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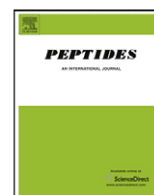

Blockade of early and late retinal biochemical alterations associated with diabetes development by the selective bradykinin B_1 receptor antagonist R-954

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ABSTRACT

The chronic hyperglycemia measured alongside diabetes development is associated with significant long-term damage and failure of various organs. In the present study it was shown that hyperglycemia induced early and long term increases in nitric oxide (NO) levels, kallikrein activity and vascular capillary permeability measured as plasma extravasation, and decreases of Na/K ATPase activity in diabetic rat retina 4 and 12 weeks after streptozotocin (STZ) injection. Treatment of the animals for 5 consecutive days with a novel selective bradykinin B_1 receptor (BKB_1-R) antagonist R-954 (2 mg/kg s.c) at the end of the 4 and 12 week periods highly reduced NO, kallikrein and capillary permeability and increased Na/K ATPase activity in the retina. These results suggest that the BKB_1-R receptor subtype is over-expressed during the streptozotocin-induced development of diabetes in rat retina as evidenced by the inhibitory effects of the BKB_1-R antagonist R-954 on NO, kallikrein and vascular permeability increases as well as Na/K ATPase decreases. The beneficial role of the BKB_1-R antagonist R-954 for the treatment of the diabetic retinopathy is also suggested.

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1. Introduction

Diabetic retinopathy, the leading cause of acquired blindness in young adult, is a multifactorial disease. Type II diabetes, a life long progressive disease, is the result of insulin resistance i.e. the body's inability to respond to the insulin commands, and is characterized by high circulating glucose. The development of diabetic hyperglycemia has been postulated to initiate early and long-term damages and failure of various organs. Being a chronic disease, diabetes induces a sustained hyperglycemia which leads to both systemic microvessel and macrovessel damages. Blood vessel swelling and leaking induce further microvasculature damages which in turn favor new vessel growth and ultimately to retina detachment. Hyperglycemia appears to be a critical factor in the etiology of diabetic retinopathy and initiates downstream events including basement membrane thickening [14], pericyte drop out [17], and decreased retinal capillary perfusion [7]. The diabetic retinal alterations, retinal hemorrhage and fractional retinal detachment, can eventually lead to blindness. Results obtained with animal models such as the streptozotocin-treated rats suggest that long-term hyperglycemia is necessary to elicit changes to the retinal vasculature [3]. In pathological conditions such as diabetes in humans, structural complications in retina were shown to be associated with increased vascular permeability, oxidative stress and vascular endothelial growth factor (VEGF) release as well as changes in activity of protein kinases [1] and kallikrein–kinin system [25]. The present study was undertaken to investigate: (1) Alterations in retinal homeostasis associated with short and long-term diabetes as well as the modifications on capillary permeability, nitric oxide (NO) level, kallikrein and Na/K ATPase activities and (2) the protective effect of the BKB_1-R antagonist on these alterations in rat diabetic retinopathy.

2. Materials and methods

Male Wistar rats weighing between 200–230 g were used. All experiments were carried out in accordance with the ethical recommendations and guidelines of the University of Argentina John F Kennedy Council of Animal Care. Rats were given a single i.p. dose (55 mg/kg) of streptozotocin (STZ). Diabetes was confirmed

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by measuring blood glucose level 72 h after STZ treatment, using blood samples from the tail vein. Blood glucose averaged 11.2 ± 2.5 and 28.2 ± 3.1 mM/L in rats 4 and 12 weeks after the STZ treatment, respectively.

2.1. Experimental protocol

Rats were divided into four groups, each made of six to seven animals: (a) Control injected with saline s.c.; (b) Control treated with R-954 (2 mg/kg s.c); (c) Diabetics (for 4 and 12 weeks) treated with saline and (d) Diabetics treated with R-954 (2 mg/kg s.c.) for 5 days before the end of the 4 week and 12 week periods. Control and diabetic animals were killed 4 or 12 weeks following the STZ treatment and the retina was dissected out. The collected retina tissues were washed with 50 mM Tris–HCl (pH 7.4) at 4 °C, blotted on filter paper and homogenized in cold 10 mM Tris–HCl, pH 8.2. The homogenates were centrifuged (10,000 × *g*) at 4 °C for 30 min and the supernatants collected for biochemical determinations. Proteins were determined according to the method of Bradford [6]. Nitrites were determined with the Griess reagent, according to Green et al. [13]. Na/K ATPase was measured by the method of Ottolenghi [21]. Briefly retina homogenates were suspended in a medium containing 120 mM NaCl, 30 mM KCl, 5 mM $MgCl_2$, 30 mM histidine, 0.2% BSA and incubated at 37 °C at a pH adjusted to 7.4. ATP (sodium salt) was added to have a 3 mM final concentration. The incubation was stopped in an ice bath adding 50 μl of 50% trichloroacetic acid (TCA). After centrifugation the Pi content of the supernatant was assayed according to the method of Baginski et al. [4]. Tissue kallikrein was determined using p-Val-Leu-Arg-paranitroaniline (S 2266, Chromogenic, Sweden) (abreviated as pNA in Fig. 2) as substrate according to Catanzaro et al. [8].

2.2. Capillary permeability

EB dye contents of the retina were determined according to Lawson et al. [19]. In brief unanesthetized rats were given a caudal venous injection of EB dye (20 mg/kg). The dye was injected 10 min before the rats were sacrificed by decapitation. Thereafter, the chest cavity was opened, and the rats were perfused through the right ventricle to pulmonary artery with 15 ml of heparinized saline (4 U/ml) using a peristaltic pump (10 ml/min). A section of the retina of both eyes were dissected, weighed and one retina were desiccated at 60 °C for 24 h. The remaining tissues were immersed in formamide (4 ml/g wet weight) at 24 °C for 24 h. The absorbance of EB dye extracted in formamide was then measured by spectrophotometry at 620 nm using a plate reader. The concentration of EB was then calculated from a standard curve and expressed as microgram of EB per gram of dry tissues.

2.3. Drugs

Streptozotocin (STZ; SIGMA, St. Louis, MO, USA) was dissolved in saline and injected i.p.; Evans Blue (EB) dye was dissolved in saline and injected in the tail vein; BKB_1-R antagonist R-954 (Ac-Orn-[Oic^2,$\alpha MePhe^5$,D-βNal^7,Ile^8]desArg^{9}BK; IPS Pharma, Sherbrooke, PQ, Canada, was dissolved in saline and administered s.c.

2.4. Statistical analysis

The data are presented as means ± SEM. Multiple comparisons between the various groups were done with ANOVA followed by the Bonferroni test. The level of hyperglycemia in the control group and the streptozotocin-treated groups (in table) were compared with the Student's *t*-test. A probability value $P < 0.05$ was considered significant.

Table 1
Effect of R-954 on blood glucose levels in controls and in groups of rats 4 and 12 weeks following streptozotocin.

Rats	Hyperglycemia (mM/L)
Control	5.6 ± 1.2
Diabetic 4 weeks	11.2 ± 2.5*
Diabetic 4 weeks + R-954	13.1 ± 2.8*
Diabetic 12 weeks	28.2 ± 3.1*
Diabetic 12 weeks + R-954	29.8 ± 2.1*

Data are means ± SEM; $n = 6–7$ rats.
* $P < 0.05$ compared with control group.

3. Results

Four and 12 weeks after the induction of diabetes by the STZ treatment, the rats had a marked hyperglycemia as compared to control animals (Table 1). Blood glucose levels were 5.6 ± 1.2 mM/L in control rats whereas they increased to 11.2 ± 2.5 after 4 weeks and to 28.2 ± 3.1 mM/L after 12 weeks. The BKB_1-R antagonist treatment did not modify blood glucose levels in the 4- or 12-week groups of control animals.

Total NO concentrations in retina homogenates of the 4 and 12-week groups of control animals averaged 3.2 ± 0.5 μmol/mg proteins. As shown in Fig. 1 the total NO concentration in the retina of the 4 and 12-week groups of diabetic rats increased to 4.3 ± 0.9 (NS) and 11.8 ± 1.3 μmol/mg proteins ($P < 0.05$), respectively. The R-954 treatment markedly reduced NO levels in the retina of the 4 and 12-week groups of diabetic rats to 2.7 ± 0.6 and 6.7 ± 0.9 μmol/mg proteins respectively. The changes were only statistically significant in the retina homogenates of the 12-week group of animals.

Kallikrein activity in retina homogenates averaged 32 ± 1.3 nmol pNA/mg proteins whereas in the 4 and 12-week groups it increased to 56 ± 2.3 and 102 ± 2.6 nmol pNA/mg proteins respectively. As shown in Fig. 2, the enzyme activity increased by 175% at 4 weeks of diabetes and by more than 300% at 12 weeks as compared to the levels in control animals ($P < 0.05$). Treatment with the antagonist R-954 lowered the enzyme activity in the retina homogenates to values very close to controls (22 ± 1 and 48 ± 5.2 nmol pNA/mg proteins in the respective groups) ($P < 0.05$).

The vascular capillary permeablility was also measured in control and diabetic rats. In control animals the vascular permeability averaged 17 ± 1.3 μg/g dry tissue. These values increased to 36 ± 1.3 and 68.1 ± 3.8 μg/g dry retina tissue in the 4 and 12-week groups of diabetic rats, respectively ($P < 0.05$). Following the five days administration of R-954, vascular permeability was highly reduced in the retina of diabetic rats and reached 24.2 ± 5.2 and

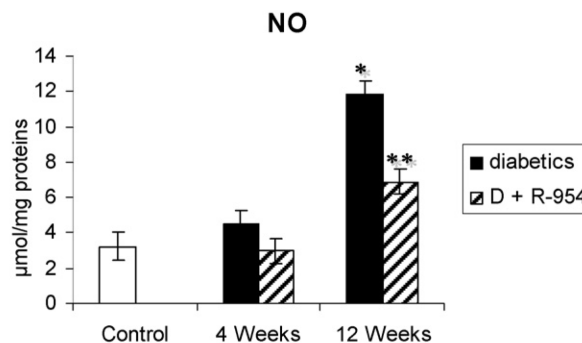

Fig. 1. Effect of BKB_1-R antagonist R-954 on nitric oxide concentration (μmol/mg proteins) in retina homogenates of controls and of groups of rats 4 and 12 weeks following streptozotocin treatment. Results are means ± SEM of 6–7 animals. * $P < 0.05$ diabetics vs. Controls; ** $P < 0.05$ diabetics + R-954 vs. diabetics; $P < 0.05$ diabetics 12 weeks vs. diabetics 4 weeks.

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