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Probucol restores the defective leukocyte–endothelial interaction in experimental diabetes

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Abstract

Defective leukocyte–endothelial interactions have been observed in experimental type 1 diabetes. One of the mechanisms involved in the late complications of diabetes mellitus is the formation of free radicals species. Antioxidant treatment has been demonstrated to have beneficial effects on the complications observed in this pathology. Using intravital microscopy to visualize venules of the internal spermatic fascia, we demonstrated that the defective leukocyte–endothelial interactions in alloxan-induced diabetic rats could be corrected by probucol treatment. The defects were quantitated by the number of leukocytes rolling along the venular endothelium, sticking to the venular wall after topical application of zymosan-activated plasma (10%–0.1 ml) or leukotriene B_4 (1 μM/0.1 ml) and migrated after the application of a local irritant stimulus (carrageenan, 100 μg/0.1 ml). Leukocyte counts, erythrocyte velocity and venular shear rate, unaltered in diabetic rats, were not modified by this treatment. Reactive oxygen species formation by endothelial cells increased in diabetic preparations, and the reduced expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and P-selectin in cross-sections of the whole testis of the animals, were both restored by the antioxidant agent. Therefore, antioxidant treatment improves leukocyte–endothelial interaction in diabetic rats at least in part by restoring the expression of adhesion molecules in venules of diabetic rats.

Keywords: Diabetes mellitus; Probucol; Leukocyte–endothelial interaction

1. Introduction

Enhanced oxidative stress and changes in antioxidant capacity observed in both clinical and experimental diabetes mellitus have been implicated in the etiology of chronic diabetic complications (Baynes, 1991; Strain, 1991; Thompson and Godin, 1995; Van Dam et al., 1995). Several studies have demonstrated the beneficial effects of probucol, an antioxidant, in the complications of diabetes, such as lipoprotein oxidation (Parthasarathy et al., 1986; Morel and Chisolm, 1989), impaired chemotaxis (Hara et al., 1992) and diabetic cardiomyopathy (Kaul et al., 1996). This agent scavenges superoxide (Bridges et al., 1991; Parthasarathy et al., 1986). In addition to its antioxidant properties, probucol has also been shown to enhance endogenous antioxidant enzyme activity (Siveski-Iliskovic et al., 1994, 1995).

Polymorphonuclear neutrophils are cells with multiple functions. They constitute an essential defense barrier of the organism. In vivo and in vitro studies have indicated that the events of inflammatory response such as rolling, adherence and migration are depressed in type 1 diabetes induced experimentally (Sannomiya et al., 1990, 1997; Fortes et al., 1991). We recently demonstrated that ascorbic acid supplementation restores the defective leukocyte–endothelial interaction in diabetic rats (Zanardo et al., 2003), indicating that antioxidant treatment might be beneficial in diabetes-induced alterations.

We designed the present study to investigate whether other antioxidant agents were also effective to reverse the defective leukocyte–endothelial interactions in diabetic rats. For this, we chose probucol, that is a lipid soluble instead of a hydro-soluble antioxidant and structurally different from ascorbic acid, another antioxidant. The mechanism(s) involved in the probucol effect was also explored.

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2. Methods

2.1. Animals

Male Wistar rats weighting between 150 and 180 g (age 8 weeks) were obtained from our breeding colony at the Institute of Biomedical Sciences, São Paulo, SP, Brazil.

2.1.1. Induction of diabetes mellitus

Diabetes mellitus was induced with an i.v. injection of alloxan (40 mg/kg) dissolved in physiological saline. Control rats were injected with saline alone. After administration of alloxan, the animals were allowed free access to food and water. Thirty days thereafter, the presence of diabetes was verified by blood glucose concentrations >11.2 mM measured with a blood glucose monitor (Biobras type TA80GL) in samples obtained from the cut tip of the tail according to Jarrett et al. (1970).

2.1.2. Animal preparation and treatment

Rats were randomized into four groups that were age- and weight-matched, with at least five animals per group. The groups consisted of the following: (i) alloxan-diabetic rats; (ii) non-diabetic controls; (iii) alloxan-diabetic rats treated with probucol; (iv) non-diabetic controls treated with probucol. In the treated group, probucol, as a highly lipid soluble compound, was suspended in saline with 2% Tween 80, and was administered by gavage at a daily dose of 20 or 200 mg/kg body weight. Diabetic and non-diabetic control groups received the same volume of saline alone, since it was demonstrated that Tween 80 administered by gavage does not have any effect in leukocyte–endothelial interactions (Cruz et al., 2000). Probucol treatment was initiated 30 days after diabetes induction and was administered for 12 days. The experimental protocols were approved and performed in accordance with the guidelines of the Ethics Committee of our Institute.

2.2. Total and differential leukocyte counts

Leukocyte counts were performed on blood samples collected at the time of killing. Total leukocytes were counted with a Neubauer chamber. Stained blood films were used for differential leukocyte counts (Kaplow, 1965). Blood samples for these measurements were collected from the tail while the rats were anesthetized.

2.3. Intravital microscopy

2.3.1. Surgical procedure

The animals were anesthetized with an i.p. injection of sodium pentobarbital (40 mg/kg). Additional anesthetic was given as required to maintain the same depth of anesthesia, usually not more than 10% of the required dose. The internal spermatic fascia of the wall of the scrotal chamber was exteriorized for microscopic examination in situ. This was done through a longitudinal incision of the skin and dartos muscle in the midline over the ventral aspect of the scrotum and opening of the cremaster muscle to expose the internal fascia. This procedure does not require extensive surgical manipulation for observation of the vascular network and provides a valuable means of transilluminating a tissue for quantitative studies of the microcirculation. In addition, respiratory movements of the animals do not affect the preparation, and its microcirculatory characteristics remain basically unchanged throughout the experiment. The animals were maintained on a special board thermostatically controlled at 37 °C, which included a transparent platform on which the tissue to be transilluminated was placed. The preparation was kept moist and warmed by irrigating the tissue with warmed (37 °C) Ringer Locke's solution, pH 7.2–7.4, containing 1% gelatin. The composition of the solution was (in mM): 154 NaCl; 5.6 KCl; 2 $CaCl_2$.2 H_2; 6 $NaHCO_3$ and 5 glucose. The rate of outflow of the solution onto the exposed tissue was controlled to keep the preparation in continuous contact with a film of the liquid. A 500-line television camera was incorporated onto a triocular Zeiss microscope to facilitate observation of the enlarged image (× 2500) on the video screen. Images were recorded on a video recorder with a × 40 long-distance objective with a 0.65 numerical aperture. An image-splitting micrometer was adjusted to the phototube of the microscope as described by Baez (1969). The image splitter sheared the optical image into two separate images and displaced one with respect to the other. By rotating the image splitter in the phototube, shearing was maintained in a direction at right angles to the axis of the vessel. The displacement of one image from the other allowed the measurement of vessel diameter. Vessels selected for study were third order venules, defined according to their branch order location within the microvascular network (Gore and Bohlen, 1977; Rhodin, 1980). These vessels corresponded to post-capillary venules, with diameters ranging from 12 to 18 μm.

2.3.2. Experimental protocols

2.3.2.1. Leukocyte rolling. In a series of experiments, interaction of leukocytes with the luminal surface of the venular endothelium was studied in a segment of the vessel. Rolling leukocytes ("rollers") were defined as those white blood cells that moved at a velocity less than that of red blood cells in the bloodstream. The number of rollers was determined over a 10-min period without any stimulus except for the surgical manipulation. These leukocytes moved sufficiently slowly to be individually visible and were counted as they rolled past a 100-μm venule length (Fortes et al., 1991).

2.3.2.2. Leukocyte adhesion. Leukocyte adhesion was quantified following topical application of zymosan-activated plasma or leukotriene B_4. A leukocyte was considered adherent to the venular endothelium if it remained stationary

for >30 s (Granger et al., 1989). Adherent cells ("stickers") were expressed as the number per 100-μm venule length. Adhesion was investigated under two sets of conditions. In the first, the internal spermatic fascia, after a suitable control period of normal circulation, was exposed to 0.1 ml of a solution containing 10% zymosan-activated homologous plasma in physiological saline. Zymosan-activated plasma was prepared according to Cruz et al. (2000). Adhesion of leukocytes was assessed 10 min after topical application of zymosan. Plasma treated identically, except for the addition of zymosan, was used as a control. In the second, leukocyte adhesion was quantified using the same protocol for animals with local application of leukotriene B_4 (1 μM–0.1 ml). Only one section of the vascular bed from each animal was tested.

2.3.2.3. Leukocyte migration. The number of leukocytes that accumulated in a 2500-μm^2 standard area of connective tissue adjacent to a post-capillary venule was determined after the induction of a local inflammatory response. Cells were counted on the recorded image. Four different fields were evaluated for each animal to avoid sampling variability. The data were then averaged for each animal. The inflammatory reaction was evoked by injecting 100 μg of carrageenan in 0.1 ml of saline into the scrotum of the animals and the number of migrated cells was counted after 2 h of carrageenan injection.

2.4. Hemodynamic parameters

The left carotid artery was cannulated and mean arterial blood pressure and heart rate were measured. The catheter was filled with heparinized saline (20 IU/ml). Direct blood pressure recordings were obtained by connecting the arterial cannula to a physiograph (MK-III, Narco Bio System, Houston, TX). Indirect heart rate recordings were obtained by counting waveforms generated on the physiograph tracings. Centerline red blood cell velocity (V_{RBC}) was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, USA), that was calibrated against a rotating glass disk coated with red blood cells. Venular wall shear rate (γ) was calculated from the Newtonian definition: $\gamma = 8 \times V_{mean}/D_V$ (where D_V = vessel diameter and $V_{mean} = V_{RBC}/1.6$ for vessels >25 μm) (Davis, 1987).

2.5. Measurement of spontaneous oxidative stress

In order to assess free radical production by endothelial cells in vivo, we used hydroethidine, an oxygen radical-sensitive fluorescent probe, which easily permeates cell membranes (Bucana et al., 1986). Intracellular hydroethidine can be directly oxidized to form red fluorescent ethidium bromide, which in turn is trapped in the nucleus by intercalation into DNA (Rothe and Valet, 1990). Hydroethidine is especially sensitive to superoxide anion and to a lesser degree to hydrogen peroxide (Suzuki et al., 1995). These experiments were performed according to Zanardo et al. (2003). The internal spermatic fascia was visualized through an intravital microscope (× 20-water immersion objective lens, Zeiss) using a digital color charge device (CCD) camera (ZVS-47EC, Zeiss). Single unbranched venules with a diameter between 20 and 40 μm and approximately 250 μm in length were selected for study. A digital-gain control mode in the color CCD camera allowed suitable transmission images to be obtained. The camera sensitivity and shutter speed were set at the following values (contrast = 0, brightness = 0, manual integration = 4). To elicit fluorescent images, we illuminated the preparation with a 200-W mercury lamp. The light was passed through a quartz collector, heat filter (model KG-2, Zeiss), and excitation filter (490 nm, Zeiss) for epi-illumination. Fluorescence emission from the specimen was passed through a band-pass filter (590 nm) and onto the CCD camera. Transillumination images were also recorded immediately after the fluorescence images on a hard disk. During the intervening periods, the shutter for the excitation light was kept closed. The number of ethidium bromide-positive nuclei along venules (N_{EB}) was counted every 15 min for 60 min. At the end of the experiments, the tissue was superfused with absolute ethanol for 10 min followed by ethidium bromide superfusion to ascertain the total number of nuclei lining the vessel wall (N_T). The ethidium bromide-positive number of nuclei was computed as $(N_{EB}/N_T) \times 100$ (%).

2.5.1. Hydroethidine superfusion

During an initial 20-min stabilization period, the internal spermatic fascia was superfused (1 ml/min) with warmed (37 °C) Krebs-Henseleit bicarbonate-buffered solution saturated with 95% N_2/5% CO_2. A background image in the selected tissue area was recorded and stored in the memory of a laboratory computer (Gateway2000 DMI). The preparation was then superfused with the buffer solution containing hydroethidine (5 μM) for 60 min. Free radical production from firmly attached leukocytes was distinguished from that by endothelial cells by comparing the transillumination images with the ethidium bromide fluorographs. Endothelial cells labeled with ethidium bromide had a longitudinally oriented spindle shape positioned on the inner lining of the venular wall, differently from leukocytes that are round-shaped, brighter and loosely found in the fluorographs. The number of ethidium bromide-stained nuclei was counted per unit venule length.

2.6. Imunohistochemistry analysis for detection of ICAM-1 and P-selectin

Cross-sections of the whole testis of the animals were prepared for the immunofluorescence detection of intercellular adhesion molecule-1 (ICAM-1) or P-selectin on the endothelium of venules of the internal spermatic fascia. Under anesthesia with pentobarbital sodium (40 mg/kg, i.p.), carrageenan (100 μg–0.1 ml) or saline at the same

volume as carrageenan was injected into the scrotal chamber of the animals and 2 h later, the testis were excised. Specimens were then quick-frozen in hexane. Sections of 8-μm thickness were cut on a Reichen Jung 2800 cryostat at −20 °C and collected on glass slides coated with silane. The samples were fixed in acetone at −20 °C for 10 min. The sections were washed three times in PBS (5 min each) and then incubated with Superblock Blocking Buffer at room temperature for 2 h to block nonspecific sites in the tissue. Biotin-conjugated anti-rat ICAM-1 (CD54) monoclonal antibody (1:10 in PBS) or rabbit anti-human CD62P polyclonal antibody (1:50 in PBS) was applied overnight at 4 °C followed by three rinses in PBS for 5 min each. Negative control (NC) sections were treated in the same manner as the experimental slides except that the primary antibody was replaced with PBS. Subsequently, cross-sections were incubated with streptoavidin-fluorescein for 1 h at room temperature, followed by three rinses in PBS (5 min each). In order to preserve the fluorescence, the cross-sections received Vectashield mounting medium with propidium iodide. Fluorescence on the venular endothelial cells of the internal spermatic fascia was evaluated on a Nikon Eclipse E-800 inverted microscope at ×20/0.45 and analyzed by the software Image-Pro® Plus (Media Cybernetics, Silver Spring, MD, USA). The results are expressed as mean density of fluorescence (arbitrary units ± S.E.M.).

2.7. Reagents

The following reagents were used: alloxan hydrate, probucol, gelatin, zymosan from *Saccharomices cerevisae*, leukotriene B_4 and slides coated with silane (all from Sigma, Missouri, USA); carrageenan sodium salt, a 60,000- to 100,000-Mr polysaccharide composed of sulphated galactose units (Marine Colloids, Springfield, NJ); sodium pentobarbital (Hypnol®, Cristália, Brazil); hydroethidine (Poliscience, USA), heparin (Liquemine®, Roche, RJ, Brazil); NaCl; KCl; $CaCl_2.2H_2O$; $NaHCO_3$, glucose and ethanol (all from Merck, RJ, Brazil); hexane (Fluka Chemical, Ronkonkoma, NY, USA); superblock blocking buffer (Pierce Chemical, IL, USA); streptoavidin-fluorescein (Amersham Pharmacia Biotech, London, UK); vectashield mounting medium with propidium iodide (Vector, CA, USA); biotin-conjugated anti-rat ICAM-1 (CD54) monoclo-

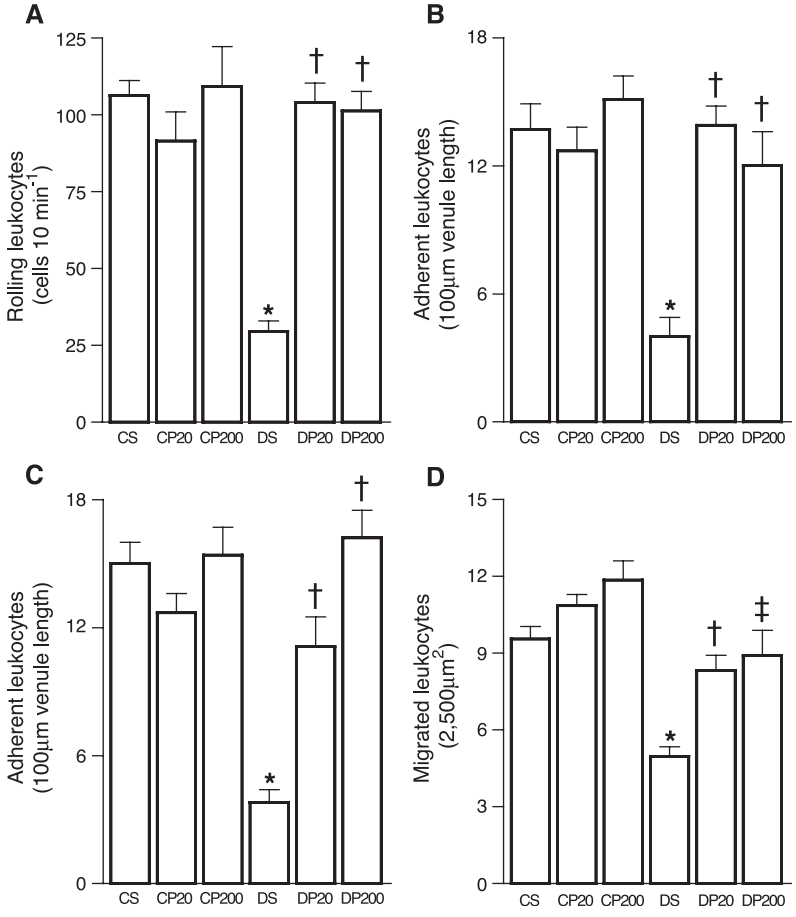

Fig. 1. Leukocyte–endothelial interactions in post-capillary venules of the internal spermatic fascia of control (C) and diabetic (D) rats treated with saline (S) or probucol 20 (P20) or 200 (P200) mg/kg/day. In A, number of rolling leukocytes in 10-min periods. In B, number of adherent leukocytes after 10 min of zymosan-activated plasma applied topically. In C, number of adherent leukocytes after 10 min of leukotriene B_4 applied topically. In D, leukocyte migration after 2 h of carrageenan injection. Values are means ± S.E.M. *$P<0.001$ vs. CS, †$P<0.01$ vs. DS and ‡$P<0.001$ vs. DS.

Table 1
Venular diameter, mean arterial blood pressure (MABP), heart rate (HR), red blood cell velocity (V_{RBC}) and venular wall shear rate (γ) from saline-treated control and diabetic rats (CS and DS, respectively), probucol (20 or 200 mg/kg/day)-treated control and diabetic rats (CP20, CP200 and DP20, DP200; respectively)

Groups	Vessel diameter (μm)	MABP (mm Hg)	HR (beats min^{-1})	V_{RBC} (mm s^{-1})	γ (s^{-1})	n
CS	16.7 ± 0.6	105.2 ± 1.4	342.0 ± 9.2	2.0 ± 0.1	971.0 ± 66.7	10
CP20	20.3 ± 1.0	92.1 ± 4.3	310.0 ± 18.4	1.8 ± 0.1	748.5 ± 40.3	6
CP200	17.9 ± 0.6	95.6 ± 6.0	295.0 ± 19.6	2.1 ± 0.1	950.6 ± 63.5	6
DS	17.2 ± 0.7	100.2 ± 3.8	308.6 ± 10.8	2.0 ± 0.1	927.9 ± 36.0	7
DP20	17.7 ± 0.6	103.9 ± 2.8	293.3 ± 12.0	2.0 ± 0.1	913.1 ± 38.7	9
DP200	19.0 ± 1.2	105.3 ± 3.1	324.0 ± 14.7	2.3 ± 0.1	982.4 ± 128.5	5

n = number of animals used.
Values are means ± S.E.M.

nal antibody (Seikagaku America, Falmouth, MA, USA) and purified rabbit anti-human CD62P (P-selectin, cross-reactivity rat) polyclonal antibody (BD Pharmingen, USA).

2.8. Statistical analysis

Data are given as mean ± S.E.M. One-way analysis of variance followed by Tukey–Kramer multiple comparisons test was used, when pertinent. The minimum acceptable level of significance was P at a value less than or equal to 0.05.

3. Results

3.1. Diabetic rats exhibited a loss of weight gain and an increase in blood glucose levels

Relative to controls, diabetic rats had a significantly reduced gain in body weight (144.1 ± 8.5 and 22.0 ± 10.3 g, in control and diabetic animals, respectively, $P < 0.001$). Blood glucose concentrations were significantly elevated in samples from diabetic in comparison with those from control animals (5.2 ± 0.4 and 19.7 ± 1.2 mM, in control and diabetic animals, respectively, $P < 0.001$).

3.2. Probucol treatment did not have a significant effect on body weight gain or blood glucose levels

Probucol treatment did not significantly interfere with body weight gain in either probucol-treated control (122.8 ± 13.0 and 115.3 ± 5.9 g for 20 and 200 mg/kg/day, respectively) or diabetic rats (24.4 ± 9.2 and 44.4 ± 6.6 g for 20 and 200 mg/kg/day, respectively). Probucol also did not significantly influence blood glucose levels in either probucol-treated controls (4.9 ± 0.5 and 4.0 ± 0.3 mM for 20 and 200 mg/kg/day, respectively) or diabetic rats (21.1 ± 1.7 and 18.3 ± 0.9 mM for 20 and 200 mg/kg/day, respectively).

3.3. Probucol treatment corrected the impaired rolling, adhesion and migration of leukocytes in diabetic rats

A marked decrease in the number of rolling cells was observed in the diabetic rats relative to controls. Treatment of diabetic rats with probucol (20 or 200 mg/kg/day) resulted in an increase in the number of rolling leukocytes. The values reached matched those observed in control animals. The results are presented in Fig. 1A. The number

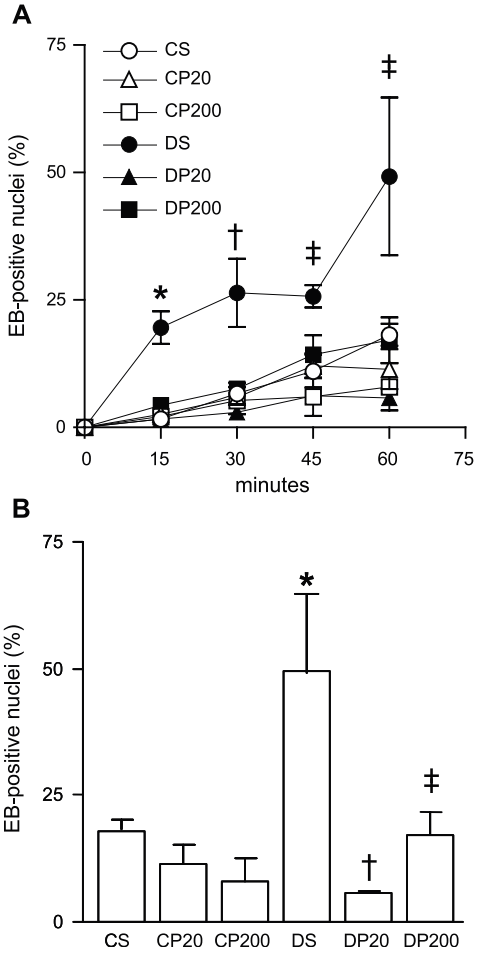

Fig. 2. In A, plot showing the time course of the number of ethidium bromide (EB)-positive nuclei along venules of the internal spermatic fascia. In B, bar graph showing the number of ethidium bromide (EB)-positive nuclei along venules of the internal spermatic fascia 60 min after the onset of hydroethidine superfusion. CS = saline-treated control rats, DS = saline-treated diabetic rats, CP20 and CP200 = probucol (20 or 200 mg/kg/day, respectively)-treated control rats, DP20 and DP200 = probucol (20 or 200 mg/kg/day)-treated diabetic rats. In A; *$P < 0.001$, †$P < 0.01$ and ‡$P < 0.05$ vs. CS. In B, *$P < 0.05$ vs. CS, †$P < 0.01$ and ‡$P < 0.05$ vs. DS.

of adherent leukocytes after zymosan-activated plasma (Fig. 1B) or leukotriene B_4 (Fig. 1C) was decreased in diabetic animals. Probucol treatment corrected the impaired adherence in diabetic rats (Fig. 1B and C). No leukocyte adhesion was observed following application of non-activated plasma to the preparations (data not shown). The number of migrated cells after carrageenan (Fig. 1D) was also decreased in diabetic animals, and as with rolling and

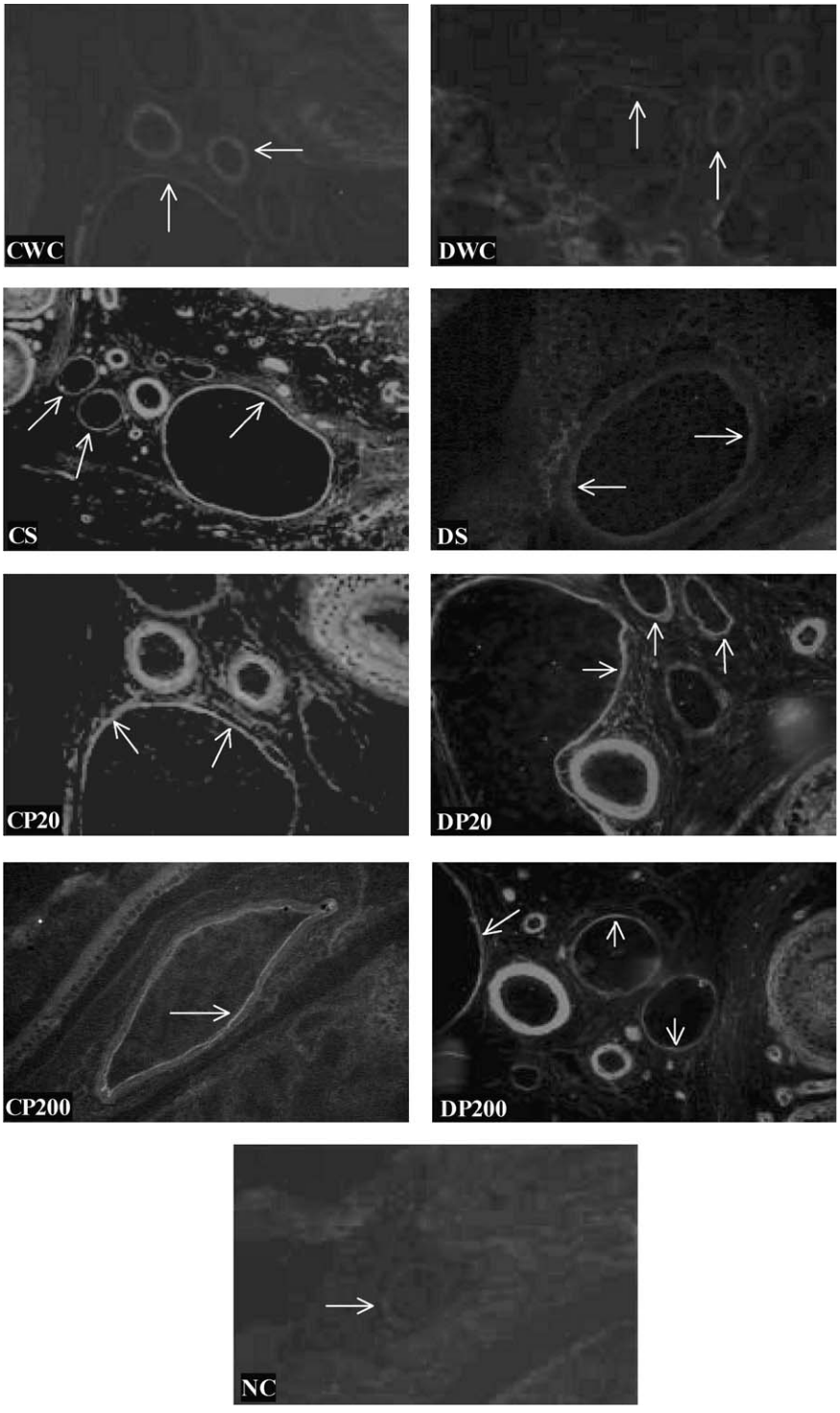

Fig. 3. Photomicrographs of immunohistochemical fluorescent staining for ICAM-1 (in grey) in venules (arrows) of the internal spermatic fascia in vitro. Fluorescent staining was largely absent if no ICAM-1 antibody was added (NC), in control and diabetic rats that did not receive carrageenan injection (CWC and DWC, respectively) and in saline-treated diabetic rats that received carrageenan (DS). Significant immunofluorescence was seen in saline-treated control rats (CS), probucol 20 (CP20) and 200 (CP200) mg/kg/day-treated control rats and probucol 20 (DP20) and 200 (DP200) mg/kg/day-treated diabetic rats.

adhesion, probucol treatment restored leukocyte migration. Probucol treatment of control rats did not interfere with rolling, adhesion, or migration (Fig. 1). Also, the observed changes were not dependent on the number of circulating leukocytes because total and differential leukocyte counts in the peripheral blood were similar in control, diabetic and probucol-treated diabetic and control rats (data not shown).

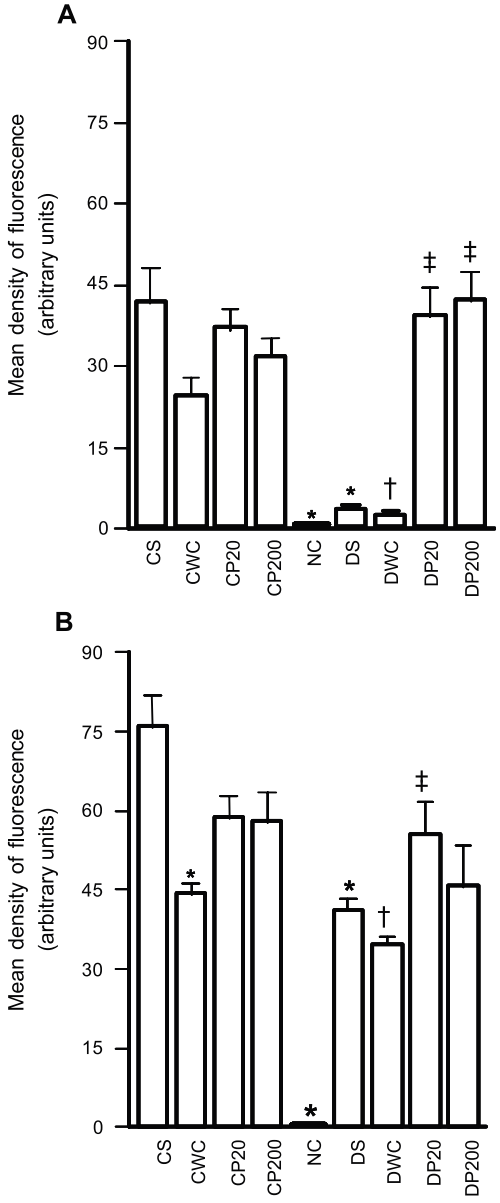

Fig. 4. Bar graph representing mean density of fluorescence (arbitrary units ± S.E.M.) of ICAM-1 (A) and P-selectin (B) in venules of internal spermatic fascia 2 h after local carrageenan injection. Fluorescent staining was largely absent if no ICAM-1 or P-selectin antibody was added (NC), in venules from control and diabetic rats that did not receive carrageenan injection (CWC and DWC, respectively) and in venules from saline-treated diabetic rats that received carrageenan (DS). Significant immunofluorescence was seen in venules from saline-treated control rats (CS), probucol 20 (CP20) and 200 (CP200) mg/kg/day-treated control rats and probucol 20 (DP20) and 200 (DP200) mg/kg/day-treated diabetic rats. In A, $*P<0.001$ vs. CS, $^{\dagger}P<0.05$ vs. CWC and $^{\ddagger}P<0.001$ vs. DS. In B, $*P<0.001$ vs. CS and $^{\dagger}P<0.05$ vs. CWC, $^{\ddagger}P<0.05$ vs. DWC.

3.4. Mean arterial blood pressure, heart rate, red blood cell velocity and wall shear rate in diabetic rats were not affected by probucol treatment

Under baseline conditions, diabetic rats had mean arterial blood pressure and heart rate similar to those of control rats. Venules of diabetic rats had a red blood cell velocity and wall shear rate similar to those of control rats. Probucol treatment of control and diabetic rats did not interfere with these parameters (Table 1).

3.5. Superoxide production in venules of diabetic rats was reduced by probucol treatment

During continuous superfusion of hydroethidine, no significant changes in microvascular velocity or in leukocyte–endothelial cell adhesive interaction were observed. Ethidium bromide fluorescence was strikingly increased in the wall of diabetic venules compared with those of non-diabetic control rats without application of a stimulus (Fig. 2). The increased level of ethidium bromide fluorescence in diabetic rats was reduced by probucol treatment (Fig. 2). Fig. 2A depicts the time course for the relative number of ethidium bromide positive nuclei (in percentage) along the diabetic venular wall. As Fig. 2B shows, the number of ethidium bromide-positive nuclei (percent) along fascia venules 60 min after the start of hydroethidine superfusion was significantly increased in diabetic venules compared with non-diabetic control ones. After probucol treatment, a lower number of ethidium bromide-positive nuclei was found in diabetic preparations. Probucol treatment of non-diabetic controls did not interfere with the number of ethidium bromide-positive nuclei.

3.6. The expression of ICAM-1 and P-selectin in venules of diabetic rats was restored by probucol treatment

Fig. 3 shows photomicrographs of immunohistochemical fluorescent staining for ICAM-1 in venules of the internal spermatic fascia in vitro. The expression of ICAM-1 (Fig. 4A) and P-selectin (Fig. 4B), as expressed in mean density of fluorescence (arbitrary units), which was reduced in diabetic preparations, was restored to levels similar to those of control animals after the lower dose of probucol (20 mg/kg). At the higher dose (200 mg/kg) of probucol, only the ICAM-1 expression was restored.

4. Discussion

The precise mechanism by which chronic hyperglycemia leads to the development of the long-term complications of

diabetes (DCCT, 1993) is not completely understood. One of these complications is the reduced leukocyte migration that may render diabetic patients and animals more prone to infections. Confirming previous findings (Cruz et al., 2000; Zanardo et al., 2003), we demonstrated a reduced number of rollers, adhered and migrated leukocytes in alloxan-diabetic rats. In the present study, we found increased reactive oxygen species production and decreased immunoreactivity to ICAM-1 and P-selectin in venular endothelium of diabetic rats. The novel and important finding of the present work is that probucol, similarly to ascorbic acid, restored the defective leukocyte–endothelial interaction and the reduced expression of ICAM-1 and P-selectin in venules of the internal spermatic fascia in diabetic rats.

The probucol restorative effect was not related to an increase in the number of circulating leukocytes, since total and differential leukocyte counts were unaltered by probucol treatment. It has been well established that alterations in red blood cell velocity (V_{RBC}) and/or wall shear rate (γ) can alter leukocyte adhesion to the vascular wall (Perry and Granger, 1991). We observed no differences among hemodynamic parameters (mean arterial blood pressure, heart rate, V_{RBC} and γ). Hemodynamic changes can, therefore, be excluded.

Oxidative stress may be involved in the complications of late diabetes. It may occur as a result of increased free radical generation, decreased levels of antioxidants and/or impaired regeneration of reduced forms of antioxidants. The present microfluorographic studies provided in vivo demonstration of oxidative stress in a diabetic animal model without any additional stimulation, similarly to that demonstrated previously (Zanardo et al., 2003). Probucol treatment, similarly to ascorbic acid (Zanardo et al., 2003), reduced oxidative stress in diabetic rats.

Excessive free radical production is one of several mechanisms responsible for the late complications of diabetes (Baynes, 1991). It has been shown that overproduction of free radicals in diabetes leads to endothelial dysfunction. In the past several years, antioxidants such as vitamin E have been demonstrated to be beneficial for diabetes (Morel and Chisolm, 1989; Keegan et al., 1995). Chronic treatment with vitamin E partially restores endothelial dysfunction in diabetic rats (Dhein et al., 2003). The same was observed for probucol (Karasu, 1998). We showed that probucol decreased superoxide production in venules of diabetic rats, which had been expected since probucol scavenges superoxide anions in vitro (Bridges et al., 1991). We suggest that improving endothelial function by decreasing superoxide production might have a positive effect on the expression of endothelial adhesion molecules.

There are conflicting data in the literature on the consequences of the probucol effect on leukocyte migration. Probucol has been considered as a potent inhibitor of atherosclerosis in rabbit models (Kita et al., 1988; Daugherty et al., 1989), reducing monocyte infiltration. Whether the same occurs in rats and in diabetes remains to be demonstrated. In human umbilical vein endothelial cells cultures, this agent was able to inhibit the expression of VCAM-1 but not ICAM-1 after tumor necrosis factor-α (TNF-α) and interleukin-1 stimulus (Zapolska-Downar et al., 2001). However, when the stimulus was low-density lipoprotein (LDL), ICAM-1 and P-selectin expression were inhibited (Liu et al., 2002). It is worth mention that the two latter studies were in vitro while ours was in in vivo, and we tested zymosan and leukotriene B_4 as stimuli instead of TNF-α, interleukin-1 or LDL.

On the other hand, enhanced chemotaxis of non-diabetic macrophages in response to stimuli has been demonstrated in response to probucol. The effect seems not to involve oxidative stress, and was inhibited by cycloheximide, suggesting that probucol-enhanced chemotaxis is not related to its antioxidant capacity, but results from de novo synthesis of cell surface proteins that respond to some particular chemoattractants (Hara et al., 1992).

In the present study, while treatment of non-diabetic rats with probucol did not enhance leukocyte recruitment, it restored the reduced leukocyte behavior in diabetic rats along with a reduction of the generation of superoxide anions, which was increased in diabetic venules. Therefore, the ameliorating effect of probucol on leukocyte migration in diabetes mellitus might involve enhancement of the expression of cell surface proteins such as cell adhesion molecules in addition to its properties as an antioxidant and activator of endogenous antioxidant enzymes. In fact, the reduced expression of ICAM-1 and P-selectin observed in diabetic preparations was restored to levels similar to the control ones after probucol treatment. However, further studies are necessary to determine whether the protective effect of probucol is a result of its primary antioxidant effect or its secondary effect on cell surface protein synthesis.

In summary, our previous and present results together suggest that oxidative stress plays a significant role in the endothelial dysfunction and in the disturbances of leukocyte behavior in diabetes. Antioxidants such as probucol are able to ameliorate these adverse effects in vivo. We suggest also that the beneficial effect of probucol on leukocyte behavior might involve the increased expression of adhesion molecules, which reduced in diabetic preparations. Regardless of the mechanism, reduction of free radical production might correct the defective leukocyte–endothelial interaction found in experimental diabetes and may have a similar effect in diabetic patients.

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