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Ischemic conditioning protects the rat retina in an experimental model of early type 2 diabetes

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ABSTRACT

Diabetic retinopathy is a leading cause of acquired blindness in adults, mostly affected by type 2 diabetes mellitus (T2DM). We have developed an experimental model of early T2DM in adult rats which mimics some features of human T2DM at its initial stages, and provokes significant retinal alterations. We investigated the effect of ischemic conditioning on retinal changes induced by the moderate metabolic derangement. For this purpose, adult male Wistar rats received a control diet or 30% sucrose in the drinking water, and 3 weeks after this treatment, animals were injected with vehicle or streptozotocin (STZ, 25 mg/kg). Retinal ischemia was induced by increasing intraocular pressure to 120 mmHg for 5 min; this maneuver started 3 weeks after vehicle or STZ injection and was weekly repeated in one eye, while control eyes were submitted to a sham procedure. Fasting and postprandial glycemia, and glucose, and insulin tolerance tests were analyzed. At 12 weeks of treatment, animals which received a sucrose-enriched diet and STZ showed significant differences in metabolic tests, as compared with control groups. Brief ischemia pulses in one eye and a sham procedure in the contralateral eye did not affect glucose metabolism in control or diabetic rats. Ischemic pulses reduced the decrease in the electroretinogram a-wave, b-wave, and oscillatory potential amplitude, and the increase in retinal lipid peroxidation, NOS activity, TNFα, Müller cells glial fibrillary acidic protein, and vascular endothelial growth factor levels observed in diabetic animals. In addition, ischemic conditioning prevented the decrease in retinal catalase activity induced by T2DM. These results indicate that induction of ischemic tolerance could constitute a fertile avenue for the development of new therapeutic strategies to treat diabetic retinopathy associated with T2DM.

Introduction

Diabetes mellitus is a worldwide growing disease and represents a huge social and healthcare problem owing to the burden of its complications. The incidence of type 2 diabetes mellitus (T2DM) has increased significantly in the past decades, and is expected to continue to rise (Shaw et al., 2010). Chronic hyperglycemia of diabetes leads to microvascular and macrovascular circulatory impairment that has a negative impact on several organs. Diabetic retinopathy (DR) may be the most common of diabetes complications, and is a leading cause of visual impairment and blindness in people of working age (Fong et al., 2004). Current treatments for DR such as laser photocoagulation, corticosteroids, or anti-vascular endothelial growth factor agents are not fully efficacious, and may have significant adverse effects. Therefore, the development of resources to protect the retina against diabetic damage is a goal of vast clinical importance.

Understanding the molecular mechanisms of retinal damage associated with T2DM should help identify therapies to treat/postpone this sight-threatening complication of diabetes. For this purpose, some genetically modified animal models including transgenic, generalized knockout, and tissue specific knockout mice have been employed. However, a typical diabetic profile is not always seen in these genetically induced models, nor do they absolutely mimic the pathogenesis of human T2DM (Matsuura et al., 2005; Movassat et al., 1995), since these gene mutations are extremely rare in human populations. In a similar way, animal models of T2DM induced by removal of a portion of the pancreas (Portha et al., 1989) are not representative of T2DM etiology in humans which is typically preceded by obesity (Bray, 2004; Goralski and Sinal, 2007). In order to better understand the events

Abbreviations: AUC, area under the curve; DR, diabetic retinopathy; ERG, electroretinogram; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; IPC, ischemic preconditioning; IPGTT, intraperitoneal glucose tolerance; IPITT, intraperitoneal insulin tolerance test; MDA, malondialdehyde bis-dimethyl acetal; NOS, nitric oxide synthase; OPs, oscillatory potentials; PostC, ischemic postconditioning; STZ, streptozotocin; TBARS, thiobarbituric acid reactive substances; T2DM, type 2 diabetes mellitus; TNF-α, tumor necrosis factor α; VEGF, vascular endothelial growth factor.

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which precede and precipitate the onset of T2DM, some nutritional animal models have been also developed (Mühlhausler, 2009; Surwit et al., 1988).

Initially, the natural history of T2DM includes a period of normal or near-normal fasting plasma glucose levels and marked postprandial glycemic excursions. Historically, it was believed that microvascular complications of diabetes including T2DM develop only after ~10 to 15 years of active disease (UKPDS Group, 1995, 1998). However, it is increasingly clear that complications may begin at lower glucose concentrations or during sporadic increases in glucose levels, rather than after current thresholds for the diagnosis of T2DM are consistently reached (Stolar, 2010). Recently, we have developed an experimental model of T2DM through a combination of diet-induced insulin resistance and a slight secretory impairment resulting from a low-dose streptozotocin (STZ) treatment. This model mimics some features of human T2DM at its initial stages, such as slight fasting hyperglycemia, hyperinsulinemia, and elevated postprandial (nocturnal) glycemic levels (Salido et al., 2012). Noteworthy, only animals exposed to this combined treatment develop significant retinal alterations, whereas each one of these maneuvers *per se* (a sucrose-enriched diet or the injection of a low dose of STZ) does not induce significant retinal changes (Salido et al., 2012). Thus, this model could offer the opportunity to investigate DR at an early stage in a setting of moderately altered glucose metabolism.

DR is the most common ischemic disorder of the retina (Stitt et al., 2011). Ischemic retinopathy develops when retinal blood flow is insufficient to match the metabolic needs of the retina, one of the highest oxygen-consuming tissues. Although at present there is no effective treatment against retinal ischemic injury, it is possible to activate an endogenous protection mechanism that protects from retinal ischemic damage by ischemic preconditioning (IPC) or ischemic postconditioning (PostC) (Fernandez et al., 2009; Roth et al., 1998). IPC and PostC require a brief period of ischemia applied before or after ischemic injury, respectively, which do not produce any damage *per se*, and trigger yet incompletely described mechanisms that result in tolerance to the subsequent or previous severely damaging ischemic event (Gidday, 2006). It was shown that IPC and PostC afford the retina a very high degree of protection against acute ischemic damage (Fernandez et al., 2009; Roth et al., 1998). In this context, the aim of the present work was to analyze the effect of brief ischemia pulses on retinal alterations observed in an experimental model in rats which mimics early stages of human T2DM.

Materials and methods

Animals

Male Wistar rats (400 ± 50 g) derived from stock supplied by Charles River Breeding Laboratories (Wilmington, MA, USA) were purchased from a local dealer. Rats were housed in a standard animal room with food and water *ad libitum* under controlled conditions of humidity, temperature (21 ± 2 °C), and luminosity (200 lux), under a 12-hour light/12-hour dark lighting schedule (lights on at 7:00 AM). All the experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The ethic committee of the School of Medicine, University of Buenos Aires (Institutional Committee for the Care and Use of Laboratory Animals, (CICUAL)) approved this study.

Feeding and treatments

Experimental groups included in this study are depicted in Fig. 1. Rats were randomly assigned to receive either a standard commercial rat chow and tap water (control groups), or a standard commercial rat chow and 30% sucrose in the drinking water, and an i.p. injection of STZ (25 mg/kg in 0.1 M citrate buffer, pH 4.5) 3 weeks after receiving 30% sucrose in the drinking water (T2DM groups). Three weeks after vehicle or STZ-injection, weekly ischemia pulses were applied in one eye, whereas the contralateral eye was submitted to a sham procedure. For retinal ischemia induction, animals were anesthetized with ketamine hydrochloride (150 mg/kg) and xylazine hydrochloride (2 mg/kg) administered intraperitoneally. After topical instillation of proparacaine, the anterior chamber was cannulated with a 30-gauge needle connected to a pressurized bottle filled with sterile saline solution. Retinal ischemia was induced by increasing intraocular pressure (IOP) to 120 mm Hg for exactly 5-min, as previously described (Fernandez et al., 2011). With

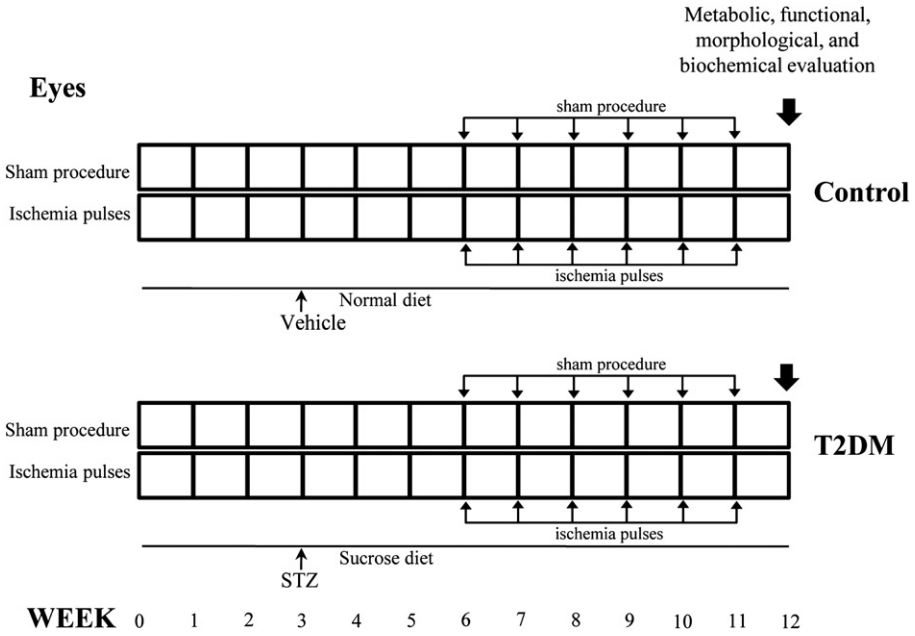

Fig. 1. Experimental groups for retinal studies. Animals were submitted to a normal diet (control) or a sucrose enriched diet (T2DM) for 12 weeks. At third week of diet, animals were i.p. injected with vehicle (control) or STZ (T2DM). Three weeks after vehicle or STZ-injection, animals received a 5-min ischemia pulse in one eye and a sham treatment in the contralateral eye. This maneuver was weekly repeated until week 11. For metabolic studies, groups of control and diabetic animals whose eyes remained intact were included. Retinal and metabolic studies were performed at 12 weeks of sucrose treatment.

this maneuver, complete ocular ischemia was produced, characterized by the loss of ERG b-wave and the cessation of flow in retinal vessels, determined by funduscopic examination. The contralateral eye was cannulated without raising IOP. During ischemia pulses, animals were kept normothermic with heated blankets. These maneuvers were weekly repeated until week 11 (Fig. 1). For the metabolic studies, groups of control and diabetic animals in which both eyes remained intact were included. For the retinal studies, sham-treated eyes served as the control group, because in preliminary studies we found that in comparison with intact eyes, the sham procedure did not affect the electroretinogram (ERG) or retinal morphology (data not shown).

Glucose level assessment

Plasma glucose levels were determined in tail vein blood samples using the Accu-Check Performa blood glucose meter (Roche Diagnostic, Mannheim, Germany). In some experiments, animals were fasted for 4 h before the measurements, whereas in other experiments, plasma glucose levels were assessed at 11.00 or 23.00 h in non fasted animals.

Intraperitoneal glucose tolerance (IPGTT) and intraperitoneal insulin tolerance test (IPITT)

IPGTT and IPITT were performed at 12 weeks of sucrose treatment, as previously described (Salido et al., 2012). Animals were restricted of food and water 4 h before the experiments. For the IPGTT, rats received an i.p. injection of glucose (1 g/kg body weight). Blood samples for plasma glucose measurement were collected from the tail vein at time 0 (before glucose injection) and 30, 60, and 120 min after glucose administration. The area under the curve (AUC) was calculated using the trapezoidal rule estimation, as previously described (Salido et al., 2012).

For the IPITT, rats were intraperitoneally injected with regular human insulin (0.75 IU/kg body weight). Blood samples were taken from the tail vein at time 0 (before insulin injection) and 15, 30, 45, and 60 min after insulin administration. Insulin sensitivity was estimated from the slope of the 0–60 min glucose disappearance rate. This slope was determined by linear regression as previously described (Salido et al., 2012).

Electroretinography

The electroretinographic activity was assessed as previously described (Salido et al., 2012). Briefly, after 6 h of dark adaptation, rats were anesthetized under dim red illumination. Phenylephrine hydrochloride and tropicamide were used to dilate the pupils, and the cornea was intermittently irrigated with balanced salt solution to maintain the baseline recording and to prevent keratopathy. Rats were placed facing the stimulus at a distance of 20 cm. All recordings were completed within 20 min and animals were kept warm during and after the procedure. A reference electrode was placed through the ear, a grounding electrode was attached to the tail, and a gold electrode was placed in contact with the central cornea. A 15 W red light was used to enable accurate electrode placement. This maneuver did not significantly affect dark adaptation and was switched off during the electrophysiological recordings. ERGs were recorded from both eyes simultaneously and ten responses to flashes of unattenuated white light (5 ms, 0.2 Hz) from a photic stimulator (light-emitting diodes) set at maximum brightness (9 cd s/m^2 without filter) were amplified, filtered (1.5-Hz low-pass filter, 1000 high-pass filter, notch activated), and averaged (Akonic BIO-PC, Buenos Aires, Argentina). The a-wave was measured as the difference in amplitude between the recording at onset and the trough of the negative deflection, and the b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave. Runs were repeated 3 times with 5 min-intervals to confirm consistency. Mean values from each eye were averaged, and the resultant mean value was used to compute the group means a- and b-wave amplitude ± SEM. The mean peak latencies and peak-to-peak amplitudes of the responses from each group of rats were compared.

Oscillatory potentials (OPs) were assessed as previously described (Fernandez et al., 2011). Briefly, the same photic stimulator with a 0.2 Hz frequency and filters of high (300 Hz) or low (100 Hz) frequency were used. The OP amplitude was estimated by measuring the heights from the baseline drawn between the troughs of successive wavelets to their peaks. The sum of three OPs was used for statistical analysis.

Histological examination

Rats were killed and their eyes were immediately enucleated, immersed for 24 h in 4% formaldehyde in 0.1 M phosphate buffer (pH 7.2), and embedded in paraffin. Eyes were sectioned (5 μm) along the vertical meridian through the optic nerve head. Microscopic images were digitally captured with a Nikon Eclipse E400 microscope (illumination: 6-V halogen lamp, 20 W, equipped with a stabilized light source) via a Nikon Coolpix s10 camera (Nikon, Abingdon, VA, USA). Sections were stained with hematoxylin and eosin (H&E), and analyzed by masked observers. Measurements (×400) were obtained at 1 mm dorsal and ventral from the optic disk.

Immunohistochemical studies

Antigen retrieval was performed by heating (90 °C) slices for 30 min in citrate buffer (pH 6.3) and then preincubated with 2% normal horse serum, 0.1% bovine serum albumin, and 0.4% Triton X-100 in 0.01 M PBS for 1 h. For immunodetection of glial cells, sections were incubated overnight at 4 °C with a mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody conjugated to Cy3 (1:1200; Sigma Chemical Co., St Louis, MO, USA). After immunostaining, the sections were mounted with antifade medium with the fluorescent nuclear stain DAPI (Vector Laboratories, Burlingame, CA, USA). For vascular endothelial growth factor (VEGF) immunodetection, paraffin sections were treated with 0.3% H_2O_2 in PBS for 20 min (for blocking endogenous peroxidase activity) and incubated overnight at 4 °C with a rabbit polyclonal anti-VEGF antibody (1:800; Calbiochem, La Jolla, CA, USA). Immunohistochemical detection was performed using the LSAB2 System-HRP (Dako, California, USA), based on biotin–streptavidin–peroxidase, and visualized using 3,3′-diaminobenzidine as cromogen. An Olympus BX50 microscope (Olympus, Tokyo, Japan) was used for

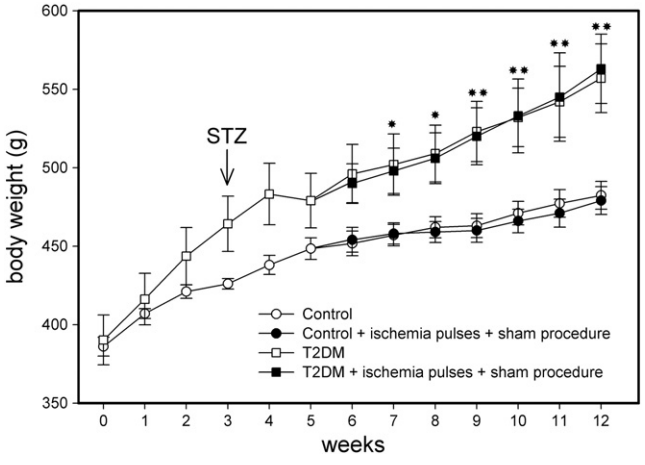

Fig. 2. Time-course of body weight for all the experimental groups. The body weight significantly increased throughout the experiment in all groups, but from week 7 to 12, it was significantly higher in groups receiving sucrose and STZ than in those which did not. Weekly ischemia pulses in one eye and a sham procedure in the contralateral eye did not affect this parameter in control or diabetic rats. Data are mean ± SEM (from week 0 to week 5: $n = 20$ animals/group; from week 7 to week 12: $n = 10$ animals/group). $^{*}P<0.05$, $^{**}P<0.01$ for diabetic groups vs. control (intact eyes), by Tukey's test.

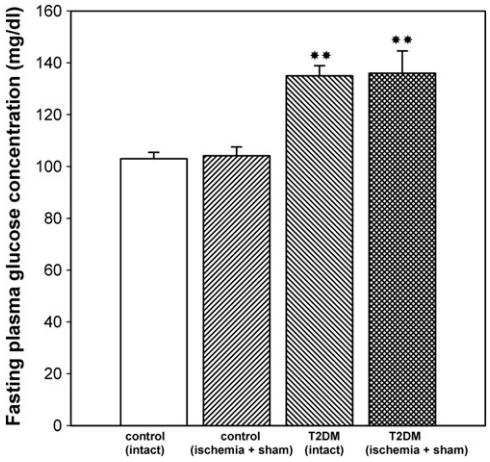

Fig. 3. Fasting plasma glucose levels. At 12 weeks of sucrose treatment, fasting plasma glucose levels were significantly higher in diabetic than in control groups. Weekly ischemia pulses in one eye and a sham procedure in the contralateral eye did not affect this parameter in control or diabetic rats. Data are the mean ± SEM ($n = 10$ animals/group); $^{**}P<0.01$ vs. control (intact eyes), by Tukey's test.

microscopic observations. Comparative digital images from different samples were grabbed using identical time exposition, brightness, and contrast settings.

Measurement of thiobarbituric acid reactive substances (TBARS) levels

TBARS levels in retinal tissue were analyzed as described (Belforte et al., 2010). Retinas were homogenized in 15 mM potassium buffer plus 60 mM KCl, pH 7.2. The homogenate (300 μL) was mixed with 75 μL 10% SDS and 1.4 mL 0.8% thiobarbituric acid dissolved in 10% acetic acid (pH 3.5). This solution was heated to 100 °C for 60 min. After cooling, the flocculent precipitate was removed by centrifugation at 3,200 *g* for 10 min. After addition of 1.0 mL water and 5.0 mL of n-butanol-pyridine mixture (15:1, vol/vol), the mixture was vigorously shaken and centrifuged at 2000 *g* for 15 min. The absorbance of the organic layer was measured at an emission wavelength of 553 nm by using an excitation wavelength of 515 nm with a Jasco FP 770 fluorescence spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo, Japan). The range of the standard curves of malondialdehyde bis-dimethyl acetal (MDA) was 10–2000 pmol. Results were expressed as nanomoles MDA per milligram protein.

Catalase activity

Retinal catalase activity was assessed as previously described (Moreno et al., 2004). Individual retinas were homogenized in 50 mM phosphate buffer, pH 7.4. Two hundred microliters of the homogenate were mixed with 25 μL of 20% triton X-100 and the mixture was incubated for 5 min at 4 °C and centrifuged at 10,000 *g* for 1 min. The supernatant (50 μL) was diluted with 900 μL of 50 mM phosphate buffer, and the reaction was started by adding 50 μL of 200 mM H_2O_2 in 50 mM phosphate buffer. Absorbance at 240 nm was recorded at 37 °C for 100 s every 10 s. The enzymatic activity was calculated by using the extinction coefficient of H_2O_2 at 240 nm, 0.0394 mM^{-1} cm^{-1} and results were expressed as nmol H_2O_2/min. milligram of protein.

NOS activity assessment

Retinal NOS activity was assessed as previously described (Salido et al., 2012). Each retina was homogenized in 100 μL of buffer solution containing 0.32 m sucrose and 0.1 mM EDTA (adjusted to pH 7.4 with Tris base). Reaction mixtures contained 50 μL of the enzyme source, 50 μL of a buffer stock solution (final concentrations: 10 mM HEPES, 3 mM $CaCl_2$, 1 mM NADPH, 5 μM FAD, 1 mM β-mercaptoethanol, L-^{3}H-arginine 5 μCi/mL, purity greater than 98%), and 1 μM L-arginine. After incubation at 37 °C for 30 min, the reaction was stopped by adding 200 μL of stop buffer (50 mm HEPES, 10 mm EDTA, and 10 mm EGTA, pH 5.5) and cooling the tubes for 5 min. The solution was mixed with 600 μL of resin Dowex AG50W-X8 (Na^+ form) to remove L-arginine and centrifuged at 10,000 *g* for 5 min. L-^{3}H-citrulline in the supernatant was quantified by liquid scintillation counting. Nonenzymatic conversion of L-^{3}H-arginine to L-^{3}H-citrulline was tested by adding buffer instead of the enzyme source.

Assay for TNF-α level assessment

Retinas were homogenized in 150 μL of phosphate saline buffer (PBS) pH 7.0 supplemented with 10% fetal bovine serum heat inactivated and a cocktail of protease inhibitors. Samples were cleared by centrifugation for 10 min at 13,000 rpm. TNF-α levels were determined as previously described (Salido et al., 2012), using specific rat enzyme-linked immunosorbent assays (ELISA) using antibodies and standards obtained from BD Biosciences, Pharmingen, San Diego, CA, USA, according to the manufacturer's instructions. The reaction was stopped and absorbance was read immediately at 450 nm on a microplate reader (Model 3550, Bio-Rad Laboratories, California, USA).

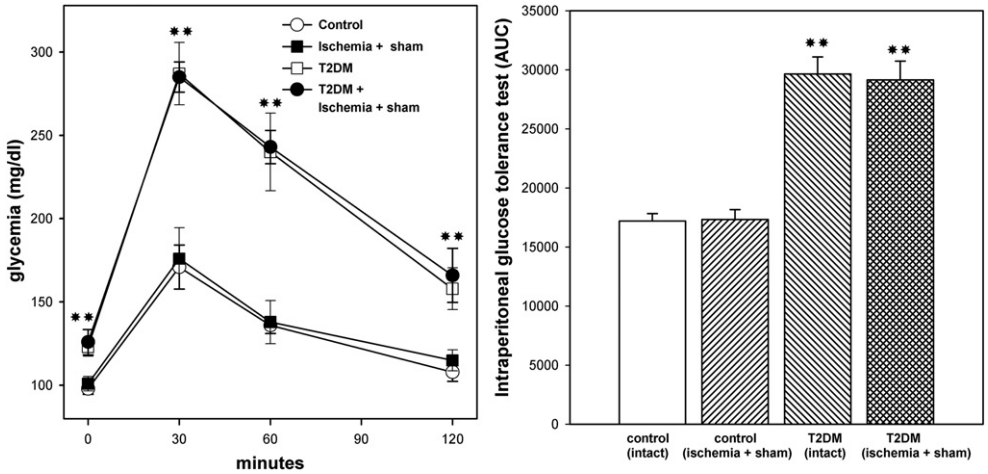

Fig. 4. Intraperitoneal glucose tolerance test. Left panel: mean plasma glucose concentrations in response to an intraperitoneal glucose challenge assessed before and 30, 60, and 120 min after 1 g/kg glucose injection for all the experimental groups at 12 weeks of treatment. Right panel: average AUC of the IPGTT. Higher values of the AUC were observed in diabetic than in control groups. Weekly ischemia pulses in one eye and a sham procedure in the contralateral eye did not affect the IPGTT in control or diabetic rats. Data are the mean ± SEM ($n = 10$); $^{**}P<0.01$ vs. control (intact eyes), by Tukey's test.

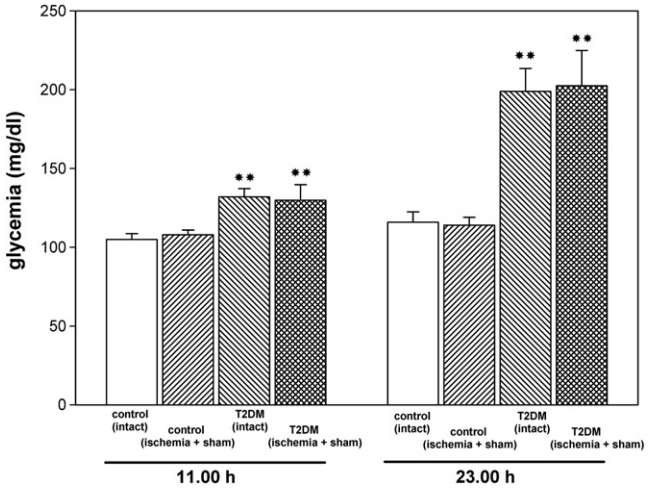

Fig. 5. Diurnal and nocturnal non-fasting glycemia. At 12 weeks of sucrose treatment, diurnal (i.e. at 11.00 h) and nocturnal (i.e. at 23.00 h) plasma glucose levels were significantly higher in diabetic than in control groups. Weekly ischemia pulses in one eye and a sham procedure in the contralateral eye did not affect diurnal or nocturnal non-fasting plasma glucose levels in control or diabetic animals. Data are the mean ± SEM ($n = 10$ animals per group); $^{**}P<0.01$ vs. control (intact eyes), by Tukey's test.

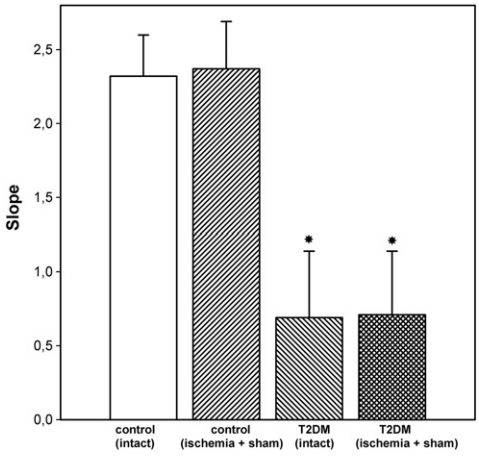

Fig. 6. Intraperitoneal insulin tolerance test. Rats were fasted for 4 h and then intraperitoneally injected with insulin (0.75 U/kg of body mass). Blood glucose levels were assessed before and 15, 30, 45, and 60 min after insulin administration. Insulin sensitivity was measured by the glucose disappearance rate within 60 min, evident from the average slope in the fitting curve. Higher insulin resistance was observed in diabetic than in control groups. Weekly ischemia pulses in one eye and a sham procedure in the contralateral eye did not affect this parameter in control or diabetic rats. Data are means ± SEM ($n = 10$ animals/group), $^{*}P<0.05$ vs. control (intact eyes), by Tukey's test.

Protein level assessment

Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Statistical analysis

Data are shown as mean ± SEM. Results were tested for normality using the Kolmogorov–Smirnoff approach. Statistical analysis was then performed by a two-way ANOVA, followed by a Tukey's *post hoc* test, as stated. For all analyses, a value of $P<0.05$ was considered significant.

Results

Metabolic studies

Fig. 2 depicts the time-course of the body weight from control or diabetic animals whose eyes remained intact, and control and diabetic animals which were submitted to weekly ischemia pulses in one eye, and a sham procedure in the contralateral eye. The body weight significantly increased throughout the experiment in all groups, but from week 7 to 12, it was significantly higher in diabetic than in control animals. The sham procedure in one eye and ischemia pulses in the contralateral eye did not affect the body weight in control or diabetic rats at any of the time points examined. Fasting plasma glucose levels were significantly higher in animals receiving sucrose + STZ than in control animals, while the sham procedure in one eye and ischemia pulses in the contralateral eye did not affect this parameter in control or diabetic animals (Fig. 3). Results of the IPGTT are shown in Fig. 4. At 12 weeks of treatment, glucose levels assessed before, and 30, 60, and 120 min after an i.p. administration of 1 g/kg glucose, and the average AUC was calculated for all the experimental groups. Higher values of AUC were observed for diabetic groups as compared with control groups, while ischemia pulses and the sham procedure did not affect this parameter. Plasma glucose levels were assessed at 11.00 and 23.00 h in non fasted animals (Fig. 5). Diurnal (i.e. at 11.00 h) and nocturnal (i.e. at 23.00 h) glycemia was significantly higher in diabetic animals as compared with control animals. Ischemia pulses and a sham procedure did not modify diurnal or nocturnal glycemia in control or diabetic animals. Insulin resistance was significantly higher in diabetic animals with intact eyes or submitted to ischemia pulses (in one eye) and a sham procedure (in the other eye) than in control animals, as shown in Fig. 6.

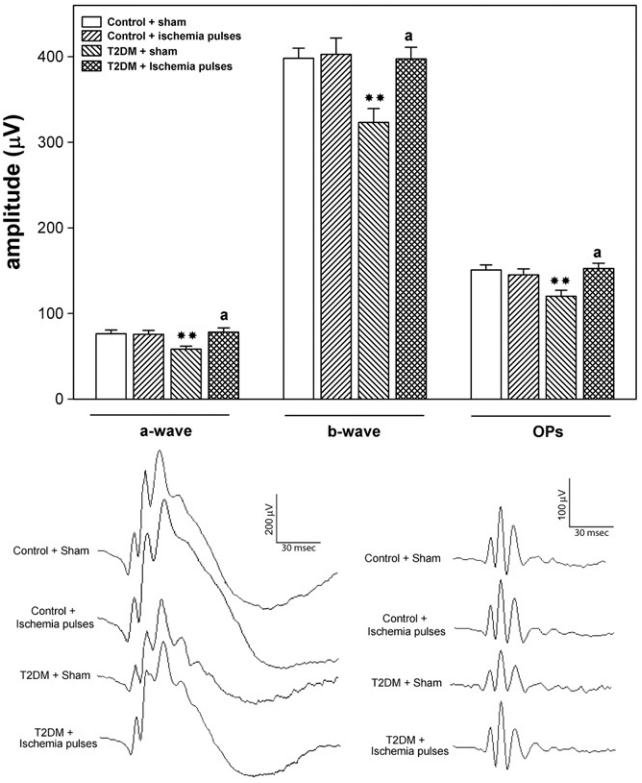

Fig. 7. Effect of ischemic tolerance on retinal function. Upper panel: average amplitudes of scotopic ERG a-wave, b-wave and OPs. At 12 weeks of treatment, a significant decrease in the amplitude (but not their latencies) of ERG a-wave, b-wave, and OP amplitude was observed in eyes from diabetic rats submitted to a sham procedure, as compared with eyes from control rats submitted to a sham procedure or ischemia pulses. Ischemia pulses significantly prevented the retinal dysfunction induced by experimental T2DM. Lower panel: representative scotopic ERG traces from all experimental groups. Data are the mean ± SEM ($n = 15$ eyes/group), $^{**}P<0.01$ vs. control + sham procedure; a: $P<0.05$ vs. T2DM + sham procedure, by Tukey's test.

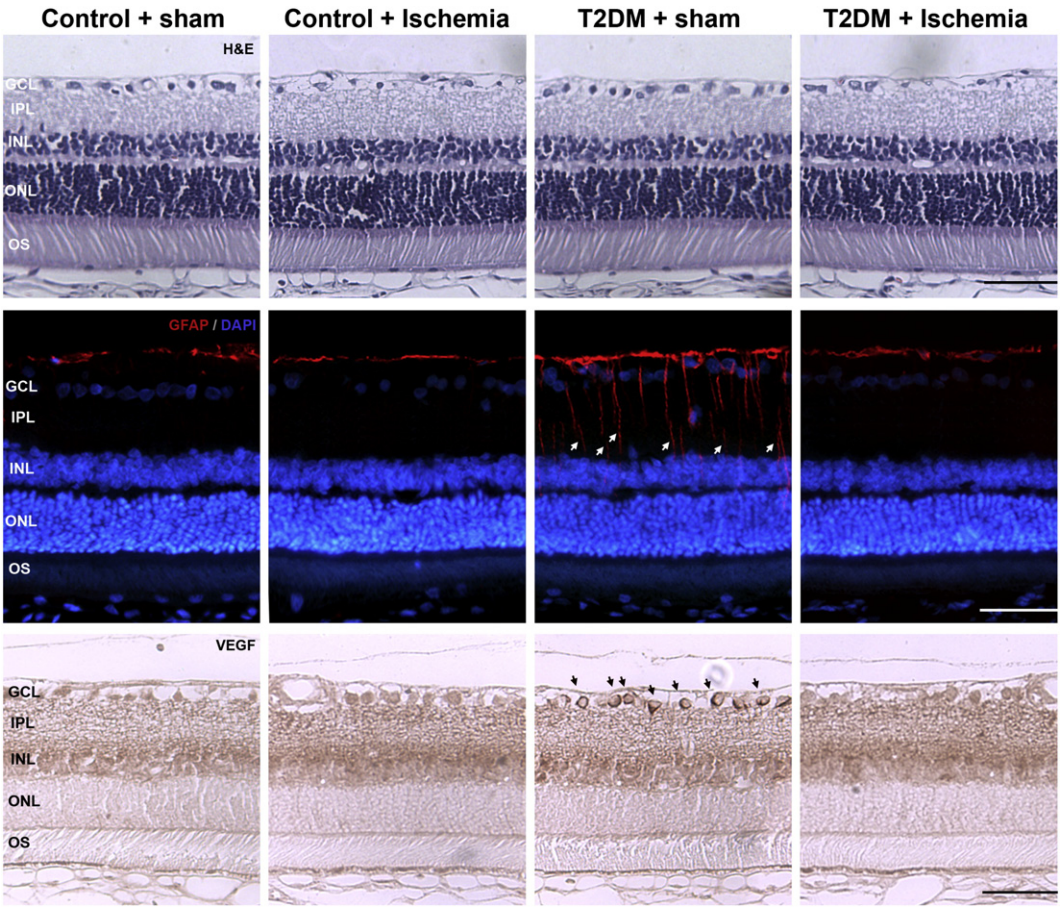

Fig. 8. Effect of ischemia pulses on retinal histology. Upper panel: representative photomicrographs of retinal sections from all the experimental groups stained with H&E. No evident changes in retinal morphology were observed among groups. Middle panel: immunohistochemical detection of GFAP. In eyes from diabetic rats submitted to a sham procedure, an intense GFAP (+) immunoreactivity was observed in astrocytes and glial processes from the inner limiting membrane to the outer retina associated with activated Müller cells (white arrows), whereas in the other groups, GFAP immunostaining was restricted to astrocytes localized in the nerve fiber layer. Lower panel: VEGF in retinal sections. In eyes from control rats (sham procedure or ischemia pulses) and diabetic rat eyes submitted to ischemia pulses, a weak VEGF-immunoreactivity was diffusely observed throughout the inner retina. In eyes from diabetic animals submitted to a sham procedure, intense immunoreactivity in ganglion cell layer (GCL) cells (black arrows) was observed. Scale bar: 50 μm. Shown are photomicrographs representative of 6 retinas/group. IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; OS, outer segments of photoreceptors.

Retinal studies

The functional state of the retinas was analyzed by scotopic electroretinography. The average amplitude of scotopic ERG a-wave, b-wave, and OP amplitudes at 12 weeks of sucrose treatment and representative ERG traces are shown in Fig. 7. These parameters were significantly reduced in eyes from diabetic animals submitted to a sham procedure as compared with control eyes submitted to a sham procedure or ischemia pulses. Ischemia pulses significantly decreased the effect of experimental T2DM on these parameters. No differences in the ERG a-wave, b-wave, and OP latencies were observed among groups (data not shown).

At 12 weeks of treatment, the retinal structure was examined by light microscopy. The integrity of the laminar structure of the retina was intact in all groups (Fig. 8, upper panel). The immunohistochemical assessment of GFAP is shown in the middle panel of Fig. 8. In retinas from control eyes (submitted to a sham procedure or ischemia pulses) and in retinas from diabetic animals submitted to ischemia pulses, astrocytes localized in the nerve fiber layer were GFAP-immunopositive, whereas in retinas from diabetic animals submitted to a sham procedure, an increase in retinal GFAP levels in glial processes from the inner limiting membrane to the outer retina associated with activated Müller cells was observed. VEGF levels in the ganglion cell layer (GCL) from diabetic animals submitted to a sham procedure increased in comparison with retinas from the control, ischemia pulses, T2DM + ischemia pulses groups (Fig. 8, lower panel).

Retinal TBARS levels (an index of lipid peroxidation), and catalase activity were assessed at 12 weeks of sucrose treatment. Experimental T2DM increased lipid peroxidation and decreased catalase activity, whereas ischemia pulses reduced retinal oxidative stress in diabetic animals (Fig. 9). Retinal NOS activity and TNFα levels were significantly higher in diabetic than in control eyes, whereas ischemia pulses, which showed no effect *per se*, reduced the increase in these parameters, as shown in Fig. 9.

Discussion

The present results indicate that a weekly application of 5-minute retinal ischemia pulses, which showed no effect *per se*, decreased retinal alterations observed in an experimental model in rats which mimics early stages of human T2DM. Although the influence of duration and frequency of ischemia pulses on retinal protection deserves to be further examined, these results indicate that a significant restoration of retinal alterations associated with experimental T2DM can be achieved by the procedure performed herein. Notably, the retinal protection induced by ischemia pulses was independent from the metabolic alteration, as shown by the fact that ischemic pulses in one eye and a sham procedure in the contralateral eye did not affect the body weight gain, fasting hyperglycemia, diurnal and nocturnal hyperglycemia in non fasted animals, glucose tolerance, and insulin resistance.

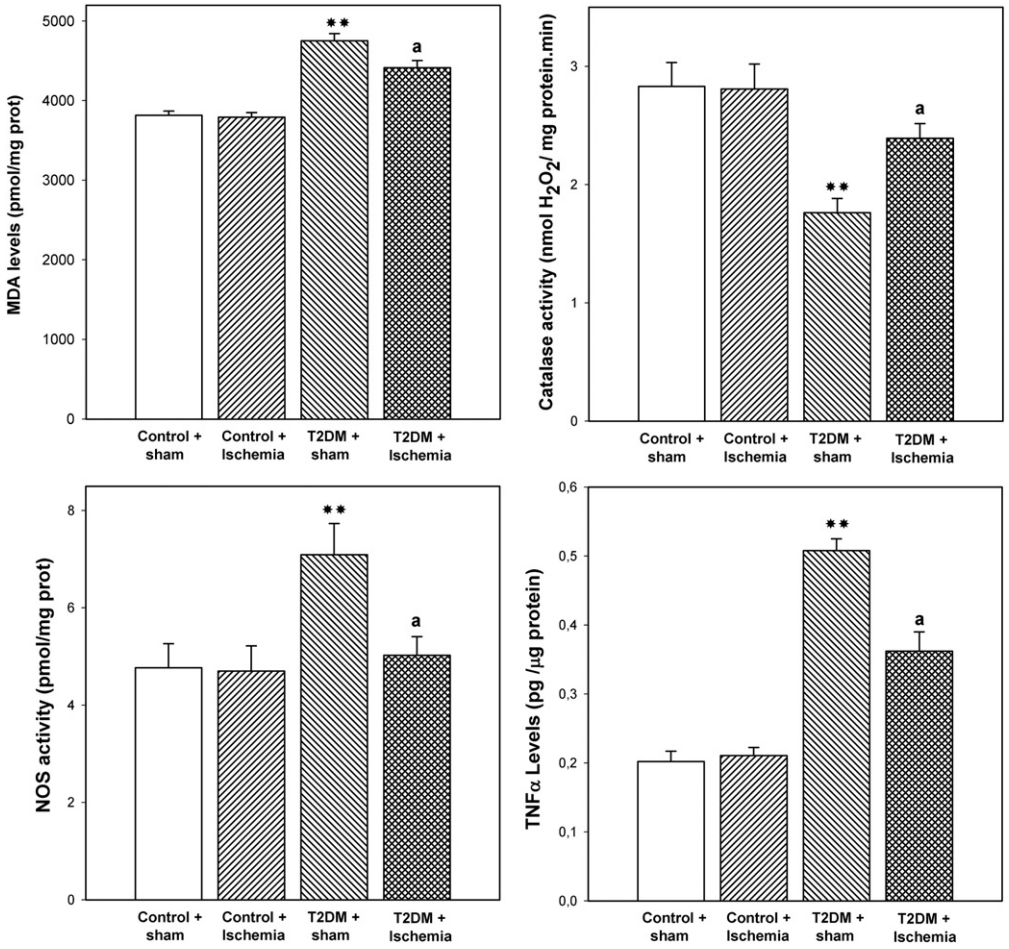

Fig. 9. Effect of ischemic tolerance on retinal TBARS, catalase and NOS activity, and TNFα levels. Retinal lipid peroxidation, NOS activity, and TNFα levels were significantly higher, and catalase activity was significantly lower in eyes from diabetic animals submitted to a sham procedure than in the other groups. Data are mean ± SE ($n = 6$ retinas/group), $^{**}P<0.01$ vs. control + sham procedure; a: $P<0.05$ vs. T2DM + sham procedure, by Tukey's test.

In a previous report, we showed that ischemic tolerance prevents retinal changes in the widely used STZ-induced type 1 diabetes rat model (Fernandez et al., 2011). High doses of STZ rapidly lead to destruction of pancreatic β-cells with acute insulin deficiency, body weight loss, and a pronounced reduction of lifespan, which markedly differ from the clinical and metabolic features that characterize human T2DM, and from the metabolic alterations induced by this experimental model of early stages of T2DM. Notably, despite that different pathogenic mechanisms could be involved in retinal alterations induced by type 1 and type 2 experimental diabetes, the application of ischemia pulses was effective in reducing retinal changes in both experimental models (i.e. STZ-induced type 1 diabetes and T2DM), supporting that retinal ischemia could play a key role in both types of diabetes-induced retinopathy.

The ERG is considered a sensitive marker of early neuronal abnormalities, long before DR can be clinically detected (Tzekov and Arden, 1999). As shown herein, experimental T2DM induced significant alterations in the retinal function which were reduced by ischemic tolerance. It has been postulated that Müller cells may be critical in the initial steps of retinopathy in T2DM (Cunha-Vaz and Bernardes, 2005). GFAP is an intermediate filament that is normally expressed in astrocytes but not in retinal Müller cells. However, in a variety of retinal injuries, including STZ-induced DR (Hammes et al., 1995), GFAP expression in retinal Müller cells became evident. Therefore, GFAP expression in Müller cells is widely used as a molecular indicator for retinal stress. The combined treatment provoked alterations in Müller cells (as shown by the increase in GFAP immunoreactivity), whereas brief ischemia pulses decreased the reactive expression of GFAP in Müller cells.

Although the pathogenesis of DR is still far from being fully understood, VEGF is recognized as a major contributor to its development, and is implicated as the initiator and mediator of nonproliferative and proliferative DR (Aiello and Wong, 2000; Lutty et al., 1996). In fact, clinical trials using anti-VEGF therapies are showing promising results against advanced stages of DR (Nicholson and Schachat, 2010). Our results indicate that ischemic tolerance reduced retinal VEGF levels in diabetic rats.

Enhanced oxidative stress and declines in antioxidant capacity are considered to play important roles in the pathogenesis of chronic diabetes mellitus and its complications (Mokini et al., 2010; Zheng and Kern, 2009). A significant increase in retinal lipid peroxidation and NOS activity, as well as a decrease in catalase activity was observed in diabetic animals, which were reduced by ischemic tolerance. NO is synthesized by constitutive and inducible NOS isoforms. Previous studies have suggested that constitutive NOS mediates some early events in DR (El-Remessy et al., 2003; Joussen et al., 2002); however, given that an inflammatory component has been shown to occur in early DR (do Carmo et al., 1998; Joussen et al., 2001), the iNOS isoform could also be an important potential contributor to NO generation. Thus, the relative relevance of the different NOS isoforms in this particular model of T2DM-induced DR is an important issue that will be examined in the near future.

In DR, abnormalities in vascular and neuronal function are closely related to the local production of inflammatory mediators (Kaul et al., 2010). In that context, a significant increase in retinal TNFα levels was observed in rats submitted to the combined treatment, whereas brief ischemia pulses abrogated the increase in this parameter.

Oxidative, nitrosative, and inflammatory mechanisms appear as a unified pathway leading to metabolic deterioration resulting from hyperglycemia, and insulin resistance (Korkmaz et al., 2012). The present results indicate that ischemic tolerance can behave as an antioxidant, antinitridergic, and anti-inflammatory, and anti-VEGF therapy. Notwithstanding, the involvement of other mechanisms which have been implicated in retinal IPC (such as heat shock proteins (Biermann et al., 2011), hypoxia-inducible factor −1 alpha and heme oxygenase-1 (Zhu et al., 2007), mitogen-activated protein kinase p38 (Dreixler et al., 2009a), protein kinase B/Akt (Dreixler et al., 2009b), and mitochondrial K^+/ATP channels (Roth et al., 2006), among others), and that are crucial to retinal protection against acute ischemic damage, cannot be excluded. Therefore, further studies to determine the mechanisms behind ischemic tolerance will provide a more complete picture of how neuroprotection is achieved in the context of retinal diabetic damage.

Conclusions

These results suggest that ischemic tolerance reduced retinal alterations observed in an experimental model which mimics early stages of human T2DM. Many exogenously delivered chemical preconditioning agents (e.g., inflammatory cytokines, anesthetics, and metabolic inhibitors) can also induce ischemic tolerance, raising the hope that in the future, IPC could be pharmacologically mimicked *in vivo* (Gidday, 2006), leading to advancements in treatments against diabetic retinopathy associated to T2DM.

Disclosures

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