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Antioxidant properties of calcium dobesilate in ischemic/reperfused diabetic rat retina

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Abstract

Calcium dobesilate possesses antioxidant properties and protects against capillary permeability by reactive oxygen species in the rat peritoneal cavity, but whether a similar action can take place in the diabetic rat retina is unknown. We investigated the oral treatment of diabetic rats with calcium dobesilate on the prevention of free radical-mediated retinal injury induced by ischemia/reperfusion (90 min ischemia followed by 3 min and/or 24 h of reperfusion). Streptozotocin-induced diabetic rats were orally treated with 50 and 100 mg/kg of calcium dobesilate for 10 days ($n = 12$ in each group). In the first series of studies, calcium dobesilate was found to significantly reduce the maldistribution of ion content in diabetic ischemic/reperfused rat retina. Thus, in diabetic rats treated with 100 mg/kg/day calcium dobesilate, ischemia/reperfusion provoked: (i) 27.5% increase in retinal Na^+ content compared to 51.8% in the vehicle-treated group ($P < 0.05$), and (ii) 59.6% increase in retinal Ca^{2+} content compared to 107.1% in vehicle-treated animals ($P < 0.05$). In the second series of studies, calcium dobesilate was found to significantly protect diabetic rat retina against inhibition of Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities by ischemia/reperfusion (54% and 41% reduction, respectively, with 100 mg/kg of calcium dobesilate) and also against changes in retinal ATP, reduced glutathione (GSH), and oxidized glutathione (GSSG) contents. In the third series of experiments, rats treated with 100 mg/kg of calcium dobesilate reduced the hydroxyl radical signal intensity to 41% (measured by electron paramagnetic resonance), induced by ischemia/reperfusion in diabetic rat retina. Finally, 100 mg/kg calcium dobesilate significantly reduced retinal edema (measured by the thickness of the inner plexiform layer) in diabetic rats. In conclusion, oral treatment with calcium dobesilate significantly protected diabetic rat retina against oxidative stress induced by ischemia/reperfusion. Whether the antioxidant properties of calcium dobesilate explain, at least in part, its beneficial therapeutic effects in diabetic retinopathy deserves further investigation.

Keywords: Calcium dobesilate; Diabetic retinopathy; Na^+; K^+; Ca^{2+}; Antioxidant; Retina; Ischemia; Reperfusion, (Rat)

1. Introduction

Calcium dobesilate stabilises blood–retinal barrier permeability in diabetic retinopathy, but its mechanism of action is poorly understood (Leite et al., 1990; Vojnikovic, 1991; Van Bijsterveld and Janssen, 1981; Beyer et al., 1980). It has been recently shown that calcium dobesilate possesses antioxidant properties in vitro (Brunet et al., 1998a) and protects against capillary permeability by reactive oxygen species in the rat peritoneal cavity (Brunet et al., 1998b). On the other hand, there is evidence to indicate that hyperglycemia may induce diabetic microangiopathy through the generation of oxidative stress and glycoxidation (for recent reviews see Watts and Playford, 1998; Baynes and Thorpe, 1999). In diabetic retinopathy, several authors reported evidence of oxidative stress (Abu el-Asrar et al., 1995; Losada and Alio, 1996; Sano et al., 1998; Verdejo et al., 1999). Moreover, antioxidant therapy with trolox (Ansari et al., 1998) or nicanartine (Hammes et al., 1997) reduces pericyte loss and other abnormalities in diabetic rat retina, and vitamin E normalizes retinal blood

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flow in humans with insulin-dependent diabetes (Bursell et al., 1998).

We have previously shown in diabetic rat retina that ischemia and reperfusion aggravates biochemical changes resulting from oxidative stress, particularly an increase in retinal Na^+ and Ca^{2+} contents (Szabo et al., 1991, 1995, 1997). Then, we decided to investigate whether oral treatment of diabetic rats with calcium dobesilate was able to prevent the maldistribution of retinal ion contents induced by ischemia/reperfusion. The finding of a protective effect by calcium dobesilate led us to investigate further protective actions on: (i) retinal Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities and (ii) retinal ATP, reduced glutathione (GSH) and oxidized glutathione (GSSG) contents. Finally, the free radical scavenging potential of calcium dobesilate in diabetic rat retina was studied by electron paramagnetic resonance, together with its potential protective action against edema formation measured by the thickness of the inner plexiform layer in the retina.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats weighing 300–320 g were used for all studies. All animals received care in compliance with the principles of laboratory animal care formulated by the National Society for Medical Research and the guide for the care and use of laboratory animals prepared by the National Academy of Sciences and published by the National Institute of Health (publication No. 85–23, revised 1985).

2.2. Induction of diabetes

Rats were injected intraperitoneally with a single dose of streptozotocin (45 mg/kg) and non-diabetic age-matched control rats concurrently received citrate buffer (Tosaki and Das, 1997). Then, animals were fed a regular rat chow and drank water ad libitum for 8 weeks. Diabetic rats manifested glucosuria, polydipsia and polyphagia. The development of diabetes was confirmed by measuring plasma glucose levels at the time of sacrifice. In some rats (about 15%), the induction of diabetes with streptozotocin was not successful. These rats were immediately replaced by other diabetic rats. Ischemia/reperfusion studies were performed with diabetic rats, 8 weeks after the induction of diabetes.

2.3. Ischemia and reperfusion

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and the central retinal artery was occluded as described in detail elsewhere (Szabo et al., 1992). The pupils were maximally dilated with some drops of atropine sulfate (1%) and the lids were retracted by sutures. At the onset of each experiment, peritomy was performed and a traction-type occluder consisting of a silk suture threaded through a polyethylene guide cannula was used for retinal artery occlusion. The suture was positioned behind the globe loosely around the optic nerve, central retinal artery, ciliary arteries, and the retrobulbar connective tissue using an operating microscope. Regional ischemia could be induced at any time by pulling the suture while pressing the tube against the surface of the optic nerve. The ischemia could then be maintained for any desired period by clamping the tube and the suture. After the designated period of ischemia, the occluder was released and the eye was reperfused. The successful induction of ischemia and the adequacy of reperfusion were confirmed visually by an ophthalmoscope.

2.4. Treatment protocols

Calcium dobesilate or vehicle oral treatments were initiated 10 days before the ischemia/reperfusion studies. Four series of experiments were carried out, as follows.

2.4.1. Effects of calcium dobesilate on ion shifts induced by ischemia / reperfusion in diabetic rat retina

Thirty-six diabetic rats were divided into three groups ($n = 12$ per group): (i) animals receiving vehicle p.o. (sodium chloride 0.9% in 5% of arabic gum) for 10 days, (ii) rats receiving 50 mg/kg/day calcium dobesilate p.o. for 10 days, and (iii) rats receiving 100 mg/kg/day calcium dobesilate p.o. for 10 days. A group of 12 non-diabetic age-matched rats receiving vehicle for 10 days was used for comparison. Twenty-four hours after the last treatment, eyes were subjected to 90 min of regional ischemia followed by 24 h of reperfusion. Such ischemic/reperfusion periods were selected according to our previous publications (Szabo et al., 1995, 1997). Then, animals were anesthetized and retinal ion contents were measured by atomic absorption spectrophotometry according to the method described below (Section 2.5).

2.4.2. Effects of calcium dobesilate on the disturbances in Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities and in ATP, GSH and GSSG contents induced by ischemia / reperfusion in diabetic rat retina

Eighty-four diabetic rats were divided into seven treatment groups ($n = 12$ per group): (i) animals receiving vehicle p.o. (sodium chloride 0.9% in 5% of arabic gum) for 10 days, (ii) rats receiving vehicle p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 3 min of reperfusion, (iii) rats receiving vehicle p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 24 h of reperfusion, (iv) rats receiving 50 mg/kg/day of calcium dobesilate p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 3

min of reperfusion, (v) rats receiving 50 mg/kg/day of calcium dobesilate p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 24 h of reperfusion, (vi) rats receiving 100 mg/kg/day calcium dobesilate p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 3 min of reperfusion and (vii) rats receiving 100 mg/kg/day calcium dobesilate p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 24 h of reperfusion. A group of 12 non-diabetic rats receiving vehicle for 10 days was included for comparison as the age-matched control group. At the end of the reperfusion period, animals were anesthetized and retinal Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities and retinal ATP, GSH and GSSG contents were measured according to the protocols described below (Sections 2.6–2.8).

2.4.3. Effects of calcium dobesilate on the free radical generation induced by ischemia / reperfusion in diabetic rat retina

Forty-eight diabetic rats and twenty-four non-diabetic rats were divided into six groups ($n = 12$ per group): (i) non-diabetic animals receiving vehicle p.o. (sodium chloride 0.9% in 5% of arabic gum) for 10 days, (ii) non-diabetic rats receiving vehicle p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 3 min of reperfusion, (iii) diabetic rats receiving vehicle p.o. (sodium chloride 0.9% in 5% of arabic gum) for 10 days, (iv) diabetic rats receiving vehicle p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 3 min of reperfusion, (v) rats receiving 50 mg/kg/day calcium dobesilate p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 3 min of reperfusion and (vi) rats receiving 100 mg/kg/day calcium dobesilate p.o. for 10 days and then subjected to 90 min of retinal ischemia followed by 3 min of reperfusion. The ischemic period and reperfusion time points were selected according to our previous publications (Szabo et al., 1995, 1997). At the end of the reperfusion period, animals were reanesthetized, eyes were examined by operating microscope and ophthalmoscope and free radical production was measured by electron paramagnetic resonance according to the protocol described below (Section 2.9).

2.4.4. Effects of calcium dobesilate on the histology of retina induced by ischemia / reperfusion in diabetic rat retina

Non-diabetic and diabetic retinas were subjected to ischemia/reperfusion in the presence or absence of calcium dobesilate. Fixation of retina for histology is described below (Sections 2.10 and 2.11).

2.5. Measurement of retinal Na^+, K^+, Ca^{2+} and Mg^{2+} contents

Animals were anesthetized with pentobarbital (60 mg/kg i.p.), the chest was opened, and a perfusion cannula was inserted through the heart into the aorta. The right ventricle of the heart was opened and the blood was allowed to escape. Cold (6–11 °C) Na^+, K^+, Mg^{2+} and Ca^{2+}-free solution containing 100 mM of trishydroxymethylamino-methane and 220 mM of sucrose (pH adjusted to 7.4 by HCl, pO_2 and osmolality were 0–4 kPa and 300–330 mosM/g, respectively) was used to wash out ions and blood from the vasculature and extracellular space and to stop or at least reduce the activity of membrane enzymes responsible for the membrane ion transport. Two hundred milliliters of cold buffer washed out $> 90\%$ of the ions from the extracellular space (Tosaki et al., 1988). As described previously (Szabo et al., 1995), a short (30 s) washing out period was optimal since a prolonged cold perfusion may cause artifacts and destruction of retinal tissue. Immediately after the washing out period, the eye was enucleated and rapidly opened, and retinal tissue was progressively detached from the retinal epithelium and finally dissociated by cutting from the optic nerve (Doly et al., 1984). Retinal tissue was dried for 48 h at 100 °C and then placed at 550 °C for 24 h to make ash. The ash was dissolved in 0.2 ml of 3 M nitric acid and diluted 10-fold with deionized water. Tissue Na^+ was measured at a wavelength of 330.3 nm, K^+ at 404.4 nm, Mg^{2+} at 286 nm, and Ca^{2+} at 422.7 nm in an air acetylene flame by Perkin-Elmer atomic absorption spectrophotometer. The washout perfusion method and the determination of tissue ion contents have been described previously in different tissue (Tosaki et al., 1988). Since a small amount of extracellular ions can contaminate the samples after washing out the extracellular space (Alto and Dhalla, 1979), the values obtained in our studies are termed retinal rather than intracellular ion contents.

2.6. Measurement of retinal Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities

In nonischemic and ischemic/reperfused eyes, retinal tissue was progressively detached from the epithelium and dissociated by cutting from the optic nerve (Doly et al., 1984). Retina was homogenized in 200 mM sucrose medium containing 20 mM Tris–HCl (pH = 7.5) and 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at $1000 \times g$ for 5 min in order to remove cell debris, and the resulting homogenate was used for assay. Homogenate was subjected to three cycles of freezing (dry ice) and thawing (at 37 °C) to permeabilize membranes. Na^+/K^+-ATPase activity (ouabain sensitive) was measured as described previously (Kowluru et al., 1989) with some minor modifications. The assay mixture (50 μl final volume) contained 10 mM HEPES buffer (pH 7.4), 50 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 50 mM each of EDTA and EGTA, 1 mM [γ-^{32}P]ATP (20,000–30,000 cpm/nmol), and retinal homogenate with 0.5 mM ouabain. Ca^{2+}/Mg^{2+}-ATPase activity was measured using the same reaction mixture (final volume, 50 μl) containing ouabain

and 90 mM calcium chloride (Chambers et al., 1990). Free Ca^{2+} concentration was calculated according to Fabiato (1988) and constants were determined at 25 °C, in our triplicate samples routinely using approximately 3–6 mg retinal protein. The reaction was initiated by the addition of the ATP, and the mixture was incubated at 37 °C for 10 min. Hydrolysis of ATP was linear for both Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase for up to 20 min and 15 mg of protein. Nucleotides were separated from $^{32}P_i$ using activated charcoal (Luthra et al., 1976). Na^+/K^+-ATPase activity was calculated as the difference between the activities in the presence and absence of ouabain. Ca^{2+}/Mg^{2+}-ATPase activity was defined by the difference in activities obtained in the presence and absence of calcium chloride.

2.7. Measurement of ATP contents

Retinal ATP contents were measured by using a bioluminescent assay kit from Sigma (605-10S Hitachi spectrophotometer, Japan) and, for comparison, by using an enzymatic method (kit 366 UV, Sigma) measuring NADH oxidation by glyceraldehyde phosphate dehydrogenase.

2.8. Measurement of reduced and oxidized glutathione contents

Reduced glutathione (GSH) and oxidized glutathione (GSSG) contents were measured fluorimetrically using *o*-phthalaldehyde. Eyes were enucleated at fixed time-points (before ischemia, after ischemia, and after 3 min, and 24 h of reperfusion), and GSH and GSSG contents were measured in the retina. Fresh retina was sonicated in 25% (wt./vol.) phosphoric acid containing 2 mM EDTA. Homogenate was centrifuged at $40{,}000 \times g$ for 20 min, and the supernatant was used for measuring GSH and GSSG. Protein content was measured in pellet dissolved in 0.1 N of NaOH. GSH was assayed with *o*-phthalaldehyde (in a total volume of 1.3 ml), as previously described by Mokrasch and Teschke (1984) after 5 min preincubation of the samples with formaldehyde. Fluorescence was measured at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. GSSG was also measured in 1.3 ml final volume, and the reaction was run at pH 12.0–13.0. GSSG samples were pretreated with *N*-ethylmaleimide for 30 min in the presence of 2-vinylpyridine in order to mask GSH content of the samples (Teare et al., 1993).

2.9. Electron paramagnetic resonance studies

Eyes were removed and the retina was quickly detached (3–4 s) as described by Doly et al. (1984) and put in liquid nitrogen before the induction of ischemia and after 90 min ischemia followed by 3 min of reperfusion. The frozen retina, under liquid nitrogen, was then pulverized in order to get a fine powder, and the powder was transferred to a precision electron paramagnetic resonance (EPR) tube. The EPR tube had an inside diameter of 3 mm and was filled to a height of 3 cm. EPR spectra were then recorded at a temperature of 77 °K using a Brucker CXP-200 wide-bore spectrometer (Bruker Medical Instruments, Billerica, MA, USA). Quantification of the signals was carried out by comparison of the integrated signal area with that of the commonly used free radical standard potassium peroxylamine disulfonate in frozen aqueous solution in identical EPR tubes (Zweier et al., 1987). In the EPR studies, samples were placed in a microwave-resonant cavity and irradiated with microwave frequency ($\nu = 9.278GH$). The cavity is placed between the pole pieces of a magnet. The magnetic field, H, is varied, and the absorption of microwave power as a function of magnetic field is recorded. Resonance is defined by the following: $g = h/\beta \times \nu$ (MHz)$/H(G)$, where h/β is a constant with a value of 0.714484, and g is a characteristic constant whose value serves to identify any given free radical. The usual convention in EPR spectroscopy is to show the first derivative absorption function. For a free radical in which the electron is in an anisotropic environment with axial symmetry the spectrum is defined by two g values ($g_{\|} = 2.033$; $g_{+} = 2.005$). The magnitude of the splitting is defined by the hyperfine coupling constant $a_n = 24G$ ($1G = 0.1$ mT). Thus, EPR spectroscopy can provide information about the chemical structure of free radicals, in particular, the symmetry of the electron environment and the presence of nuclei in the vicinity of the electron (Zweier et al., 1987; Van der Kraaij et al., 1989). The half-life of oxygen free radicals is between 10^{-9} and 10^{-12} s, the ischemic reperfused tissue intensively produces free radicals during the first few minutes of ischemia (Tosaki et al., 1990); therefore, the procedure regarding the detachment of retina (a few seconds) does not substantially influence free radical signal intensity in retinal tissue.

2.10. Fixation of eyes and retina for histology

For fixation of the eye, a cannula was introduced through the heart into the aorta, and the right ventricle of the heart was opened in order to allow the blood to escape. For washing out the blood prior to fixation, a buffered solution of 0.9% NaCl (100 ml) was used. A short (approximately 20 s) washing out period was optimal since prolonged perfusion prior to fixation may cause artifacts in the nervous tissue. One hundred milliliters of the fixative solution (Bouin's solutions) followed immediately without any interruption of the NaCl perfusion. Then the eye was enucleated and rapidly cut open and divided into two halves by coronal section through the ora serrata. The vitreous was removed and the eye was immersed into the fixative solution (Bouin's fluid). After this postfixation,

the tissue was dehydrated in graded series of ethanol and embedded in paraffin. Sagittal sections of 7 μm were cut and stained with hematoxylin-eosin.

2.11. Quantification of histology

Ischemia- and reperfusion-induced cell swelling is well-recognized and documented in the inner plexiform layer of the retina (Shakib and Ashton, 1966). The average of the retina's thickness for each eye was measured in the sagittal section near the optic nerve, and expressed in micrometers using a video-plan computer analyzer (IMSTAR, Paris, France), as we previously described (Szabo et al., 1991). The migration of neutrophils was observed after the 24-h reperfusion period, and this phenomenon was classified as the presence or absence of neutrophils.

2.12. Statistics

Values are given as mean ± S.E.M. Differences in mean values between groups were first tested for statistical significance by using a one-way analysis of variance (ANOVA). In case of significant differences, values of the drug-treated diabetic ischemic groups were compared with those of the untreated diabetic ischemic group by means of the modified *t*-test (Wallenstein et al., 1980). An analogous procedure was followed for binomially distributed variables (e.g., the presence or absence of neutrophils). To compare individual groups, an overall chi-square test for $2 \times n$ table was constructed, followed by a sequence of 2×2 chi-square tests. If $P < 0.05$, the values were considered to differ significantly.

3. Results

3.1. Retinal ion contents

The effect of calcium dobesilate was tested in diabetic rats (at 50 or 100 mg/kg/day p.o. during 10 days) for its protective action against the retinal ion shifts induced by 90 min of regional ischemia, followed by 24 h of reperfusion. Diabetic and non-diabetic age-matched untreated rats received vehicle as drug-free controls for comparison.

Table 1 shows basal values of retinal Na^+, K^+, Ca^{2+} and Mg^{2+} contents in the four groups of animals ($n = 12$ per group). It can be seen that the diabetic state was associated to modest, but significant changes in retinal ion contents, particularly in sodium and calcium ions, i.e., (i) retinal Na^+ content was significantly increased by 34%, (ii) K^+ content was significantly decreased by 9%, (iii) Ca^{2+} content was significantly increased by 33%, and (iv) Mg^{2+} content was decreased by 16%. Treatment with calcium dobesilate tended to prevent such ion content disturbances, particularly on Na^+ and Ca^{2+} contents, which remained in the normal range at the dose of 100 mg/kg/day (Table 1).

Fig. 1 shows Na^+, K^+ and Ca^{2+} contents after ischemia/reperfusion, in the three groups of diabetic rats (non-diabetic animals and Mg^{2+} contents are not given for simplicity). Comparisons with the values in Table 1 show that ischemia/reperfusion magnified the severity of the ion shifts of diabetic retina, i.e., (i) Na^+ content increased by 52%, (ii) K^+ content decreased by 24% and (iii) Ca^{2+} content increased by 107%. Moreover, Fig. 1 shows that treatment with calcium dobesilate tended to prevent such ion changes, particularly those concerning Na^+ and Ca^{2+} contents (at the dose of 100 mg/kg/day, Fig. 1). It is important to note that such significant differences were not due to the lower basal values in calcium dobesilate-treated rats (Table 1), since differences between groups remained statistically significant when expressed as a percentage change with respect to basal values. Thus, in calcium dobesilate-treated rats (100 mg/kg/day) ischemia/reperfusion provoked: (i) a 27.5% increase in Na^+ content against 51.8% in vehicle-treated animals ($P < 0.05$) and (ii) a 59.6% increase in Ca^{2+} content against 107.1% in vehicle-treated animals ($P < 0.05$).

3.2. Na^+/K^+-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities. ATP, GSH and GSSG contents

In a second series of experiments, calcium dobesilate was tested in diabetic rats (at 50 or 100 mg/kg/day p.o.

Table 1
Influence of diabetes and calcium dobesilate p.o. on basic retinal ion contents in rat

Ion species (μmol/g, dry weight)	Non-diabetic ($n = 12$)	Diabetic		
		Vehicle ($n = 12$)	Calcium dobesilate, 50 mg/kg/day ($n = 12$)	Calcium dobesilate, 100 mg/kg/day ($n = 12$)
Na^+	53.7 ± 3.1	71.8 ± 2.5[a]	69.5 ± 2.4	56.7 ± 2.5[b]
K^+	299.4 ± 4.3	273.1 ± 3.0[a]	274.8 ± 3.6	286.7 ± 3.2[b]
Ca^{2+}	2.03 ± 0.13	2.69 ± 0.16[a]	2.47 ± 0.16	1.99 ± 0.12[b]
Mg^{2+}	18.9 ± 0.7	15.8 ± 0.9[a]	16.3 ± 1.0	15.4 ± 0.9

[a] $P < 0.05$ compared diabetic and non-diabetic values.
[b] $P < 0.05$ compared treatment of diabetic rats with calcium dobesilate and vehicle. Values presented are mean ± S.E.M.

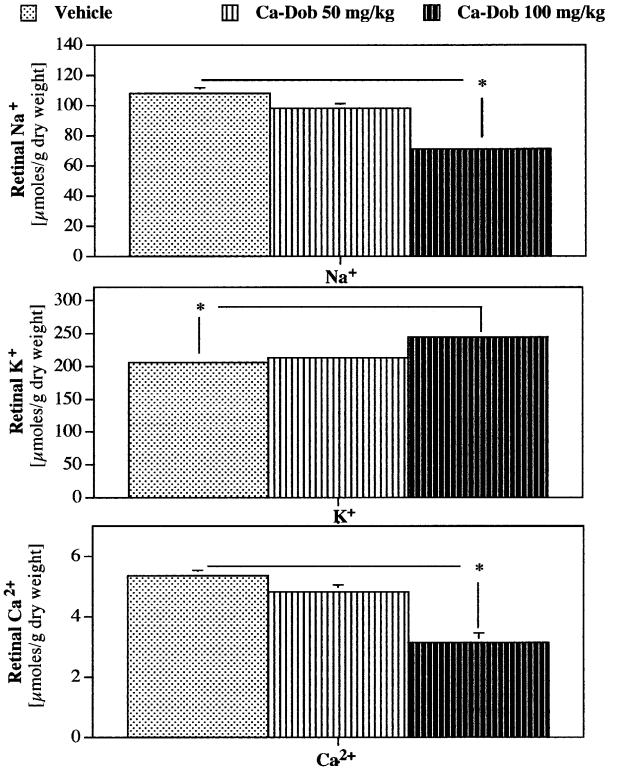

Fig. 1. Protection by calcium dobesilate p.o. against changes in ion contents induced by ischemia/reperfusion in diabetic rat retina. Values represent ion contents after ischemia, followed by 24 h of reperfusion. Protection was statistically significant at 100 mg/kg of calcium dobesilate p.o. Values are given as mean ± S.E.M. ($n = 12$ per group). (*) indicates $P < 0.05$.

during 10 days) for its protective action against the changes in retinal Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPases induced by 90 min of regional ischemia followed by 3 min or 24 h of reperfusion ($n = 12$ per group). Retinal ATP, GSH and GSSG contents were parallelly measured. Diabetic and non-diabetic rats receiving vehicle were used for comparison as drug-free controls.

Table 2 shows the basic values of retinal Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities in diabetic and non-diabetic rats. It can be seen that diabetes was associated to a modest, but significant decrease in Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities. Fig. 2 shows that ischemia/reperfusion (in retina from vehicle-treated diabetic rats) magnified the severity of such ATPase disturbances. Thus, (i) retinal Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities were reduced by 48% and 57% after 3 min of reperfusion, respectively, and (ii) the severity of such changes tended to decrease after 24 h (all changes were statistically significant). Moreover, Fig. 2 shows that oral treatment of diabetic rats with calcium dobesilate significantly protected the retina against membrane ATPase disturbances. Thus, after 90 min ischemia, followed by 3 min of reperfusion, retina from rats treated with calcium dobesilate had significantly higher Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities (this latter change was only significant at the dose of 100 mg/kg of calcium dobesilate, Fig. 2). These protective actions of calcium dobesilate on Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities were present, even after 24 h of reperfusion (Fig. 2).

Table 2 shows the drug-free values of retinal ATP, GSH and GSSG contents in diabetic and non-diabetic rats. Diabetes per se induced significant changes in retinal contents of GSH and GSSG (retinal ATP contents were slightly but not significantly decreased), indicating the development of serious retinal damage. Thus, retina from 8-week diabetic rats showed a 27% decrease in retinal GSH content, together with a 20% increase in GSSG content (Table 2). Fig. 3 shows that ischemia/reperfusion (in retina from vehicle-treated diabetic rats) magnified the severity of such disturbances. Thus, (i) retinal ATP content

Table 2
Influence of diabetes on basic values of Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities and ATP, GSH and GSSG contents in rat retina

Parameter	Non-diabetic ($n = 12$)	Diabetic ($n = 12$)
Na^+/K^+ ATPase activity (nmol Pi/min/mg prot)	103 ± 5	77 ± 4[a]
Ca^{2+}/Mg^{2+} ATPase activity (nmol Pi/min/mg prot)	30 ± 3	17 ± 2[a]
ATP content (nmol/mg prot)	1.8 ± 0.2	1.7 ± 0.2
GSH content (nmol/mg prot)	62 ± 4	45 ± 2[a]
GSSG content (nmol/mg prot)	6.1 ± 0.4	7.3 ± 0.8[a]

[a] $P < 0.05$ compared diabetic vs. non-diabetic values. Values presented are mean ± S.E.M.

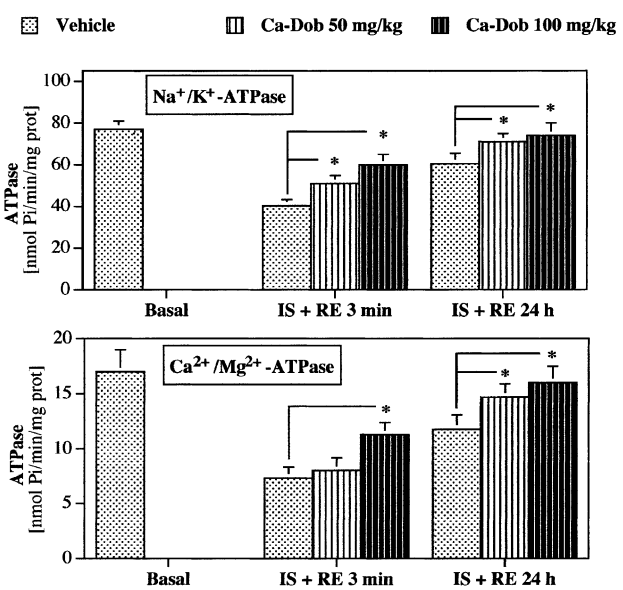

Fig. 2. Protection by calcium dobesilate p.o. against changes in Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities induced by ischemia/reperfusion in diabetic rat retina. Protection was seen at 100 mg/kg of calcium dobesilate p.o. Values are given as mean ± S.E.M. ($n = 12$ per group). (*) indicates $P < 0.05$. Ischemia + reperfusion (IS + RE).

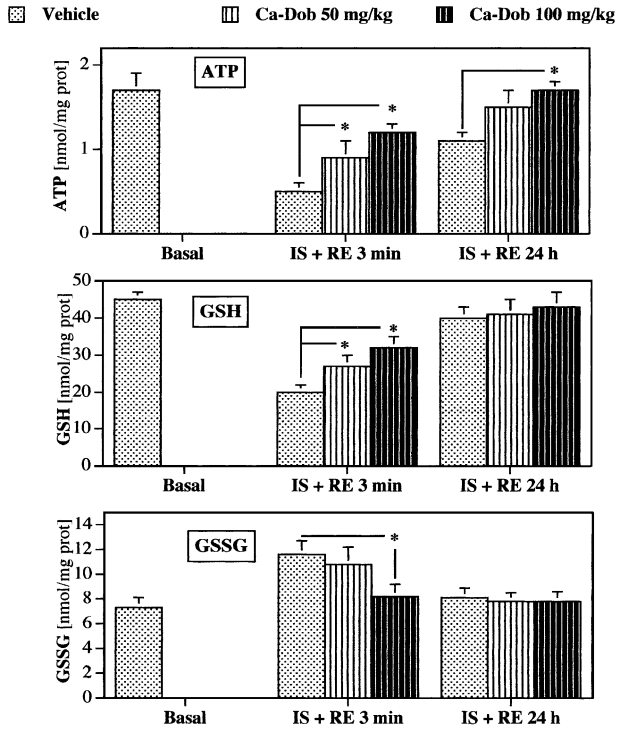

Fig. 3. Protection by calcium dobesilate p.o. against changes in ATP, GSH and GSSG contents induced by ischemia/reperfusion in diabetic rat retina. Important protection was observed: (i) after 3 min of reperfusion and (ii) at 100 mg/kg of calcium dobesilate p.o. Values are given as mean ± S.E.M. ($n = 12$ per group). (*) indicates $P < 0.05$. Ischemia + reperfusion (IS + RE).

was significantly reduced by 71% and 35% from its control (diabetic nonischemic) value after 3 min and after 24 h of reperfusion, respectively, (ii) GSH content was significantly reduced by 56% after 3 min of reperfusion (and by 11% after 24 h of reperfusion, this last change was not statistically significant), (iii) GSSG content was significantly increased by 59% after 3 min of reperfusion (and by 11% after 24 h of reperfusion, although this last change was not statistically significant). Oral treatment of diabetic rats with calcium dobesilate significantly protected against ATP, GSH and GSSG disturbances. Thus, Fig. 3 shows that after 90 min ischemia, followed by 3 min of reperfusion, retina from rats treated with calcium dobesilate had significantly higher retinal contents of ATP and GSH and lower content of GSSG (this last change was only significant at the dose of 100 mg/kg of calcium dobesilate).

3.3. Free radical production

Fig. 4 shows that the signal intensity of hydroxyl radicals in diabetic rat retina was significantly higher compared to the non-diabetic controls. On the other hand, it can be seen that ischemia, followed by 3 min reperfusion, induced a significant increase in signal intensity. Fig. 4 shows that oral treatment of diabetic rats with 100 mg/kg of calcium dobesilate protected the ischemic/reperfused retina against the above disturbances. A similar tendency was observed in retina from rats treated with 50 mg/kg of calcium dobesilate, but the differences in comparison with controls were not significant (Fig. 4). The lower panel of Fig. 4 shows representative spectra of control and ischemic/reperfused retinas treated with 50 and 100 mg/kg of calcium dobesilate. Calcium dobesilate treatment at 100 mg/kg resulted in a significant reduction

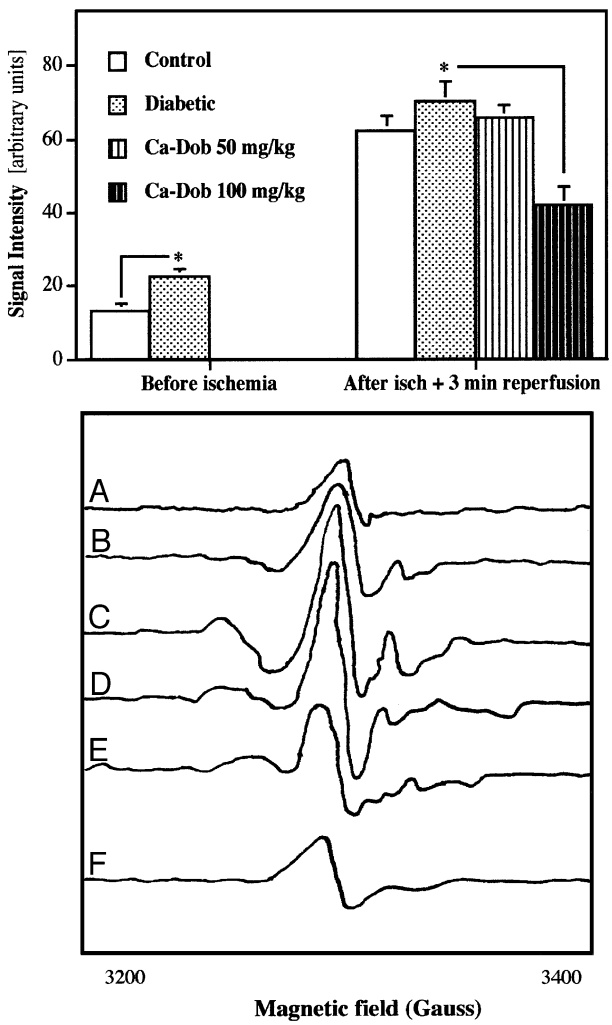

Fig. 4. Reduction by calcium dobesilate p.o. of hydroxyl radical formation induced by ischemia/reperfusion in diabetic rat retina. Ischemia, followed by 3 min reperfusion, induced a very high increase in signal intensity of hydroxyl radicals. Calcium dobesilate p.o. exerted a significant protection at 100 mg/kg. Note that the signal intensity of hydroxyl radicals in diabetic rat retina was significantly higher compared with non-diabetic controls. Values are given as mean ± S.E.M. ($n = 12$ per group). (*) indicates $P < 0.05$. The lower panel shows representative spectra obtained from nonischemic control (A) and diabetic control (B) retinas. Retinas were subjected to 90 min ischemia followed by 3 min of reperfusion in non-diabetic (C) and diabetic (D) eyes. Rats were treated with 50 mg/kg (E) and 100 mg/kg (F) calcium dobesilate (Ca-Dob), and retinas were subjected to 90 min ischemia followed by 3 min of reperfusion.

in the intensity of free radical production (Fig. 4, lower panel F).

3.4. Histology

In diabetic rat retina, ischemia/reperfusion induced histopathologic changes mostly limited to the inner part of the retina (only small alterations were detected in the photoreceptor layer using light microscope). In particular, retinal edema was well-recognized in the inner plexiform layer. Fig. 5(B) and (C) shows tissue edema in diabetic rat retina, before (B) and after a period of 90 min of ischemia, followed by 24 h of reperfusion (C). The inner nuclear layer showed more pyknotic nuclei, vacuolated spaces, and degenerative changes in the ganglion cells. Finally, diabetic retina showed migration of neutrophils from the vascular tissue (Fig. 5(B) and Fig. 5(C)). The thickness (μm) of the inner plexiform layer was measured and represented in Fig. 6. Thus, 8-week diabetic retina develops edema (column 2), and 90 min ischemia followed by 24 h of reperfusion further increased edema formation (Fig. 6, column 3).

In retina from diabetic rats orally treated with calcium dobesilate (100 mg/kg/day for 10 days), ischemia/reperfusion induced a significantly lower edema formation (Fig. 5(D) and Fig. 6, column 5). Conversely, calcium dobesilate treatment was without effect on neutrophil migration, even at 100 mg/kg (Fig. 5(D)).

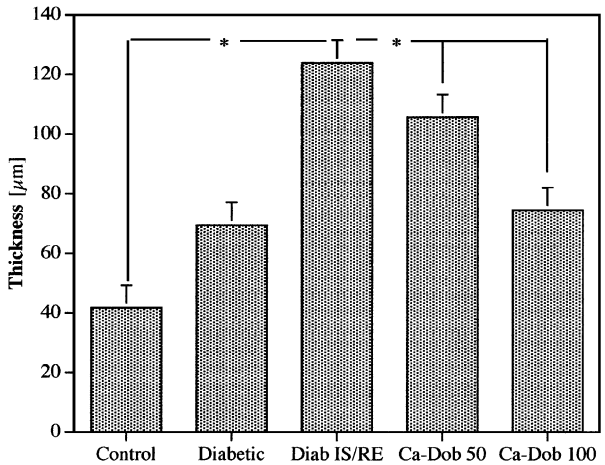

Fig. 6. Reduction by calcium dobesilate of the edema formation induced by ischemia/reperfusion in the inner plexiform layer. IS/RE: retina subjected to 90 min ischemia followed by 24 h reperfusion. Ca-Dob: dobesilate-treated diabetic rats submitted to IS/RE. Values are given as mean ± S.E.M. ($n = 12$ per group). (*) indicates $P < 0.05$.

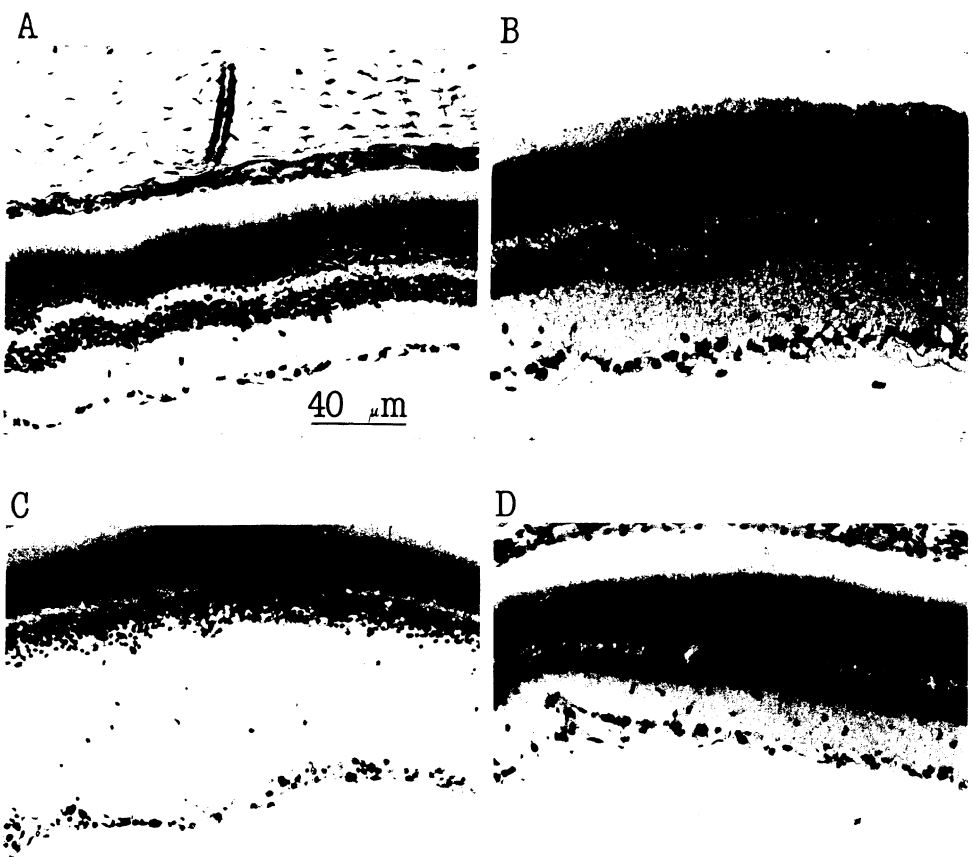

Fig. 5. Sagittal sections of rat retina showing layer structure of: non-diabetic nonischemic time-matched control (A), diabetic nonischemic control (B), diabetic retina subjected to 90 min ischemia followed by 24 h of reperfusion (C), and diabetic rats treated with 100 mg/kg of calcium dobesilate and subjected to 90 min ischemia, followed by 24 h of reperfusion (D). ns: neutrophils.

4. Discussion

Calcium dobesilate, orally given to diabetic rats, significantly protected against Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase inactivation and the associated ionic disturbances induced by ischemia/reperfusion in retinal tissues. These protective effects were dose-dependent, since ischemia/reperfusion induced less important disturbances in animals treated with 100 mg/kg of calcium dobesilate, as compared with diabetic rats treated with 50 mg/kg of calcium dobesilate. Moreover, electron paramagnetic resonance showed a reduction in hydroxyl radical production in ischemic/reperfused retina from treated animals; thus confirming the antioxidant action of calcium dobesilate (Brunet et al., 1998a,b).

To which extent our model of free radical generation by reperfusion of an ischemic retina is representative of diabetic retinopathy is unknown. Nevertheless, there is strong evidence that retinal blood flow is raised in the early stages of diabetes even before the onset of diabetic retinopathy (for recent review, see Schmetterer and Woltz, 1999), and Takahashi et al. (1998) have recently found reperfusion of occluded capillary beds in diabetic retinopathy. Moreover, Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities become insufficient in diabetes (Garner, 1993) and a further reduction in enzyme activity was observed here in diabetic retina subjected to an insult of ischemia/reperfusion. Therefore, our model of ischemia/reperfusion is perhaps enhancing a pathological process involved, at least in part, in the biochemical disturbances of diabetic retinopathy.

The loss of retinal ATPase activities in diabetes and ischemia is associated with the retinal GSH status. Glutathione in non-diabetic nonischemic cells is mainly in the reduced form, and GSH is known to be a potent antioxidant protecting SH groups in membrane proteins and enzymes including ATPases. With the decrease of retinal GSH concentration, the amount of GSSG is increased (Garner, 1993). In our study, a reduction in retinal GSH was associated with an increase of GSSG concentration in diabetes and ischemia/reperfusion, indicating an impairment of glutathione production and increased oxidative stress in the retina. A loss in GSH may contribute to the observed reduction in retinal Na^+/K^+- and Ca^{2+}/Mg^{2+}-ATPase activities. Calcium dobesilate prevented the loss of cellular GSH leading to the prevention, in part, of diabetes and ischemia/reperfusion-induced oxidative stress.

The protection observed in retinal Na^+/K^+-ATPase activities, ATP, GSH and ion contents reflected in the improvement of histology in the drug-treated groups. Although calcium dobesilate failed to significantly reduce the migration of neutrophils into the retinal tissue, cellular edema formation indicated by the thickness of the inner plexiform layer was significantly reduced in diabetic ischemic/reperfused retina treated with calcium dobesilate. This finding suggests that calcium dobesilate immediately eliminates free radicals at the site of the formation, produced by neutrophils leading to a minor membrane damage caused by these radical species. Thus, it is of interest to note that agents which are able to reduce the leukocyte migration could be protective against ischemia/reperfusion-induced injury because leukocytes contribute to the production of free radicals, increasing the damage in ischemic/reperfused retina (Stefansson et al., 1988).

In conclusion, oral treatment of diabetic rats with calcium dobesilate significantly protected against the oxygen free radical production and the metabolic and histological disturbances induced by ischemia/reperfusion in retinal tissue. Extrapolation of these results to humans should be done with caution because: (i) the development of diabetic retinal disease in streptozotocin-treated rats differs from that in humans, (ii) the speed of experimental reperfusion is much faster than in the human disease and (iii) in human retina, xanthine oxidase and other enzymes playing an important role in free radical formation, can be present at different levels in rats.

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