinetti, 1990). The GK rat is a model of non-insulin-dependent diabetes mellitus without obesity (Sone et al., 1997; Miyamoto et al., 1996), and Kobayashi et al. (1998) reported that the number and total length of microvessels

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budded from cultured choroidal explants is enhanced in diabetic GK rats. On the other hand, choroid is an important supplier of nutrients and oxygen for the neighboring retina. In case of diabetes, both vicinity and dependence suggest that choroidal angiogenesis could open the way to diabetic retinopathy (Hidayat and Fine, 1985; Fryczkowski et al., 1989; Cao et al., 1998). Therefore, we used cultured choroidal explants from GK rats to test calcium dobesilate for its in vitro effects on both angiogenesis and VEGF secretion. The finding of a positive result led us to continue by orally giving calcium dobesilate to diabetic GK rats and investigating choroidal angiogenesis ex vivo.

2. Materials and methods

2.1. Animals

A colony of type 2 diabetic GK rats was settled in our laboratory with progenitors issued from M&B (Ejby, Denmark). These rats spontaneously develop non-insulin-dependent diabetes at about 4 weeks of age, without obesity. Retinal circulatory abnormalities and increased VEGF production in the retinal optic nerve fiber layer, retinal pigment epithelium and choroid were observed in these animals, before apparent retinopathy with typical proliferative lesions (Sone et al., 1997; Miyamoto et al., 1996).

Animals were bred at the "Centre d'Elevage et de Conditionnement Expérimental des Modèles Animaux" (CECEMA, Université Montpellier II), housed in a temperature-regulated room at 22±1 °C and subjected to 12:12-h light/dark cycles; they had free access to standard diet and tap water. Experiments were performed with male GK rats at 12–15 weeks of age. Age-matched non-diabetic Wistar rats (Janvier, Le Genest St. Isle, France) were used as controls.

All procedures were performed in accordance with the ARVO Statement for the Use Of Animals in Ophthalmic and Vision Research. Investigations were performed according to the European Community guidelines for ethical animal care and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985). All manipulations were conducted in such a way to minimize stress, discomfort and pain.

2.2. Preparation and culture of choroidal explants

Choroidal explants were prepared according to the method of Nicosia and Ottinetti (1990), modified by Kobayashi et al. (1998). Nicosia and Ottinetti (1990) described an in vitro model of angiogenesis produced by culturing rings of rat aorta in gels of biological matrices (aortic endothelium exposed to a three dimensional matrix of collagen or fibrin switches to a microvascular phenotype generating branching networks of microvessels). Angiogenesis was triggered by the injury of the dissection procedure and did not require stimulation by exogenous growth factors. By adapting this rat aorta in vitro model to choroidal tissue explants, Kobayashi et al. (1998) reported that angiogenesis is enhanced in diabetic rats when compared to Wistar rats, in the presence of fetal bovine serum.

2.2.1. Blood and eyes collection

Fasting animals were anaesthetized with sodium pentobarbital (60 mg/kg, i.p., Sanofi-Synthelabo, France) and cardiac blood samples were obtained for glucose determination. Glucose was measured by the glucose oxidase method with a glucose analyser (Glucotred®, Roche Diagnostics, Germany). Blood glucose concentration was 13.9±1.1 and 7.8±0.6 mmol/l in diabetic GK and Wistar rats, respectively ($P<0.001$). Both eyeballs were rapidly isolated under aseptic conditions and placed in phosphate-buffered saline (PBS).

2.2.2. Preparation of choroidal tissues

Eyeball dissection and preparation of choroidal explants were performed under a stereomicroscope with an optic fiber light source in a sterile vertical laminar flow hood. Blood vessels, connective tissues and fatty tissues were removed from the outside of the eyeballs in a Petri dish containing Dulbecco's modified Eagle's medium (DMEM, 1 g/l glucose, Eurobio, France). After removing cornea, lens, corpus vitreum and retina from the inside of the globe, the posterior segment containing the sclera and the choroid was sectioned into either quadrants or thirds. After breaking any adhesions between the choroid and the sclera, the isolated segment of choroid was then sectioned into 1- to 2-mm^2 explants, and placed in the collagen gel. During dissection, tissues were frequently transferred to fresh clean DMEM in sterile conditions.

2.2.3. Collagen gel cultures

Choroidal tissue explants were cultured by a modified method of Nicosia and Ottinetti (1990). Isolated explants were placed on type I collagen gels, previously prepared by mixing 2 mg type I collagen (2.5 mg/ml) per ml culture medium on ice, neutralized with 0.1 N NaOH, plated in a 16-mm well (4-well plates, Nunc, Roskilde, Denmark), and allowed to gel at 37 °C. Each culture well housed one explant. A volume of a fresh collagen solution mixture was overlaid. After the collagen solidified at 37 °C, choroidal explants were cultured with DMEM (5.5 mmol/l glucose) containing 5% heat-inactive fetal calf serum supplemented with 2 mmol/l of L-glutamine (Eurobio) and 100 U/ml–100 μg/ml of penicillin–streptomycin (Eurobio). The cultures were kept in a humidified 5% CO_2 incubator at 37 °C. Growth medium was changed every 2–3 days.

2.3. Measurement of angiogenesis

The angiogenic response of the choroidal explants was quantitated by visual counts. Cultures were examined using

an inverted Will microscope (Wetzlar, Germany) and new microvessels were counted with ×10 objectives. Microvessels newly budded from a cultured explant of choroidal tissues were photographed with a Nikon camera mounted on a Nikon Eclipse TE300 (Tokyo, Japan) equipped with a Hoffman's phase contrast device.

In one selected experiment performed with control rats, the outgrowth of microvessels from a cultured explant of choroidal tissues were continuously visualized with a digital camera (Hamamatsu C 5985, Japan) mounted on a Leica DMIR B microscope (Wetzlar, Germany). Images were automatically taken every 5 min and recorded in a computer.

2.4. Measurement of VEGF in conditioned media

Secreted VEGF levels in the medium were evaluated by a specific enzyme-linked immunoabsorbent assay (ELISA) kit (Quantikine® M) obtained from R&D systems (United Kingdom). This assay recognizes natural and recombinant mouse VEGF and reacts with rat VEGF.

Conditioned media were collected every 2 days and stored at −20 °C. ELISA was performed according to the manufacturer's recommendations. Optical density was read at 450 nm in a Spectra Shell microplate reader (ATX Lab Systems, Inc., Virginia, USA). The standard curve was prepared with recombinant mouse VEGF. The sample values were read off the standard curve. Sensitivity of assay was 3.0 pg VEGF/ml. Results were expressed as absolute values (pg VEGF/ml culture medium).

In control experiments we verified that the fetal calf serum used in the experimental cell medium was devoid of measurable VEGF concentrations.

2.5. Treatment with calcium dobesilate

For in vitro experiments, calcium dobesilate was added into the collagen gel and in the culture medium at concentrations from 0 to 100 μg/ml. For VEGF measurements, five experiments (using each time one W and one GK rat) were performed. For each rat, eight explants were

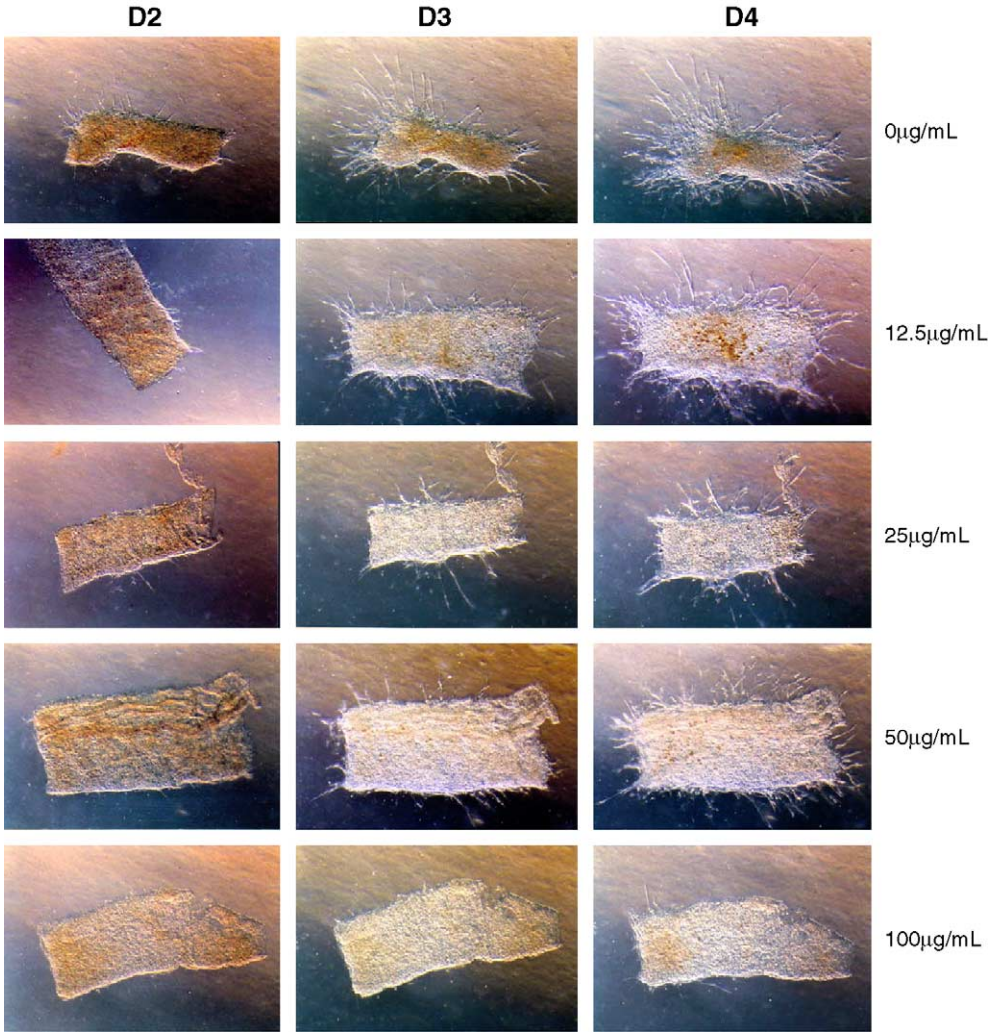

Fig. 1. In vitro antiangiogenic effects of calcium dobesilate in choroidal explants from a control rat. Calcium dobesilate concentrations are indicated on the right margin. Days of culture are given on the top. Magnification ×10.

prepared and incubated individually with the same concentration of calcium dobesilate (25, 50, or 100 μg/ml). The eight supernatants were then pooled and evaporated with a SpeedVac concentrator and reconstituted in PBS at 1/3 the original volume.

For the ex vivo experiments, 13-week-old male GK rats were divided in two groups (n=4 per group) orally receiving 100 mg/kg per day of calcium dobesilate (OM PHARMA, Meyrin, Switzerland) or vehicle (0.9% NaCl) for 10 days. Twenty-four hours after the last administration, the animals were killed and eyeballs removed for the preparation of choroidal tissues. Explants from both eyes were independently measured and the obtained values were pooled.

Calcium dobesilate treatment did not induce significant changes either in body weight or glycaemia.

2.6. Statistical analysis

Results were expressed as mean±standard error of the mean (S.E.M.) of n determinations. A Student's t-test for unpaired samples was used for statistical analysis. Significant differences were accepted for $P<0.05$.

3. Results

3.1. General aspects of choroidal explant angiogenesis

Choroidal explants from both control Wistar and diabetic GK rats were cultured, and tube formation was studied as described in Materials and methods. Figs. 1 (top row) and 2 (top row) show representative experiments with one control and one diabetic explant, respectively. Microvessel-like structures appeared spontaneously (after 5–6 h of culture), with their number and length increasing progressively up to day 4 of culture (reaching a plateau thereafter).

Fig. 3 shows mean microvessel growth values of six experiments. It can be seen that the number of microvessels progressively increased from days 2 to 4 in control (left side) and diabetic explants (right side). The

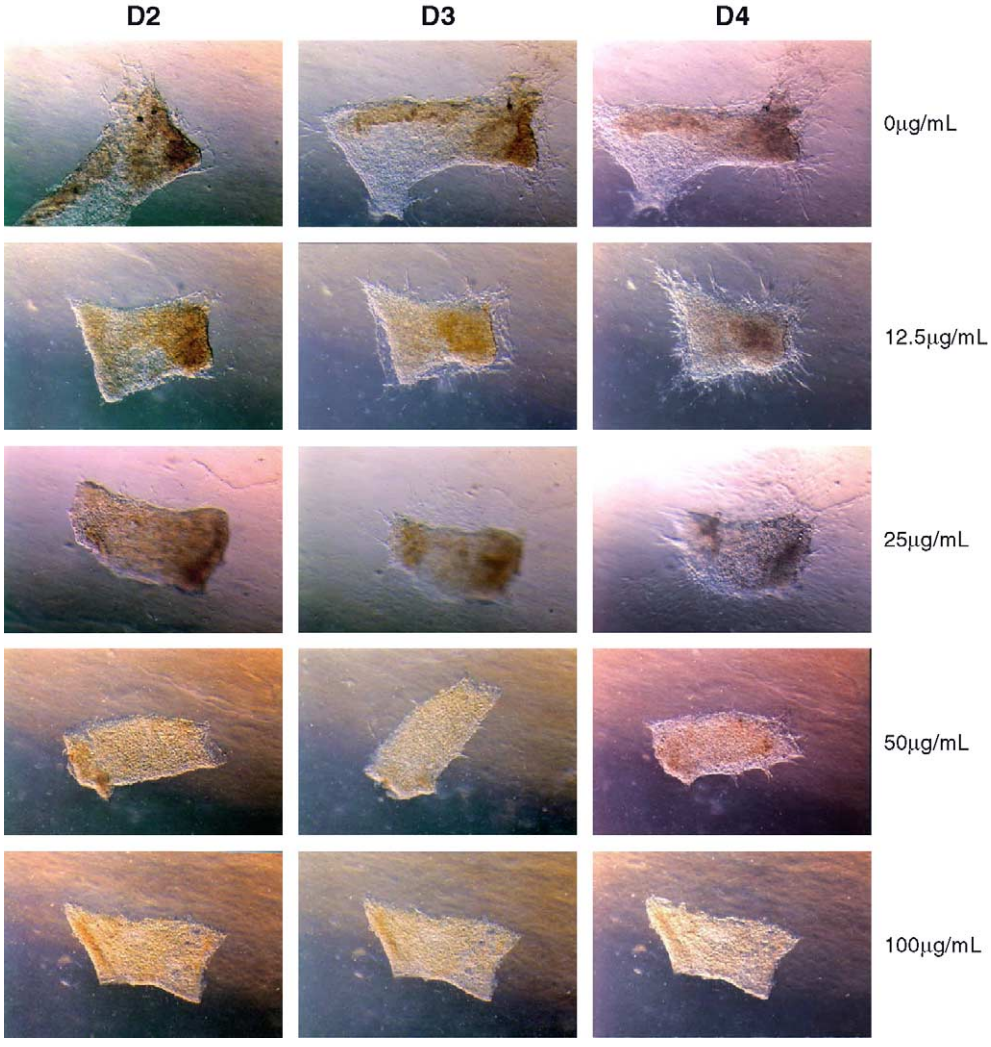

Fig. 2. In vitro antiangiogenic effects of calcium dobesilate in choroidal explants from a diabetic GK rat. Calcium dobesilate concentrations are indicated on the right margin. Days of culture are given on the top. Magnification ×10.

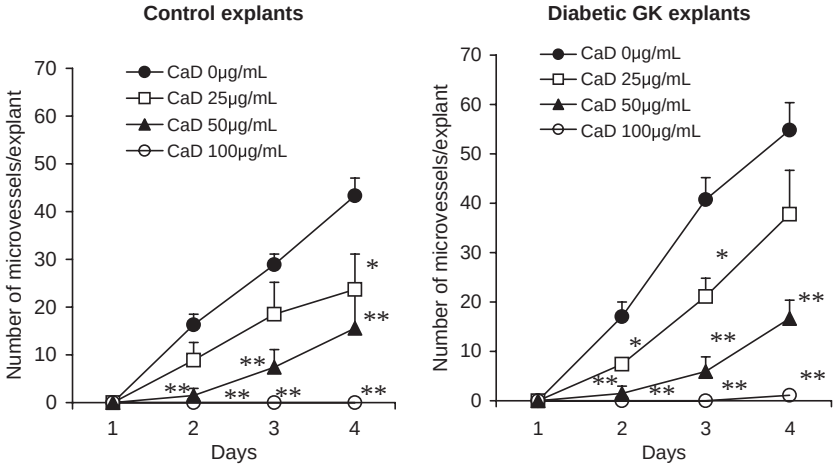

Fig. 3. In vitro effects of calcium dobesilate (25, 50 and 100 µg/ml) on angiogenesis in choroidal explants from control (left) and diabetic GK rats (right). Data are expressed as means±S.E.M. (n=6). *P<0.05 and **P<0.01 versus 0 µg/ml calcium dobesilate.

number of microvessels budded from cultured choroidal explants tended to be slightly greater in diabetic explants, but differences with controls were not statistically significant (Fig. 3, circles). Vessel length was also not significantly increased in diabetic explants (compare top rows of Figs. 1 and 2).

3.2. In vitro effects of calcium dobesilate on choroidal explant angiogenesis

Fig. 3 shows in vitro effects of calcium dobesilate on choroidal explant angiogenesis (n=6). In control explants, calcium dobesilate 25 µg/ml induced a significant decrease of tube formation (45.1±17.7%, P<0.05) after 4 days of culture (Fig. 3, left). Calcium dobesilate 50 µg/ml inhibited tube formation from day 2 of culture, with a decrease of 64.4±16.4% (P<0.01) at day 4. A concentration of 100 µg/ml fully inhibited tube formation from days 2 to 4. In diabetic explants (Fig. 3, right), 25 µg/ml calcium dobesilate significantly decreased tube formation from day 2 of culture, reaching a decrease of 30.7±16.4% at day 4 (P<0.01, n=4). Concentrations of 50 and 100 µg/ml calcium dobesilate inhibited tube formation similarly as in control explants from days 2 to 4.

Figs. 1 and 2 show representative experiments with one control and one diabetic explant, respectively. In control explants (Fig. 1), calcium dobesilate dose-dependently reduced microvascular growth at doses equal or higher than 25 µg/ml, with almost complete inhibition at 100 µg/ml. Fig. 2 shows similar results in diabetic explants. As for control explants, no effect was observed with a concentration of 12.5 µg/ml calcium dobesilate. This concentration was therefore not tested in the other in vitro experiments reported in Fig. 3.

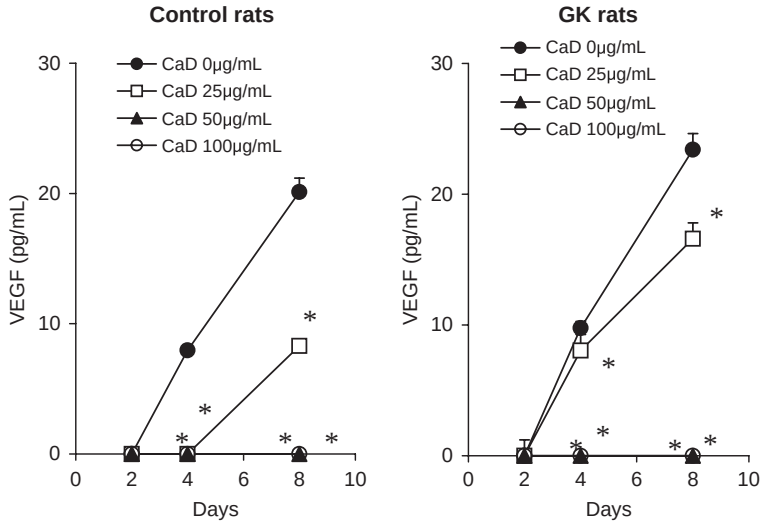

Fig. 4. In vitro effects of calcium dobesilate (25, 50 and 100 µg/ml) on VEGF (pg/ml) secreted by choroidal explants from control (left) and diabetic GK rats (right). Data are expressed as means±S.E.M. (n=5). *P<0.01 versus 0 µg/ml calcium dobesilate.

3.3. In vitro effects of calcium dobesilate on choroidal VEGF secretion

Levels of VEGF secreted into the culture medium by choroidal explants of control and diabetic GK rats increased from days 2 to 8 in a time-dependent manner (Fig. 4). For each concentration tested, VEGF levels were determined from five pooled supernatants (from eight explants). Levels of VEGF in diabetic explants were slightly but significantly greater than those of control explants (Fig. 4).

Calcium dobesilate fully inhibited VEGF secretion in both control and diabetic explants at concentrations equal or higher than 50 μg/ml (Fig. 4). In control explants, a concentration of 25 μg/ml induced complete VEGF inhibition until day 4. By contrast, in diabetic explants, 25 μg/ml CaD only decreased VEGF secretion by 16.0±0.5% (P<0.01, n=5) at day 4 and by 29.5±2.4% (P<0.01, n=5) at day 8 (Fig. 4, right).

3.4. Ex vivo effects of calcium dobesilate on choroidal angiogenesis and VEGF secretion

Diabetic GK rats were orally treated for 10 days with calcium dobesilate 100 mg/kg per day or vehicle (saline), and ex vivo tube formation in cultured choroidal explants was studied as described in Materials and methods (n=4 per group). The number of microvessels of the choroidal explants increased in a time-dependent manner, with values always lower than those of non-treated diabetic GK rats (Fig. 5, left); statistical significance was reached after 3 days of culture (decrease of 38.8±8.8%, P<0.05, n=4).

VEGF secreted into the culture medium by the above choroidal explants was measured at days 3 and 4 of culture. Fig. 5 (right) shows that choroidal explants from rats treated with calcium dobesilate tended to secrete less VEGF, although differences with vehicle-treated rats did not reach statistical significance.

4. Discussion

Calcium dobesilate was investigated both in vitro and ex vivo for its action on tube formation in choroidal explants from diabetic GK and control rats. In all circumstances, calcium dobesilate inhibited tube formation. Moreover, the in vitro inhibitory action of calcium dobesilate was associated to a profound inhibition of VEGF production.

Choroidal angiogenesis was unaffected by diabetes and VEGF production was only marginally increased in choroidal explants from GK rats. However, Kobayashi et al. (1998) reported that microvessel growth was almost doubled in explants from diabetic GK rats as compared to explants from control Wistar rats. One possible explanation is that we used collagen gels, which seem to stimulate angiogenesis less than the fibrin gels used by Kobayashi et al. (1998) (Nicosia and Ottinetti, 1990).

Calcium dobesilate inhibited tube formation to a similar extent in both control and diabetic explants. In vitro, significant inhibition of tube formation was found for calcium dobesilate concentrations ≥25 μg/ml (≥ 60 μM). Similarly, calcium dobesilate concentrations≥25 μg/ml were required to inhibit VEGF production in vitro. To our knowledge, nothing is known about choroidal contents of calcium dobesilate following chronic administration in patients with diabetic retinopathy. In humans, after a single oral administration of 1000 mg, plasmatic values of calcium dobesilate reached a plateau of about 25 μg/ml (C. Chiavaroli, personal communication; see also Benakis et al., 1974). On the other hand, Rota et al. (2004) found that oral treatment of diabetic rats with calcium dobesilate (100 mg/kg/day) for 10 days reduced retinal VEGF

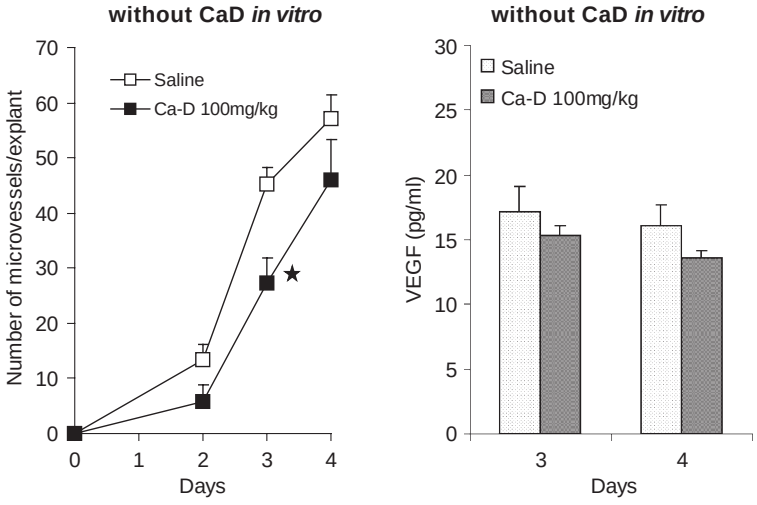

Fig. 5. Angiogenesis (left) and VEGF formation (right) in choroidal explants from diabetic GK rats orally treated with 100 mg/kg per day calcium dobesilate or vehicle (saline) for 10 days. No calcium dobesilate was added to the culture medium. Explants from both eyes were independently measured and the obtained values were pooled. Data are expressed as means±S.E.M. (n=4 per group). *P<0.05 versus vehicle group. CaD indicates calcium dobesilate.

expression by 69.4%. These arguments suggested to us that chronic treatment allows calcium dobesilate to accumulate enough in choroidal tissue to reach therapeutic antiangiogenic concentrations. Therefore, we performed an ex vivo experiment in diabetic GK rats, by using the same treatment protocol (standard protocol in diabetic rats, see for instance Szabo et al., 2001).

Ex vivo, calcium dobesilate inhibited tube formation by 38.8%. This can be compared with the 30.7% inhibition obtained in vitro with calcium dobesilate 25 μg/ml (see Fig. 3). Therefore, chronic treatment of diabetic GK rats with calcium dobesilate seems to ensure choroidal dobesilate concentrations able to exert antiangiogenic actions. Of course, the above numbers can be strongly influenced by species differences and several other factors. In particular, by the fact that explants were cultured for 4 days without calcium dobesilate. Thus, calcium dobesilate deserves to be further investigated for antiangiogenic properties in the in vivo model of Wang et al. (2003), a complementary tool for screening the actions of VEGF-lowering drugs on the angiogenic process in diabetic retinopathy.

Choroidal explants from GK rats treated with calcium dobesilate tended to secrete less VEGF, but differences with vehicle-treated rats did not reach statistical significance. This contrasts with the finding of Rota et al. (2004), who found that calcium dobesilate reduces retinal VEGF expression by 50–60% in streptozotocin-diabetic rats. Several factors can explain the difference in the results. First, VEGF was only marginally increased in choroidal explants from GK rats, whereas it was increased fourfold in streptozotocin-diabetic rat retina (Rota et al., 2004). Second, there was a culture period of 4 days were calcium dobesilate was not added to the incubation media, whereas Rota et al. (2004) measured VEGF expression in retinal samples of rats where the last oral dose of dobesilate was given 2–3 h before sacrifice. Finally, calcium dobesilate may perhaps interfere with other growth factors involved in choroidal neovascularization, such as connective tissue growth factor (CTGF) (He et al., 2003), basic fibroblast growth factor (bFGF) (Frank et al., 1996), angiopoietin 1 and 2, which are known to stimulate retinal angiogenic responses via an increased expression of metalloproteinase-9 (MMP-9), (Das et al., 2003) and transforming growth factor-β which induces VEGF secretion by retinal pigment epithelial cells, subsequently inducing choroidal neovascularization (Nagineni et al., 2003).

No attempt was done here to characterize the mechanism by which calcium dobesilate inhibits angiogenesis in choroidal explants. Nevertheless, several arguments suggest that calcium dobesilate may act via its antioxidant properties. First, reactive oxygen species can induce endothelial dysfunction, resulting in the release of growth factors that stimulate vascular cell proliferation (vascular growth factor expression is mainly up-regulated by hypoxia; for review, see Tardif et al., 2002; Maulik and Das, 2002). Second, antioxidants can inhibit angiogenesis, as well as the formation of vessel-like tubular structures of endothelial cell cultures on Matrigel (Ashino et al., 2003). Finally, the diabetic status is strongly associated with endothelial dysfunction, and calcium dobesilate was found to increase endothelium-dependent aorta relaxation, perhaps via its antioxidant properties (Tejerina and Ruiz, 1998).

Retinal and choroidal neovascularization are the most common causes of severe visual loss in developed countries (Campochiaro and Hackett, 2003). On the other hand, tight glycemic control and laser therapy reduce vision loss and blindness, but do not reverse existing ocular damage and only slow the progression of the disease. Therefore, complementary, effective pharmacological treatments are urgently needed. Indeed, a series of new inhibitors of vascular proliferation are currently under development (Speicher et al., 2003).

In conclusion, calcium dobesilate inhibited in vitro and ex vivo angiogenesis of choroidal explants, at least partly by inhibiting VEGF production. Antiangiogenesis can be involved in the therapeutic benefit of calcium dobesilate in diabetic retinopathy. Therefore, the present results deserve confirmation in other in vivo animal models of choroidal angiogenesis.

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