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Reduction of retinal albumin leakage by the antioxidant calcium dobesilate in streptozotocin-diabetic rats

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Abstract

Calcium dobesilate stabilizes blood–retinal barrier in patients with diabetic retinopathy and possesses antioxidant properties in the retinas of rats with streptozotocin-induced diabetes, exposed ex vivo to ischemia–reperfusion. Here we investigated the action of calcium dobesilate on retinal albumin leakage in streptozotocin-diabetic rats, together with relevant in vivo retinal antioxidant and permeability markers, i.e., carboxymethyl-lysine-advanced glycation end product (CML-AGE) formation and vascular endothelial cell growth factor (VEGF) overexpression. Twenty days after streptozotocin administration, diabetic rats were treated for 10 days with calcium dobesilate (100 mg/kg/day per os) or vehicle. Retinal albumin leakage, CML-AGE formation, and VEGF overexpression were evaluated by immunohistochemistry of frozen eye sections. Diabetic rats exhibited dramatic increases in: (i) retinal albumin leakage (31% of positive vessels vs. 0.2% in nondiabetic rats, $P<0.008$), (ii) CML-AGE retinal occurrence ($40 \pm 3\%$ vs. *undetectable* positive vessels), and (iii) retinal VEGF protein expression (14.6 ± 1.1 vs. 3.5 ± 0.5 VEGF-positive spots/field, $P<10^{-4}$). Calcium dobesilate significantly reduced: (i) retinal albumin leakage (by 70%, $P<0.008$), (ii) retinal CML-AGEs contents (by 62%, $P<0.008$), and (iii) retinal VEGF expression (by 69.4%, $P<0.008$). In conclusion, calcium dobesilate orally given to diabetic rats markedly reduced retinal hyperpermeability, CML-AGE contents, and VEGF overexpression. These results strongly suggest that calcium dobesilate stabilizes blood–retinal barrier in diabetic retinopathy via an in situ antioxidant action. Further studies in patients are required to confirm such view.

Keywords: Diabetic retinopathy; Calcium dobesilate; Advanced glycation end product; Antioxidant; Vascular permeability; VEGF (vascular endothelial cell growth factor)

1. Introduction

Vision loss in diabetic retinopathy is strongly correlated with increased vascular permeability (Antonetti et al., 1999). Calcium dobesilate (2,5 dihydroxybenzenesulfonate) improves visual acuity and stabilizes blood–retinal barrier in patients with diabetic retinopathy (Leite et al., 1990; Grignolo et al., 1979; Nemeth et al., 1975; Vojnikovic 1991; see also van Bijsterveld and Janssen, 1981; for review about calcium dobesilate, see Berthet et al., 1999).

Chronic hyperglycemia is the major determinant of diabetic retinopathy (for review, see Watkins, 2003). Animal studies showed that hyperglycemia produces oxidative stress in the diabetic retina and accelerated formation of advanced glycation end products (AGEs; for review, see Kowluru and Kennedy, 2001; Wautier and Guillausseau, 2001; Stitt, 2003). Hyperglycemia-induced oxidative stress contributes to the accelerated death of retinal vascular cells, particularly pericytes (Ansari et al., 1998; Kowluru and Koppolu, 2002; Romeo et al., 2002), leading to acellular capillaries (Hammes et al., 1997) and increased vascular permeability (Ellis et al., 1998).

In vitro studies showed that calcium dobesilate is an oxygen free radical scavenger (Lozovskaia et al., 1990; Brunet et al., 1994, 1998a; Ruiz et al., 1997). This was confirmed by animal studies showing that orally given calcium dobesilate possesses antioxidant actions in normal rats (Brunet et al., 1998b) and ex vivo in the retinas of rats

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with streptozotocin-induced diabetes, exposed to ischemia–reperfusion (Szabo et al., 2001). However, whether calcium dobesilate stabilizes blood–retinal barrier in diabetic rats and whether it displays in vivo antioxidant actions in the diabetic rat retina are unknown.

Several efforts have been made in order to identify early markers of retinal oxidative injury (for references, see Obrosova et al., 2000). The deregulated overexpression of vascular endothelial cell growth factor (VEGF) plays a key role in the retinal neovascularization and macular edema of diabetic retinopathy (for recent review, see Caldwell et al., 2003; for retinal VEGF in streptozotocin-diabetic rats, see Sone et al., 1997; Hammes et al., 1998). Several studies showed that AGEs increase the VEGF expression of retinal cells (Hirata et al., 1997; Lu et al., 1998; Segawa et al., 1998; Endo et al., 2001; Treins et al., 2001; Mamputu and Renier, 2002). Evidence has been accumulated showing that antioxidants prevent VEGF up-regulation (Obrosova et al., 2001; El-Remessy et al., 2003). Specifically, Obrosova et al. showed that the antioxidants taurine and DL-alpha-lipoic acid significantly reduced the early up-regulation of retinal VEGF in rats. Thus, taken together, these findings suggest that oxidative stress could accelerate both retinal AGE formation and AGE-dependent VEGF expression.

Here we investigated if calcium dobesilate reversed retinal albumin leakage in rats with streptozotocin-induced diabetes. The finding of a positive result allowed us to examine in vivo effects of calcium dobesilate on relevant markers of antioxidant actions, i.e., retinal AGE contents and VEGF expression.

2. Materials and methods

The investigation was performed according to the European Community Guidelines for Ethical Animal Care and the Guide for Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 85-23, revised 1985).

2.1. Animals

Male Wistar rats, weighing 280–320 g were adapted to a humidity- and temperature-controlled room for 3–4 days before the protocol was initiated. Rats were fed a standard diet and tap water was given ad libitum.

2.2. Induction of diabetes

A preliminary experiment in rats showed that streptozotocin doses of 60–70 mg/kg induced rapid hyperglycemia in a high proportion of animals. These hyperglycemic rats exhibited body weights lower than controls, and 80–100% lived for as long as 3 months without insulin supplementation (for doses, route, and frequency of administration of streptozotocin in rats, see Tomlinson et al., 1992; Murata et al., 1996; Obrosova et al., 2001; Szabo et al., 2001). On the basis of this experiment, a single intraperitoneal dose of 65 mg/kg streptozotocin was selected for all the experiments, together with a period of 20 days to induce uncompensated diabetes in a high proportion of rats.

Sixteen rats were given a single intraperitoneal (i.p.) dose of 65 mg/kg streptozotocin and 10 other rats received vehicle (5 mM citrate buffer, 1 ml) and were used as nondiabetic controls. After 20 days, 10 streptozotocin-treated rats exhibited glycosuria, hyperglycemia (nonfasting glycemia was 26.7 ± 3.4 vs. 6.1 ± 1.2 mM in control rats), body weights significantly lower than controls (256 ± 18 vs. 428 ± 12 g in control rats), and were considered as diabetic rats. The remaining six streptozotocin-treated rats were nonglycosuric, and their body weight (427 ± 11 g) was not different from that of control rats (428 ± 12 g) and were therefore excluded from the study.

2.3. Treatment protocol

Treatment with calcium dobesilate was started 20 days after streptozotocin (or vehicle) injection. Calcium dobesilate was dissolved daily in 1-ml saline, and it was orally given through gentle esophageal canulation. Nondiabetic control and diabetic rats were given calcium dobesilate or vehicle (1-ml saline) for 10 days, according to the following scheme:

1. Group I ($n = 5$): Nondiabetic rats (receiving saline).
2. Group II ($n = 5$): Nondiabetic rats treated with 100 mg/kg calcium dobesilate.
3. Group III ($n = 5$): Streptozotocin-diabetic rats (receiving saline).
4. Group IV ($n = 5$): Streptozotocin-diabetic rats treated with 100 mg/kg calcium dobesilate.

The final administration of calcium dobesilate (or saline) was performed on the day of the experiment, 2–3 h before sacrifice. On the day of sacrifice, rats were sequentially anesthetized with pentobarbital before careful removal of the eyes, followed by thoracotomy and by blood sample withdrawing by cardiac puncture.

2.4. Preparation of retinas

For each animal, the left and right eyes were carefully removed, embedded in OCT® compound (Tissue-Tek, Sakura Finetek Europe, The Netherlands), immediately frozen in liquid nitrogen, and subsequently stored at −80 °C. Frozen eyes were serially sectioned (six 4-μm-thick sections were cut per eye). Sections were placed in poly-L-lysin coated slides and fixed with 0.3% H_2O_2/methanol for 20 min, a procedure followed by incubation in bovine serum

albumin (2% in phosphate buffer saline, PBS) for 1 h at room temperature.

2.5. Immunohistochemical detection of retinal AGE, VEGF, and albumin

Retinal sections were incubated for 1 h at room temperature with either (i) mouse anti-rat carboxymethyl-lysin(CML)-protein adduct antibody diluted 1:200 [monoclonal immunoglobulin G (IgG)1, clone 6D12, Kumamoto Immunochemical Laboratory, Japan] or (ii) polyclonal rabbit anti-VEGF IgG against 1–21 N-terminal portion of human VEGF (Santa-Cruz Biotechnology) diluted 1:100 and previously used to detect rat VEGF (Murata et al., 1996; Gilbert et al., 1998; Hammes et al., 1998) or (iii) polyclonal rabbit anti-rat albumin antibody (Dakopatts, Glostrup, Denmark) diluted 1:200. The streptavidin–biotin complex/immunoperoxidase method was subsequently carried out using an LSAB2 Kit from Dako (Dako, Carpintera, CA), as follows. After washing with PBS, sections were incubated for 30 min at room temperature with anti-mouse or anti-rabbit biotinylated immunoglobulins (LSAB2 Kit®, Dako). Sections were then washed with PBS and incubated with streptavidin-horseradish peroxidase (LSAB2 Kit®, Dako) for 20 min at room temperature, followed by three careful washings in PBS. Color development was carried out in 3-amino-9-ethylcarbazole solution (Dako) for 3–5 min at room temperature, followed by gentle washing in tap water and sealed in mounting media (for each type of immunolabeling, at least one section was counterstained with hæmatoxylin before mounting). The immunohistochemical specificity of antibodies was confirmed by two different negative controls: (i) substituting nonimmune IgG for the primary antibody, and (ii) omitting the primary antibody in the staining protocol; in both cases, no final staining was observed.

2.6. Immunohistochemical quantification

Following immunohistochemical staining, one or more cryosections from the left and right eyes (optimal histological and microscopical criteria) for each rat were examined and photos were taken under a light microscope (Zeiss Axioplan 2 Imaging, Carl Zeiss, Jena, Germany). Immunohistochemical quantification was performed at 400× magnification counting positive spots defined as follows: (i) carboxymethyl-lysine-advanced glycation end product (CML-AGE)-positive vessels demonstrating stained vascular/perivascular area (on 100 vessels scored), (ii) VEGF-positive cells per optical field (four to six fields scored/section), and (iii) albumin-positive vessels showing stained perivascular areas per vessel (on 100 vessels scored). Individual spot countings of the two eyes from each rat were averaged before averaging for the experimental group ($n=5$). Retinal sections were scored by two independent investigators blinded to sample identity.

2.7. Plasma VEGF

Blood samples were withdrawn at the time of sacrifice. After centrifugation and elimination of red blood cells and buffy coat, plasma samples were rapidly frozen and kept at −80 °C. For each animal, plasma 165-VEGF levels were measured by an enzyme-linked immunosorbent assay (ELISA; ELISA kit, R&D Systems), based on the double-antibody "sandwich" technique. Total protein content of samples was determined by using the Bio-Rad Bradford assay (Bio-Rad); because no significant differences were detected in the plasma total protein contents between the four experimental groups, results were expressed as absolute values (pg VEGF/ml plasma).

2.8. Statistical analysis

Results are given as means ± S.E.M. (n indicates the number of animals). Differences between treatment groups were assessed with the nonparametric Mann–Whitney rank sum test. A P value ≤ 0.01 was taken as statistically significant.

2.9. Reagents

Calcium dobesilate (calcium dihydroxy-2-5 benzenesulfonate) was provided by OM PHARMA. Other chemicals were from Merck-Prolabo, Sigma-Aldrich, Bio-Rad, Dako, and Santa-Cruz. Experimental solutions were prepared on a daily basis.

3. Results

3.1. General aspects

Diabetic and normal rats were treated for 10 days with calcium dobesilate or vehicle as described in the Materials and methods section. Table 1 shows endpoint body weights, glycemia, and 165-VEGF plasma levels in the four groups of rats. Diabetic rats had lower body weights and marked hyperglycemia in comparison with normal rats ($P<0.05$). Treatment of diabetic rats with calcium dobesilate was without significant effect on body weight and hyperglycemia (it was also without significant action on body weight and glycemia of control rats). Plasma 165-VEGF levels were also not significantly modified by calcium dobesilate (Table 1).

3.2. Retinal albumin leakage

In the four groups of rats, eyes were removed and serial retinal sections were prepared for immunohistochemical

Table 1
Endpoint body weight, glycemia, and plasma 165-VEGF values in diabetic and control rats, treated with calcium dobesilate or vehicle

Group	Weight (g)	Glycemia (mM)	Plasma VEGF (pg/ml)
Control	471 ± 14	6.1 ± 1.0	23.5 ± 1.0
Control + CaDob	459 ± 11	5.6 ± 1.5	21.1 ± 0.7
Diabetic	296 ± 17[a]	20.7 ± 1.4[a]	21.6 ± 1.0
Diabetic + CaDob	277 ± 24[a]	24.7 ± 1.5[a]	20.9 ± 1.9

Data are means ± S.E.M. CaDob, calcium dobesilate.
[a] $P < 0.0083$ for *Diabetic* vs. *Normal* rats (nonparametric Mann–Whitney rank sum test).

measurements as described in the Materials and methods section. Figs. 1 and 2 show albumin-positive perivascular areas, a marker of retinal vascular permeability, in the different groups of rats. It can be seen that:

- In nondiabetic rats, no immunoreactivity was seen at the abluminal side of the vascular wall in any of the retinas examined, thus indicating the absence of albumin extravasation (specific albumin immunoreactivity was only found *within* the vessels). This pattern was not changed in nondiabetic rats receiving calcium dobesilate.
- In diabetic rats receiving vehicle, a clear-cut perivascular labeling was observed between the outer plexiform layer and the inner nuclear layer, indicating substantial extravasation of albumin. The number of positive spots in this group (31.4 ± 2.2% positive perivascular spots) was significantly higher than in normal rats (0.2 ± 0.4% positive spots; $P < 0.008$).
- In diabetic rats treated with calcium dobesilate, the number of positive perivascular areas was significantly lower than in vehicle-treated diabetic rats (11.0 ± 1.9% vs. 31.4 ± 2.2% positive spots, $P < 0.008$). Thus, calcium dobesilate reduced ≈ 70% of the albumin leakage of diabetic rats.

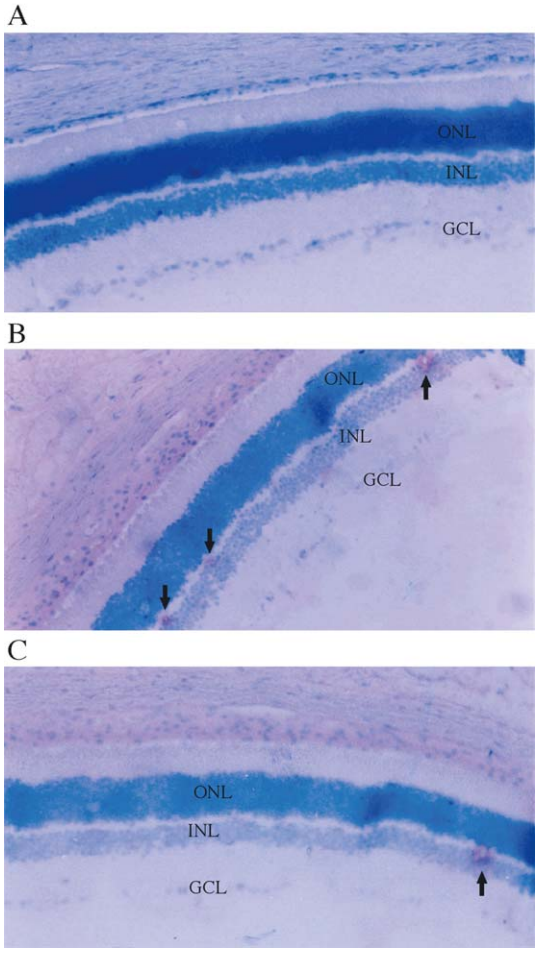

Fig. 1. Representative rat retinal sections immunostained for albumin. (A) Normal. (B) Diabetic. (C) CaD-treated diabetic. No albumin extravasation was observed in normal rats. Capillaries showing albumin leakage are indicated by arrows. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (Magnification of panels A, B, and C: 150×).

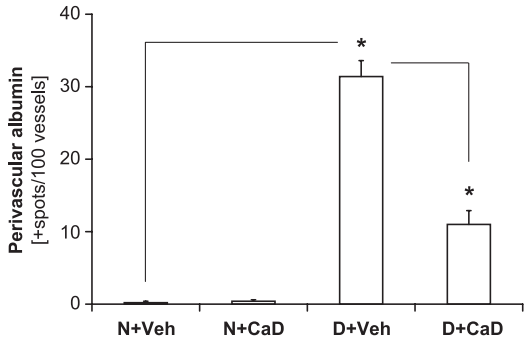

Fig. 2. Retinal albumin leakage in diabetic or normal rats treated for 10 days with CaD (100 mg/kg/daily, PO) or vehicle. N, normal; D, diabetic; VEH, vehicle; CaD, calcium dobesilate. Data are expressed as means ± S.E.M. ($n = 5$). *$P < 0.0083$ (nonparametric Mann–Whitney rank sum test).

3.3. Retinal advanced glycation end products

Figs. 3 and 4 show the retinal occurrence of advanced glycation end products (AGE) as assessed with a specific antibody against carboxymethyl-lysin(CML)-protein adducts (6D12 clone, IgG1). It can be seen that:

- Nondiabetic rat retina was exempt of CML-AGE labeling (*all* sections were examined).
- In retinal sections from diabetic rats, striking vascular and perivascular labeling for CML-AGE was found (defined hereafter as positive spots). The fraction of positive spots in diabetic rats was 40.0 ± 3.0%.
- Diabetic rats treated with calcium dobesilate exhibited a very significant decrease in the number of CML-AGE-positive spots. Only 15.0 ± 2.0% positive spots appeared in calcium dobesilate-treated animals against the 40% ($P < 0.008$) in vehicle-treated diabetic rats. Thus, calcium dobesilate reduced retinal CML-AGE in diabetic rats by 62%.

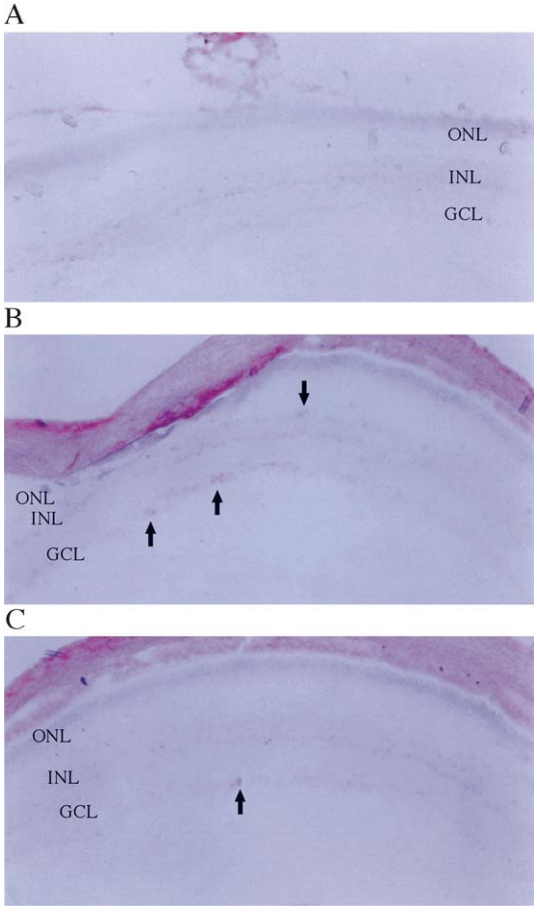

Fig. 3. Representative rat retinal sections immunostained for AGEs. (A) Normal. (B) Diabetic. (C) CaD-treated diabetic. Capillaries stained for AGEs are shown by arrows. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (Magnification of panels A, B, and C: 100×).

3.4. Retinal VEGF protein expression

Figs. 5 and 6 show VEGF-positive retinal cells, as detected by immunohistochemistry in the four groups of rats. It can be seen that:

- Nondiabetic rat retina exhibited very few, scattered VEGF-positive spots in the inner nuclear layer and ganglion cells (3.5 ± 0.2 VEGF-positive cells/field). This pattern was not changed in nondiabetic rats receiving calcium dobesilate. (3.6 ± 0.1 positive cells/field).
- In diabetic rats receiving vehicle, the number of VEGF-positive cells (14.6 ± 1.1) was significantly higher in the inner nuclear layer and in the ganglion cell layer (about fourfold higher) as in nondiabetic rats ($P < 0.008$).
- In diabetic rats treated with calcium dobesilate, a significantly lower VEGF-immunolabeling was detected in the inner nuclear layer and the ganglion cell layer (6.9 ± 0.5 positive cells/field, $P < 0.008$). This represents a 69.4% reversal of the increase in VEGF retinal

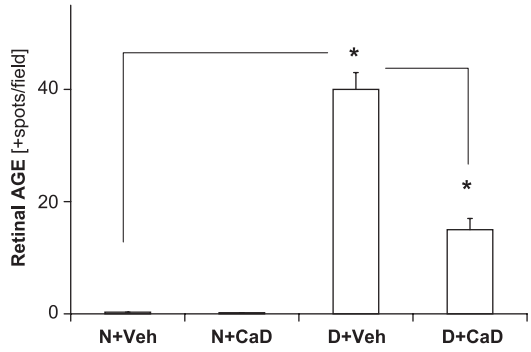

Fig. 4. Retinal CML-AGE occurrence in diabetic or normal rats treated for 10 days with CaD (100 mg/kg/daily, PO) or vehicle. N, normal; D, diabetic; VEH, vehicle; CaD, calcium dobesilate. Data are expressed as means ± S.E.M. ($n = 5$). *$P < 0.0083$ (nonparametric Mann–Whitney rank sum test).

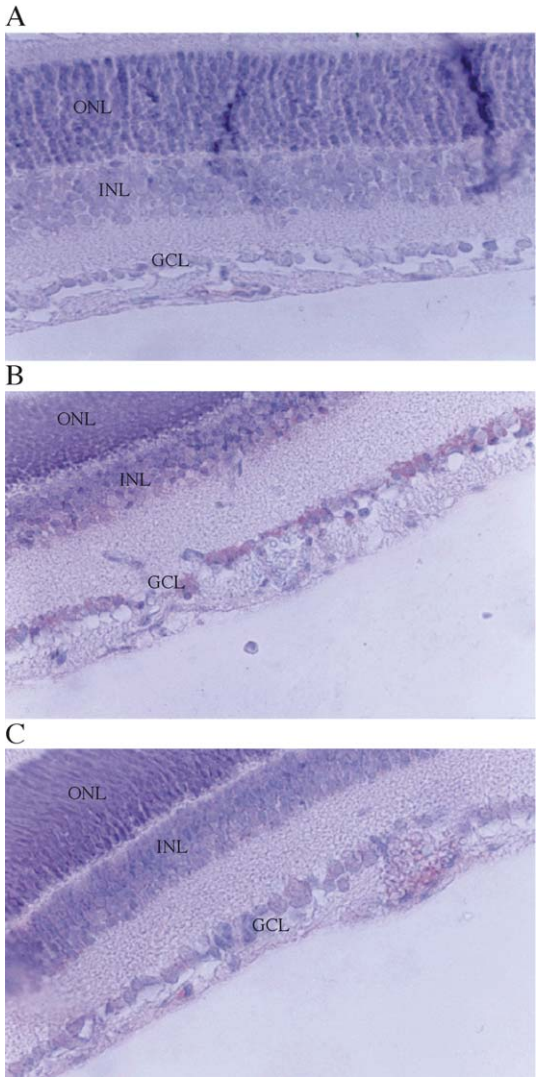

Fig. 5. Representative rat retinal sections immunostained for VEGF. (A) Normal. (B) Diabetic. (C) CaD-treated diabetic. Strong brown staining was seen in the ganglion cell layer (GCL) and in the inner nuclear layer (INL) of diabetic rats. Reduced staining was observed in CaD-treated diabetic rats. ONL, outer nuclear layer. (Magnification of panels A, B, and C: 200×).

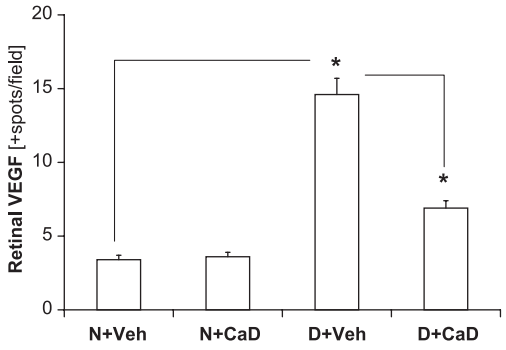

Fig. 6. Retinal VEGF protein expression in diabetic or normal rats treated for 10 days with CaD (100 mg/kg/daily, PO) or vehicle. N, normal; D, diabetic; VEH, vehicle; CaD, calcium dobesilate. Data are expressed as means ± S.E.M. ($n = 5$). *$P < 0.0083$ (nonparametric Mann–Whitney rank sum test).

expression of diabetic rats (i.e., a residual twofold increase with respect to control rats for both layers).

4. Discussion

Oral administration of calcium dobesilate to rats with streptozotocin-induced diabetic retinopathy markedly reduced retinal hyperpermeability, in association with major and significant reductions in retinal AGE contents and VEGF protein overexpression. This confirms in streptozotocin-diabetic rats previous observations in patients with diabetic retinopathy showing that calcium dobesilate stabilizes blood–retinal barrier (Nemeth et al., 1975; Grignolo et al., 1979; Leite et al., 1990; Vojnikovic, 1991).

Abnormal AGE accumulation is strongly linked to diabetes-associated oxidative stress, catalyzing hyperglycemia-accelerated sugar reactions with amino groups of proteins (Maillard reactions; see Brownlee, 2000), and evidence has been accumulated suggesting a role for AGEs in the development of diabetic vascular complications (Yamamoto et al., 2003). Here we investigated carboxymethyl-lysin(CML)-protein adduct as a marker of AGEs. CML is an AGE formed on protein by combined nonenzymatic glycation and oxidation (glycoxidation) and lipid peroxidation reactions (Fu et al., 1996).

Calcium dobesilate did not modify plasma glucose neither in diabetic nor in control rats (see Table 1). Therefore, the important reversal of retinal CML-AGE by calcium dobesilate (− 62%) could be likely related to its antioxidant properties. This view is further supported by the previous observation that calcium dobesilate reduces membrane lipid peroxidation produced in vitro by oxygen free radicals (Brunet et al., 1998a). Further studies are required to investigate if calcium dobesilate reduces retinal lipid peroxidation and/or protein glycoxidation in the diabetic rat retina.

In retinal endothelial cells, AGEs increase VEGF expression through generation of oxidative stress and downstream activation of the protein kinase C pathway (Mamputu and Renier, 2002). Increased levels of both CML-AGE and VEGF were found in the aqueous humor of patients with diabetic retinopathy (Endo et al., 2001) and Urata et al. (2002) found that the CML-bovine serum albumin adduct induced VEGF overexpression in the RAW264.7 macrophage cell line (see also Inagi et al., 1999). Therefore, the important reduction of CML-AGE accumulation by calcium dobesilate (− 62%) can be involved in the associated reduction of VEGF overexpression (− 69.4%).

No significant differences were found here in plasmatic 165-VEGF levels between groups. Similarly, Malamitsi-Puchner et al. (1998) found no significant differences in serum VEGF levels between diabetic and nondiabetic subjects (see also Shinoda et al., 1999).

It is important to stress that our plasmatic VEGF values (Table 1) were 10 times lower as compared with the aqueous VEGF values obtained by Shinoda et al. (1999). Thus, (i) VEGF protein overexpression in the short-term diabetic retina is not due to the VEGF entry from plasma, and (ii) it appears that the local control of VEGF levels (e.g., retinal vs. plasma) is important to maintain a nonpathogenic status.

Our results compare well with those of Obrosova et al. (2001), showing that the antioxidants taurine and DL-alpha-lipoic acid significantly reduced the early up-regulation of retinal VEGF in streptozotocin-induced rat diabetes. Conversely, Hammes et al. (1997) showed that nicarnitine was without significant effect on retinal AGE but significantly reduced pericyte loss. Such differences could reflect different scavenging potency, profile, and/or tissue distribution of the antioxidant studied or differences in the animal models of diabetic retinopathy investigated.

The sequence of physiopathological events going from hyperglycemia to disruption of the blood–retinal barrier in streptozotocin-rat diabetic retinopathy is still a matter of investigation. Although no attempt was done here to investigate the full mechanism by which calcium dobesilate reverses retinal vascular hyperpermeability, it is interesting to mention that:

- In vivo blockade of receptor for AGE (RAGE) with solubilized RAGE suppresses vascular hyperpermeability in diabetic rats (Bonnardel-Phu et al., 1999; Kislinger et al., 1999).
- VEGF is a potent permeabilizing factor (four to five orders of magnitude more potent than histamine) which promotes the extravasation of plasma proteins, including albumin. Overexpression of retinal VEGF appears to be a causal agent in preproliferative diabetic retinopathy (Gilbert et al., 1998; Ishibashi, 2000; Sone et al., 1997), contributing to blood–retinal barrier disruption (Murata et al., 1996; Mathews et al., 1997; Treins et al., 2001; Yamagishi et al., 2002) and to progression toward proliferative retinopathy, i.e., retinal neovascularization.

Thus, it seems likely that the increased VEGF levels observed here in the diabetic rat retina contributed to the increase in retinal albumin leakage and that the partial reversal of retinal hyperpermeability by calcium dobesilate could be secondary to the partial reversal of VEGF overexpression, via an antioxidant anti-AGE action.

It has been reported that oxidant stress can induce apoptosis and pericyte loss, an earliest histopathological hallmark of diabetic retinopathy, and antioxidant treatment reduced retinal pericyte loss in diabetic rats (Ansari et al., 1998; Kowluru and Koppolu, 2002; Romeo et al., 2002). Moreover, Losa et al. (1999) have found that calcium dobesilate prevents oxidation and apoptosis in human peripheral blood mononuclear cells.

In conclusion, the oral administration of calcium dobesilate (100 mg/kg) for 10 days significantly and profoundly reversed albumin leakage in the retina from rats with streptozotocin-induced diabetes. This was associated with an important and significant reversal of both CML-AGE formation and VEGF protein expression, strongly suggesting an in situ antioxidant action. Further investigation is required to see if calcium dobesilate is also able to reverse other physiopathological disorders of diabetic retinopathy (pericyte loss, membrane lipid peroxidation) and whether a similar antioxidant mechanism explains blood–retinal barrier stabilization by calcium dobesilate in patients with diabetic retinopathy.

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