RESEARCH PAPER

Expression, distribution and function of kinin B1 receptor in the rat diabetic retina

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BACKGROUND AND PURPOSE
The kinin B₁ receptor contributes to vascular inflammation and blood-retinal barrier breakdown in diabetic retinopathy (DR). We investigated the changes in expression, cellular localization and vascular inflammatory effect of B₁ receptors in retina of streptozotocin diabetic rats.

EXPERIMENTAL APPROACH
The distribution of B₁ receptors on retinal cell types was investigated by immunocytochemistry. Effects of B₁ receptor agonist, R-838, and antagonist, R-954, on retinal leukocyte adhesion, gene expression of kinin and VEGF systems, B₁ receptor immunoreactivity, microgliosis and capillary leakage were measured. Effect of B₁ receptor siRNA on gene expression was also assessed.

KEY RESULTS
mRNA levels of the kinin and VEGF systems were significantly enhanced at 2 weeks in streptozotocin (STZ)-retina compared to control-retina and were further increased at 6 weeks. B₁ receptor mRNA levels remained increased at 6 months. B₁ receptor immunolabelling was detected in vascular layers of the retina, on glial and ganglion cells. Intravitreal R-838 amplified B₁ and B₂ receptor gene expression, B₁ receptor levels (immunodetection), leukostasis and vascular permeability at 2 weeks in STZ-retina. Topical application (eye drops) of R-954 reversed these increases in B₁ receptors, leukostasis and vascular permeability. Intravitreal B₁ receptor siRNA inhibited gene expression of kinin and VEGF systems in STZ-retina. Microgliosis was unaffected by R-838 or R-954 in STZ-retina.

CONCLUSION AND IMPLICATIONS
Our results support the detrimental role of B₁ receptors on endothelial and glial cells in acute and advanced phases of DR. Topical application of the B₁ receptor antagonist R-954 seems a feasible therapeutic approach for the treatment of DR.

Abbreviations
BK, bradykinin; DR, diabetic retinopathy; GCL, ganglion cell layer; GFAP, glial fibrillary acid protein; Iba-1, ionized calcium-binding adapter molecule 1; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; qRT-PCR, real-time quantitative RT-PCR; R-838, Sar-[D-Phe⁸]-desArg⁹-BK; R-954, AcOrn [Oic², (αMe) Phe⁸, DβNal⁹, Ileβ⁺] desArg⁹-BK; RECA-1, rat endothelial cell antigen-1; STZ, streptozotocin

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Kinin B1 receptor in STZ-diabetic retina

Introduction

Diabetic retinopathy (DR) is one of the most frequent complications of diabetes. Approximately 75% of patients living with diabetes for 20 years show clinical signs of retinopathy and over 10% of them are affected by a vision loss (Frank, 2004; Hernandez et al., 2017). Vision loss is the result of slow and gradual alterations in the microvasculature of the retina due to hyperglycaemia and an inflammatory response, which lead to the overexpression of VEGF, breakdown of the blood-retinal barrier, pathological proliferation of blood vessels and the formation of fibrous tissue in the vitreous cavity leading to retinal detachment (Wilkinson-Berka, 2006). In addition to changes in the vascular bed, pathological mechanisms in DR are associated with activation of glial cells and dysfunction of neurons. Principally, reactive microglia, astrocytes and Müller glial cells in the retina produce VEGF (Wang et al., 2010; 2015) and inflammatory mediators that amplify the inflammatory response (Chang et al., 2007; Sorrentino et al., 2016) and could play an important role in the breakdown of the haemato-retinal barrier (Antonetti et al., 1998; Wang et al., 2010). Recent findings suggest a role for the kallikrein-kinin system in the development of inflammation in DR (Wilkinson-Berka and Fletcher, 2004; Phipps and Feener, 2008; Feener, 2010; Bhat et al., 2014). All the components of the kallikrein-kinin system are present in the retina and several studies suggest that metabolic changes associated with diabetes can cause the production of kinins in the retina (Ma et al., 1996; Takeda et al., 1999; Phipps et al., 2009). Although retinal vascular permeability was decreased following systemic and intravitreal injection of a selective plasma kallikrein inhibitor in diabetic rats (Clermont et al., 2011), the relative contribution of tissue kallikrein in DR remains elusive.

Kinins are vasoactive autacoid peptides involved in a variety of biological effects including vasodilatation, inflammation and pain. Kinins are among the first mediators to be released into the injured tissue (Couture et al., 2001). Kinins undergo rapid metabolic degradation by amino-, endo- and carboxypeptidases found in blood, tissues and biological fluids. The aminopeptidases, carboxypeptidases, neutral endopeptidase and angiotensin converting enzyme (also known as kininase II) are the main enzymes responsible for the metabolism of bradykinin (BK) and kallidin (Lys-BK) into active and inactive peptide fragments. These enzymes ensure that the half-life of kinins is only a few seconds (Décarie et al., 1996; Gabra et al., 2003). Kinins exert their biological effects by activating two GPCRs: the bradykinin B1 receptor and B2 receptor (Regoli et al., 2001; Leeb-Lundberg et al., 2005). The constitutive B2 receptor mediates most of the effects of BK and kallidin while the B1 receptor is activated by the C-terminal metabolites des-Arg9-BK and Lys-des-Arg9-BK generated by kininase I (carboxypeptidase M/N). The B1 receptor is weakly expressed under physiological conditions, yet it is strongly induced and up-regulated during tissue injury or after exposure to oxidative stress, bacterial endotoxins, growth factors and pro-inflammatory cytokines such as TNF-α and IL-1β (Zhou et al., 1998; Couture et al., 2014). The induction of B1 receptors by cytokines is regulated by MAPK and the nuclear transcription factor NF-kB (Larriueva et al., 1998; Campos et al., 1999). The B1 receptor is overexpressed in the retina of type 1 and type 2 diabetic patients (Bhat et al., 2014). In the retina of streptozotocin (STZ)-diabetic rats, the B1 receptor is readily up-regulated by a mechanism involving oxidative stress, and contributes to the vasodilatation of microvessels and increased vascular permeability (Abdouh et al., 2003; Abdouh et al., 2008). The non-peptide B1 receptor antagonist LF22-0542 reverses numerous of the pathological processes, occurring after two weeks in retina from STZ-diabetic rats, such as vascular hyperpermeability, leukocyte infiltration and the up-regulation of inflammatory mediators notably VEGF (Pouliot et al., 2012). To better define the physiopathological role of the B1 receptor in DR, we investigated its cellular distribution and its expression during the course of diabetes. For this study, we focused on the nonproliferative stage of DR, and selected 2 weeks of diabetes in most experiments to enable comparison with our previous studies using LF22-0542 (Pouliot et al., 2012). The impact of B1 receptor gain-of-function secondary to intraocular treatments with a highly selective and peptidase resistant B1 receptor agonist (R-838, Sar-[D-Phe6]-des-Arg9-BK) was examined on the main inflammatory events in DR, that is, leukostasis and microgliosis and on the endothelial dysfunction, that is, vascular permeability. The phenotype of B1 receptor activation with R-838 was compared with that of B1 receptor deletion with siRNA on gene expression of kinin receptors and the VEGF system. Lastly, the peptidase resistant B1 receptor antagonist R-954, (AcOrn [Oic2, (aMe) Phe5, DjNal7, lle6] desArg9-BK) was administered topically by eye drops to determine its therapeutic value in this model of DR.

Methods

Animal model and procedures

All experimental methods and animal care procedures were approved by the animal care committee of the Université de Montréal (Protocol 15-063) in accordance with the Canadian Council on Animal Care. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015). Male Wistar rats (200–250 g, 6–8 weeks old) were purchased from Charles River Laboratories (St-Constant, QC, Canada) and housed two per cage using heated wood chip litter as bedding material in a pathogen-free environment and under standard conditions of temperature (22°C) and humidity (43%) on a 12 h light/dark cycle and allowed free access to normal chow diet and tap water. The rats were made diabetic by a single i.p. injection of STZ (Zanosar 65 mg·kg⁻¹; Sigma-Aldrich, Oakville, ON, Canada). The concentration of glucose was measured in blood samples from the tail vein with a glucometer (Accusoft; Roche Diagnostics, Laval, QC, Canada). Only STZ-treated rats with glycaemia above 20 mmol·L⁻¹ were considered diabetic and included in the study. Blood glucose and body weight were recorded three times per week and on the day of the experiment. STZ-diabetic rats were humanely killed with isoflurane when they reached the following ethical endpoints: glycaemia ≥30 mmol·L⁻¹, loss of body weight ≥20%, severe diarrhoea, loss of locomotor activity and rearing. STZ-diabetic rats were randomly divided for each pharmacological
treatment, and the experimenter was blinded to assess immunohistological parameters (B₁ receptor distribution, microglisis and leukostasis) and to perform data analysis. One rat was killed in the STZ treated group with R-838 because the glycaemia was higher than the endpoint.

Pharmacological treatments
The highly selective and metabolically protected peptide B₁ receptor agonist R-838 (Audet et al., 1997) synthesized at the Research Institute of Biotechnology, National Research Council of Canada (Montréal, QC, Canada) was injected intravitreally (100 ng·5 µL⁻¹) twice, to rats anaesthetized with 3% isoflurane, the second injection being given 48 h prior to the experiments. The vehicle (sterile saline solution, 0.9%) was administered to the contralateral eye. Rats with any kind of postoperative complication (cataract or infection) were excluded from analysis. A selective and metabolically protected peptide B₁ receptor antagonist R-954 (Gobeil et al., 2014), kindly provided by Dr Fernand Gobeil Jr (Pharmacology, Université de Sherbrooke, QC, Canada), was applied to unanaesthetized rats twice a day with one eye drop application (=100 µg·10 µL⁻¹) in both eyes during the last week prior to the experiments. R-954 was prepared in sterile saline solution and filtered (0.20 µm mesh). In addition, Ambion in vivo B₁ receptor siRNA (Invitrogen Life Technologies, Canada) was used to determine the efficacy of the B₁ receptor-siRNA strategy in reducing the mRNA levels of B₁ receptors, B₂ receptors, VEGF-A and VEGFR-2, in the retinas of STZ-diabetic rats by real-time quantitative RT-PCR (qRT-PCR) and to confirm the specificity of the polyclonal rabbit antiserum for rat B₁ receptors by immunocytochemistry (Supporting Information Figure S1). After anaesthesia with 3% isoflurane, a single intravitreal injection of siRNA (0.5, 1.0 or 10 nmol) on the day of STZ treatment for qRT-PCR and 2 days after STZ treatment for immunohistochemistry was performed. Rats were killed 1 week later, for the qRT-PCR or immunofluorescence analysis (see below).

No ocular irritation such as redness, porphyrin secretion or corneal opacity was seen 1 week after treatment with R-838, R-954 or B₁ receptor siRNA. In a previous study, s.c. administration of R-954 corrected the enhanced NO, kallikrein and vascular permeability in the retina of STZ-diabetic rats at 4 and 12 weeks (Catanzaro et al., 2012).

Moreover, a mixture of 10 µL of the radiolabelled [³H]-R-954 (Vitrax, 1 µg·µL⁻¹, specific activity: 225 mCi·mmol⁻¹, molar mass: 1194.42 g·mol⁻¹) with 1 µL of cold R-954 (100 µg·µL⁻¹) was applied on the surface of both eyes 1, 3 and 12 h (one rat for each time point) before the rat was killed to assess its ability to diffuse into the different structures. The cornea, vitreous, lens, retina and choroid from each eye of the STZ-treated rats were removed and digested in 300 µL of soluene (PerkinElmer, Boston, USA) for 2 h at 37°C. A total of 5 mL of scintillation fluid (UltimaGold, Perkin Elmer, Boston, USA) was added, and the radioactivity was counted in a scintillation counter (LS6500, Beckman Coulter, Mississauga, ON, Canada). Data are expressed as ng g⁻¹ of wet tissue, calculated from the DPM counts, specific activity and molar mass of [³H]-R954. The percentage of [³H]-R-954 represents the levels of radioactivity distributed within the various tissues.

Measurement of retinal inflammatory markers by quantitative RT-PCR
Control, 2 weeks, 6 weeks and 6 months STZ-treated rats were anaesthetized with urethane (1.5 g·kg⁻¹), and the eyes were dissected out. The retinas were isolated and immerged in RNAlater stabilization reagent (QIAGEN, Valencia, CA, USA). First-strand cDNA synthesized from 400 ng total RNA with random hexamer primers was used as the template for each reaction with the QuantiTect Rev Transcription Kit (QIAGEN). SYBR Green-based real-time quantitative PCR using Mx3000p device for signal detection (Stratagene, La Jolla, CA, USA) was performed as previously described (Pouliot et al., 2012). A PCR was performed in SYBR Green Master mix (QIAGEN) with 300 nM of each primer. For standardization and quantification, rat 18S mRNA was amplified simultaneously. The primer pairs were designed by Vector NTI software (Table 1). PCR conditions were as follows: 95°C for 15 min, followed by 46 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. The relative differences in gene expression between treatment groups and control were determined using the ΔΔCt method. Each sample was run in duplicate to ensure the reliability of single values.

Immunofluorescent and immunocytochemistry staining
The specificity of the polyclonal rabbit antiserum for B₁ receptors was shown previously in B₁ receptor knockout (KO) mice tissues (Lin et al., 2010; Lacoste et al., 2013) and further determined in the present study using retina from wild-type (WT) and B₁/B₂ receptor KO mice (provided by Dr Jean-Pierre Girolami, Université Paul Sabatier, Toulouse, France). In contrast to WT mice retina, B₁ receptor immunostaining was totally absent in the retina of B₁/B₂ receptor KO mice (Supporting Information Figure S1). Furthermore, the specificity of the B₁ receptor antiserum was confirmed in STZ rat retina treated with B₁ receptor siRNA (10 nmol) showing a complete absence of B₁ receptor labelling. Also no staining was found in STZ rat retina incubated with the pre-immune serum as a negative control (Supporting Information Figure S1).

For immunofluorescent staining, rats were perfused with a paraformaldehyde solution (2% PFA), and the eyes were dissected out, post-fixed in 2% PFA for 2 h and then frozen in isopentane (−55°C). Eyes were cut into 20-µm-thick sections with a cryostat and placed onto glass slides. Alternatively, they were embedded in paraffin after post-fixation, cut into 5-µm-thick sections and placed onto glass slides. The samples were treated to remove paraffin prior to the immunocytochemistry experiment. Tissue sections were incubated within a 20 µL staining dish containing sodium citrate buffer at 95°C. The citrate-based solution breaks the PFA-induced protein cross-links, therefore, unmask the antigens and epitopes and enhances the intensity of staining of the antibodies. Sections were placed at room temperature (RT) and allowed to cool down for 20 min. Then, sections were washed for 10 min in 0.1 M PBS buffer (pH 7.4) and incubated for 1 h (RT) in blocking buffers (PBS containing 10% donkey serum and 0.25% triton X-100) to prevent non-specific labelling. Sections were incubated overnight at RT with the blocking buffer containing one of the following primary antibodies:
polyclonal rabbit antiserum to rat B1 receptor 1:150, mouse monoclonal anti-endothelial cells [rat endothelial cell antigen-1 (RECA-1), ab 9774; ABCAM] 1:500; mouse monoclonal anti-glial fibrillary acid protein (GFAP) (IF03L, Millipore Sigma) 1:500 to label astrocytes, mouse polyclonal anti-ionized calcium binding adapter molecule 1 (Iba-1; Wako, Richmond, VA, USA) 1:500 (2 μg·mL⁻¹) to label microglia and chicken monoclonal anti-VEGFR-2 (GW21181, Abcam) 1:150, the sections were cleared with xylene and then mounted using DPX mounting medium.

**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Position</th>
<th>GenBank accession no</th>
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<tbody>
<tr>
<td>B1R</td>
<td>Forward 5’</td>
<td>CAGCCGCTTAACCATAACCGGAAAT</td>
<td>3’ 67–390</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’</td>
<td>CAGTTGAACCGGTCCGAGTGGT</td>
<td>3’ 454–431</td>
</tr>
<tr>
<td>B2R</td>
<td>Forward 5’</td>
<td>ACGTGCTGGAGAACAACAGAGTA</td>
<td>3’ 882–905</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’</td>
<td>TCCAGGAAGTGTGTATCTGGA</td>
<td>3’ 990–967</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Forward 5’</td>
<td>TCACAAAACCCGACACTATGGAGA</td>
<td>3’ 1219–1242</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’</td>
<td>TTACACGTCTCAGATCTTGGACA</td>
<td>3’ 371–1348</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>Forward 5’</td>
<td>AGTGGCTAAGGGGAGTGATCCTT</td>
<td>3’ 3269–3292</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’</td>
<td>GGGCAAGCGCGATGCAACGATT</td>
<td>3’ 3387–3292</td>
</tr>
<tr>
<td>18S</td>
<td>Forward 5’</td>
<td>TCACCTTCGATGATGATCCCGGT</td>
<td>3’ 363–385</td>
</tr>
<tr>
<td></td>
<td>Reverse 5’</td>
<td>TCTTTGATGATGATCCCGGTTT</td>
<td>3’ 470–447</td>
</tr>
</tbody>
</table>

**Quantification of B1 receptors and Iba-1 immunolabelling**

Microphotographs were obtained with a Leica microscope (Leica microsystems Co., Germany). Tissues from each experimental group were processed and imaged in parallel. The camera setting was identical for acquisition of images from all sections. Leica LCS Lite software was used to quantify the mean grey values in the retinal ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL). Data are expressed as the mean pixel energy ratio from an average of 10 perikarya in the GCL or 20 perikarya in the INL and ONL for each retinal image quantified in four rats.

**Measurement of retinal leukostasis**

The rats were anaesthetized with urethane (1.5 g·kg⁻¹) and a 16G cannula was inserted into the left heart ventricle. Rats were perfused with PBS over 1 min (35 mL·min⁻¹). FITC-coupled concanavalin A lectin was infused (20 μg·mL⁻¹ in PBS, 5 mg·kg⁻¹, 30 mL·min⁻¹). Then 4% PFA was perfused for 1 min followed by albumin 1% in PBS for 1 min and by PBS for 2 min (35 mL·min⁻¹). Eyes were removed, then the retinas were dissected out and flat mounted on a glass slide and imaged using a fluorescence microscope (Leica Microsystems Co., Germany).

**Measurement of retinal vascular permeability**

Retinal vascular permeability was measured using the Evans blue dye technique as described previously (Pouliot et al., 2012). The rats were anaesthetized with urethane (1.5 g·kg⁻¹) and a catheter (Micro-Renathane, I.D. 0.040, O.D. 0.020, Braintree Scientific, Braintree, MA, USA) was inserted into the right femoral vein. Evans blue dye (45 mg·mL⁻¹ in saline) (Sigma-Aldrich, Oakville, ON, Canada) was injected i.v. for 10 s, and 1.5 h later, saline (25 mL) was infused through the left heart ventricle to wash out intravascular dye. Eyes were removed, then the retina was dissected out and immediately weighed. Evans blue dye was then extracted by incubating each retina in 500 μL of formamide (Sigma-Aldrich, Oakville, ON, Canada) for 18 h at 70–75°C. The fluorescence of Evans blue was measured using a spectrophotofluorometer (Spex 1681 0.22 m, Horiba Jobin Yvon Inc, Edison, NJ, USA) using the wavelength of 620 nm (excitation) and 680 nm (emission).

**Statistical analysis**

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Data are expressed as mean ± SEM of values and ‘n’ refers to the number of rats for each set of experiments. Multiple comparisons between groups were performed using the non-parametric ANOVA Kruskal–Wallis test and post hoc Dunn’s test for leukostasis and cellular

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distribution of B_1 receptors and microglia quantification. One-way ANOVA followed by the Bonferroni test was used for vascular permeability, Table 2 and gene expression when F achieved P < 0.05. Statistical analysis was performed using Prism™ version 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Only probability values (P) less than 0.05 were considered to be statistically significant.

**Nomenclature of targets and ligands**

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017a,b).

**Results**

**Blood glucose levels and body weight**

Blood glucose concentrations were significantly increased while body weights were significantly decreased in STZ-diabetic rats at 2 weeks, 6 weeks and 6 months when compared to age-matched control rats (Table 2). One week of treatment with R-838 or R-954 had no significant effect on glycaemia and body weight in STZ-diabetic rats and control rats.

**Distribution of topically applied [3H]-R-954**

To make sure that the topically applied B_1 receptor antagonist on the ocular surface was able to reach the retina, we performed a preliminary experiment to evaluate the distribution of [3H]-R-954 within the ocular compartments of STZ-diabetic rat, at 1, 3 and 12 h post-administration. The results presented in Table 3 reveal that [3H]-R-954 was present in the retina 1 h after topical application and declined gradually thereafter. The major route of elimination of [3H]-R-954 is likely the choroid with its highest blood flow, suggesting that [3H]-R-954 crosses readily through the different tissues before reaching the choroid at 1 h and accumulating gradually in the choroid at 3 and 12 h after application. Because [3H]-R-954 is not metabolized in rat tissue homogenates kept at room temperature (Gobeil Jr. et al., 2014), only its intact form can be found in each tissue compartment.

**Effect of B_1 receptor stimulation on mRNA levels of kinin receptors and the VEGF system in the STZ-retina**

Results depicted in Figure 1 showed significant and similar diabetes-induced increases (=6-fold) of mRNA levels for B_1 receptors, B_2 receptors, VEGF-A and VEGFR-2 in the retina of 2 weeks STZ-diabetic rats compared to control retina. Retinal mRNA levels of all these markers were significantly higher at 6 weeks than at 2 weeks in vehicle-injected STZ-diabetic rats. Intravitreal injection of R-838 significantly further increased retinal mRNA levels of B_1 and B_2 receptors both at 2 and 6 weeks but not the mRNA of VEGF-A and VEGFR-2 when compared to vehicle-injected STZ-diabetic rats. The mRNA levels of the four markers in the retina of control rats were not changed by two intravitreal injections of R-838 at 48 h apart when compared to control vehicle at 2 weeks. However, R-838 caused a small but significant increase in B_1 and B_2 receptor mRNA levels in the 6 weeks control. B_1 receptors remained overexpressed (mRNA increased by ≈6-fold) at 6 months of diabetes, but the level of expression was not significantly enhanced by R-838 (Figure 1A’).

In a separate series of experiments performed in 2 weeks STZ-diabetic rats, retinal mRNA levels of B_1 receptors, B_2 receptors, VEGF-A and VEGFR-2 were significantly increased (fourfold to sixfold) compared to control rats. These increases in STZ-retinas were dose-dependently prevented by B_1 receptor siRNA. Doses of 1.0 or 10 nmol of B_1 receptor siRNA totally prevented any increases in B_1 receptor, B_2 receptor, VEGF-A and VEGFR-2 mRNA expression (Figure 1E).

| Table 2 |

Effects of diabetes and B_1 receptor agonist (R-838) and antagonist (R-954) treatments on body weight and glycaemia

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Ctl-vehicle (n = 6)</th>
<th>Ctl-R-838 (n = 6)</th>
<th>STZ-vehicle (n = 6)</th>
<th>STZ-R-838 (n = 5)</th>
<th>STZ-R-954 (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>351 ± 8</td>
<td>353 ± 4</td>
<td>264 ± 12*</td>
<td>260 ± 13*</td>
<td>260 ± 8*</td>
</tr>
<tr>
<td>6 weeks</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>358 ± 7</td>
<td>364 ± 4</td>
<td>279 ± 12*</td>
<td>268 ± 13*</td>
<td>–</td>
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<tr>
<td>6 months</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>430 ± 11</td>
<td>–</td>
<td>249 ± 5*</td>
<td>252 ± 5*</td>
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<tr>
<td>2 weeks</td>
<td></td>
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<tr>
<td>Glycaemia (mmol·L⁻¹)</td>
<td>5.2 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>26.6 ± 1.9*</td>
<td>27.4 ± 1.0*</td>
<td>25.7 ± 1.8*</td>
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<td>6 weeks</td>
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<tr>
<td>Glycaemia (mmol·L⁻¹)</td>
<td>5.0 ± 0.3</td>
<td>5.0 ± 0.1</td>
<td>27.7 ± 1.7*</td>
<td>28.0 ± 1.3*</td>
<td>–</td>
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<tr>
<td>6 months</td>
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<tr>
<td>Glycaemia (mmol·L⁻¹)</td>
<td>5.1 ± 0.4</td>
<td>–</td>
<td>28.8 ± 2.0*</td>
<td>29.5 ± 1.4*</td>
<td>–</td>
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</table>

Values are mean ± SEM of five to six rats per group. Statistical significance was determined with one-way ANOVA followed by the Bonferroni test. *P < 0.05 significantly different from control (Ctl)-vehicle.
Relative mRNA expression of kinin receptors and the VEGF system in the retina at different time points of diabetes. mRNA levels of B₁ receptors (B₁R; A, A'), B₂ receptors (B₂R; B), VEGF-A (C) and VEGFR-2 (D) in control (Ctl; fold = 1) and STZ-retina at 2 weeks (open graphs), 6 weeks (black graphs) and 6 months (A', grey graphs, B₁R only) with or without intravitreal administration of the B₁ receptor agonist R-838. (E) The impact of one intravitreal administration of B₁ receptor siRNA (0.5, 1.0 or 10 nmol) on mRNA levels of B₁ receptors, B₂ receptors, VEGF-A and VEGFR-2 in STZ-retina at 2 weeks in comparison to control (Ctl, fold = 1.0). Data are mean ± SEM of values from five to six rats in each group. Statistical comparison to Ctl-vehicle (*) and between 2 and 6 weeks (+) or between STZ-vehicle and STZ-R-838 (&) is indicated by *,+,& P < 0.05. Comparison to Ctl (*) or to STZ without siRNA (&) is indicated by*,& P < 0.05.

Table 3
Eye tissue distribution of [³H]-R-954 at 1, 3 and 12 h post-treatment

<table>
<thead>
<tr>
<th>Tissues</th>
<th>1 h post-treatment</th>
<th>3 h post-treatment</th>
<th>12 h post-treatment</th>
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<tbody>
<tr>
<td></td>
<td>ng (μCi·g⁻¹ wet tissue)</td>
<td>%</td>
<td>ng (μCi·g⁻¹ wet tissue)</td>
</tr>
<tr>
<td>Cornea</td>
<td>300.5 (56.6)</td>
<td>14</td>
<td>35.0 (6.6)</td>
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<tr>
<td>Lens</td>
<td>71.8 (13.5)</td>
<td>34</td>
<td>29.0 (5.4)</td>
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<tr>
<td>Vitreous</td>
<td>17.6 (3.3)</td>
<td>8</td>
<td>5.4 (1.0)</td>
</tr>
<tr>
<td>Retina</td>
<td>59.5 (11.2)</td>
<td>28</td>
<td>13.1 (2.5)</td>
</tr>
<tr>
<td>Choroid</td>
<td>32.6 (6.1)</td>
<td>15</td>
<td>42.3 (8.0)</td>
</tr>
</tbody>
</table>

Values are in ng of [³H]-R-954·g⁻¹ wet tissue (μCi·g⁻¹ wet tissue) calculated at 1, 3 and 12 h after eye drops application in STZ-diabetic rats (two eyes from one rat for each time point). The radioactivity was counted in each tissue, and the amount was expressed as a percentage of the total (%).
Effect of B1 receptor agonist and antagonist on B1 receptor immunoreactivity in the retina at 2 weeks of diabetes

The distribution of B1 receptors in the retina and the intensity of staining in the different experimental conditions were studied by immunocytochemistry. The immunodetection of B1 receptors was located mainly in vascularized layers of the retina, particularly in the retinal GCL, the INL and the ONL (Figure 2, upper panel). While basal B1 receptor immunostaining was virtually absent in the retina of control rats (Figure 2A), it was intense in the retina of 2 weeks STZ-diabetic rats (Figure 2B). Intravitreal treatment with R-838 further enhanced B1 receptor staining intensity in the retina of STZ-diabetic rats (Figure 2C), while eye drops application of R-954 reduced it markedly in STZ-retina (Figure 2D). Semi-quantitative analysis showed significant increases in B1 receptor immunolabeling in the three layers of the STZ-retina in comparison to control retina (Figure 2E). Treatment with R-838 did not significantly increase the B1 receptor labelling in STZ-retina compared to the STZ group receiving vehicle despite a tendency in GCL and INL. In contrast, treatment with R-954 abolished the increases in B1 receptor staining in GCL, INL and ONL in STZ-retina (Figure 2E).

Effect of B1 receptor agonist and antagonist on retinal leukostasis at 2 weeks of diabetes

The pharmacological effect of R-838 and R-954 on leukocyte infiltration, which is an inflammatory event strongly involved in the pathogenesis of DR, was evaluated (Figure 2, middle panel). The leukocytes were labelled with FITC-concanavalin A lectin (Figure 2A′–D′). The number of adherent leukocytes to the retinal vascular endothelium was significantly higher (Figure 2E′) in STZ-diabetic retina compared to control. Although treatment with R-838 did not enhance further the leukostasis in the STZ-retina compared with vehicle-injected STZ-retina, R-954 significantly decreased leukocyte adherence and infiltration in STZ-diabetic retina. Some leukocytes were occasionally seen within the retinal tissue in STZ treated with R-838 (Figure 2C′, arrowhead). This invasion of the retina by leukocytes indicates a strong inflammatory process controlled by B1 receptors.

Effect of B1 receptor agonist and antagonist on spatial distribution of microglia reactivity at 2 weeks of diabetes

Microglia cells were found chiefly within the layer of neuronal fibres (Figure 2A′–D′, lower panel). In pathological conditions, these phagocytic cells can be present throughout the retina, where they produce not only pro-inflammatory cytokines but also factors that ensure the survival of vascular cells and neurons (Chen et al., 2002). Here, we determined whether the B1 receptor has an effect on microglia reactivity, an event that occurs early in the development of DR. In the retina of control rats, microglia morphology was characterized by a small soma with dense ramifications (Figure 2A″). The microglia was distributed mainly in GCL (Figure 2A″–D″, E″). In the retina of 2 week STZ-diabetic rats, microglia cells were mostly ramified with some hypertrophic cell bodies and fewer processes (Figure 2B″–D″). The mean diameter of microglia was significantly enhanced (Figure 2F″), and the majority of microglia was distributed in the INL, GCL and the ONL in the STZ-retina (Figure 2E″). Treatment with R-838 or R-954 had no effect on microglia reactivity as shown by the intensity of Iba-1 labelling (Figure 2C″, D″), the mean diameter (Figure 2F″) and the density (Figure 2E″) of microglia cells, which were not significantly affected by B1 receptor agonism and antagonism in 2 week STZ-retina.

Effect of B1 receptor agonist and antagonist on retinal vascular permeability at 2 weeks of diabetes

Increased vascular permeability is an important dysfunction in the development of macular oedema in DR that contributes significantly to the reduction in vision. In this series of experiments, the pharmacological effect of a B1 receptor agonist and antagonist on retinal vascular permeability was determined. In 2 week STZ-diabetic rats, retinal Evans Blue dye extravasation measured by spectrofluorometry was significantly increased compared to control rats (Figure 3). R-838 induced a significantly greater increase of retinal vascular permeability compared to vehicle-injected STZ-diabetic rats (Figure 3A). In contrast, eye drops treatment with R-954 significantly reduced, to near control values, the vascular hyperpermeability in the retina of STZ-diabetic rats (Figure 3B). Thus, topical application of R-954 appears a highly promising approach for the non-invasive treatment at the early stage of DR.

Co-localization of B1 receptors on vascular endothelial and glial cells in the STZ-retina at 2 weeks of diabetes

As shown by confocal microscopy, there was a very faint B1 receptor staining in the control retina (Figures 4A, 5A, 6A, 7A). The B1 receptor immunostaining was, however, strongly pronounced in the retina of the STZ rat in the nerve fibre, GCL and INL (Figures 4D; 5D, D′; 6D, D′; 7D, G, J, M). In 2 week STZ-retina, there was a co-localization of immunofluorescence of B1 receptors (Figure 4F) with endothelial cells labelled with the antibody RECA-1 (Figure 4E). This co-localization supports the hyperglycaemia-induced expression of B1 receptors by endothelial cells in the STZ-retina. There was also a strong co-labelling of B1 receptors (Figure 5F, F′, F″) and the GFAP marker (Figure 5E, E′, E″), representative of astrocytes and activated Müller cells (Wang et al., 2010; Suzuki et al., 2014), mainly in the internal border of the retina and GCL of diabetic retina. The GFAP labelling in the control retina was less intense and limited to low ramified glial extensions (Figure 5B) compared to the STZ retina where highly ramified and hypertrophic glial extensions were seen (Figure 5E, E′, E″).

VEGFR-2 exhibited weak immunostaining in control retina (Figure 6B), yet the intensity of labelling was increased in the diabetic retina (Figure 6E). Importantly, B1 receptor staining did not show co-localization with VEGFR-2 on Müller cells and other retinal cells in both control and STZ-retina (Figure 6C, F). Intriguingly, B1 receptors and VEGFR-2 seem to be co-expressed in blood vessels in STZ-retina (Figure 6F, arrow). This might suggest their joint effects in activating the vascular endothelium in diabetic retina.
Figure 2

Effects of B₁ receptor (B₁R) stimulation and blockade on the cellular distribution of B₁ receptors, leukostasis and microgliosis in the retina at 2 weeks of diabetes. In all panels, microphotographs are representative of average observation in control-vehicle (A, A’, A”), STZ-vehicle (B, B’, B”), STZ-treated with the B₁ receptor agonist R-838 (C, C’, C”) and STZ-treated with the B₁ receptor antagonist R-954 (D, D’, D”). Upper panel: distribution of B₁ receptors shown by immunocytochemistry (A–D). (E) Mean pixel density ratio in the retinal GCL, the INL and the ONL for each group (each bar value is the mean ± SEM of 40 retinal sections per rat × four rats). Middle panel: Representative microphotographs of adherent leukocytes (A’–D’). The arrow indicates leukocyte infiltration within the retinal tissue in STZ-retina treated with R-838 (C). Total number of adherent leukocytes per retina in different groups is shown in panel E’ (mean ± SEM of values from six rats in each group). Bottom panel: Microgliosis shown by the immunostaining of microglia with anti-Iba-1 antibody (A”–D”). Diabetes induces microgliosis (B””) shown by hypertrophied and ramified microglia with intense immunostaining. (E”) Evaluation of microglial cell density in the retinal GCL, the INL and the ONL for each treatment. (F”) Evaluation of the mean diameter of microglia. Each bar value in E” and F” is the mean ± SEM of 40 retinal sections per rat × four rats. Statistical comparison with control (*) or STZ-vehicle (+) is indicated by *+/−P < 0.05. Bar scale: 75 μm (A–D; A”–D”); 50 μm (A’–D”).
Absence of co-localization of B₁ receptors on microglia in the STZ-retina

In contrast to the low immunostaining for Iba 1+ microglial cells (Figure 7B) in control retina, a strong immunostaining was shown for microglial cells in the STZ-diabetic retina (Figure 7E, H, K, N). However, B₁ receptor (left panels) and Iba1 staining did not merge together in STZ-diabetic retina (Figure 7F, I, L, O) and in control retina (Figure 7C), suggesting a distinct location of these markers. It is worth noting that the B₁ receptor was also seen on large cells within the GCL, which are most likely ganglion cells, based on their morphology (Figure 7J arrow).

Discussion

This study supports a role for kinin B₁ receptors in retinal inflammation and the development of vascular alterations through a mechanism involving the endothelial and GFAP-expressing glial cells on which the B₁ receptor is expressed and up-regulated. However, the B₁ receptor is not present on microglia and does not seem to influence its reactivity.

The B₁ receptor is proposed as a therapeutic target for the treatment of diabetic retinopathy as it is expressed at the very beginning of diabetes, an effect maintained during the progression of diabetes. This study also provides evidence that 1-week eye drops application of a water-soluble and metabolically stable peptide kinin B₁ receptor antagonist is an effective non-invasive approach to alleviate diabetes-induced retinal vascular inflammation.

Expression of B₁ receptor in various stages of diabetic retinopathy

Although earlier studies have shown that the B₁ receptor is up-regulated in the STZ-diabetic retina very early from (Figure 7E, H, K, N). However, B₁ receptor (left panels) and Iba1 staining did not merge together in STZ-diabetic retina (Figure 7F, I, L, O) and in control retina (Figure 7C), suggesting a distinct location of these markers. It is worth noting that the B₁ receptor was also seen on large cells within the GCL, which are most likely ganglion cells, based on their morphology (Figure 7J arrow).

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![Figure 3](image1)

**Figure 3**

Vascular permeability in the retina at 2 weeks of diabetes. Retinal vascular hyperpermeability induced at 2 weeks of diabetes after intravitreal injection of the B₁ receptor agonist R-838 or its vehicle (A) and after eye drops application of the B₁ receptor antagonist R-954 (B). Tissue injury caused by intravitreal injection might account for the slightly higher vascular permeability in control and STZ-diabetic retinas in (A) in comparison to (B). Data are mean ± SEM of values from five rats in each group. Statistical comparison to Ctl-vehicle (*) or STZ-vehicle (+) is indicated by **P < 0.05.

![Figure 4](image2)

**Figure 4**

Microphotographs of immunolocalization of B₁ receptors on endothelial cells. Representative microphotographs of double immunolabelling for B₁ receptor (green, A, D) and RECA-1 (red, B, E) in control (upper panels) and diabetic (lower panels) retina. All sections were counter-stained for DAPI (blue) which labels the cell nucleus. Note that B₁ receptors completely co-localized (yellow, F) with RECA-1 marker, indicating expression of this receptor by endothelial cells in STZ retina. Bar scale: 75 μm.
1–4 days to 6 weeks (Abdouh et al., 2003; 2008; Pouliot et al., 2011; 2012), this is the first study showing that it remains overexpressed until a late (6 months) phase of STZ-induced diabetes. The agonist R-838 amplified retinal vascular permeability, leukostasis and B1 receptor gene expression, suggesting that the B1 receptor is functional and can respond to its agonist. The up-regulation of B1 receptor gene expression by R-838 in STZ-retina is congruent with the auto-induction of B1 receptors by its own agonists through the production of pro-inflammatory cytokines such as TNF-α and IL-1β (Schanstra et al., 1998; Phagoo et al., 1999). This way, the B1 receptor can amplify the pro-inflammatory process through a positive feedback loop mechanism (Couture et al., 2014). Indeed, the antagonist R-954 reversed the increased vascular permeability, leukostasis and immunocytochemical B1 receptor protein expression, further suggesting that this receptor plays an important role in the pathological events of diabetes. In contrast, control rat retinal vessels did not express functional B1 receptors, which is consistent with earlier studies in control rat retina at 1, 4, 7 and 21 days (Abdouh et al., 2003; 2008; Pouliot et al., 2011, 2012).

**B1 receptor pathway seems independent of VEGF pathway**

The activation of B1 receptors by intravitreal injection of R-838 failed to result in overexpressed VEGF and increased VEGFR-2 gene expression although they were suppressed following blockade of B1 receptors with siRNA treatment or with LF22-0542 in the retina of STZ-diabetic rats (Pouliot et al., 2011).
The lack of effect of R-838 on expression of VEGFR-2 is supported by the absence of co-localization of the two markers on retinal cells (except endothelial cells). The VEGF system is well known to stimulate microvascular permeability, vasodilatation and angiogenesis (Murakami, 2015). VEGF also increases extravasation of plasma kallikrein into the retina, and plasma kallikrein is required for the full effects of VEGF on retinal vascular permeability and retinal thickening in rodents (Clermont et al., 2016). Conversely, tissue kallikrein inhibits VEGF signalling via the cleavage of VEGF165 isoform, which may reduce retinal vascular permeability (Nakamura et al., 2011). Importantly, BK-induced retinal thickening in mice was not affected by the blockade of VEGFR-2, and antagonists of B1 receptors and B2 receptors did not influence VEGF-induced retinal vascular permeability (Kita et al., 2015). The latter study concluded that the plasma kallikrein–kinin system is implicated as a VEGF-independent mediator of diabetic macular oedema. Indeed, the strong expression of B1 receptors on endothelial cells in STZ-retina is congruent with a direct effect of the B1 receptor on capillary leakage that can occur independently of VEGF. The down-regulation of the VEGF expression system by B1 receptor antagonism or B1 receptor gene deletion could merely derive from the inhibition of the inflammatory process.

**Role of B1 receptor in retinal vascular permeability and leukostasis**

The breakdown of the blood-retinal barrier is an event that occurs early in the development of DR, and it is attributed to the increased production of pro-inflammatory cytokines and growth factors (Kern, 2007). The increased vascular permeability may also be associated with changes in the expression of molecules that form the cell junctions of the blood-retinal barrier (Kern, 2007). Studies have shown a decreased expression of occludin and ZO-1 in diabetic retinal endothelial cells, proteins that constitute the tight junctions (Antonetti et al., 1998; Barber et al., 2000). In the present

**Figure 6**

Immunohistochemical distribution of VEGFR-2 and B1 receptors (B1R). In control retina (A), B1 receptor immunostaining was very weak and the overall B1 receptor immunofluorescence intensity was higher in STZ-retina (D, D'), particularly in the GCL of diabetic retina. Compared to control retina (B), VEGFR-2 immunofluorescence intensity increased in diabetic retina (E) notably in Müller cells process and in blood vessels (E' arrow). There is no co-localization between VEGFR-2 and B1 receptors in control and STZ-retina on Müller cells and in GCL layer (C, F), but both co-localized on blood vessels in STZ-retina (F'). Bar scale: 75 μm.
Figure 7
Absence of localization of B₁ receptors (B₁R) on microglia cells in the retina of STZ-diabetic rats. Representative images of immunofluorescent detection of microglial cells. All immunofluorescence sections were counter-stained for DAPI (blue). Immunofluorescent detection of B₁ receptors (green) in control retina (A) and STZ-retina (D–M). Immunofluorescent detection of Iba1 (red) in control retina (B) and STZ-retina (E–N). Iba1 and B₁ receptors showed faint immunostaining in control retina (A, B) but higher staining in GCL layer in STZ-retina (D–M, E–N). B₁ receptor staining did not show co-localization with Iba1 in control retina (C) and in STZ-retina (no yellow colour, F, I, L, O). In the STZ group, the B₁ receptor was present in Müller cells (G, M arrow) and ganglion cells (D, J, arrows). Bar scale: 75 μm.
study, 1 week eye drops application of R-954 reversed increased retinal vascular permeability in 2 week STZ-diabetic rats without affecting glycaemia. This is in agreement with previous data using the high-affinity non-peptide B1 receptor antagonist LF22-0542 (Pouliot et al., 2012). Retinal vascular permeability was also decreased following systemic and intravitreal injection of a selective plasma kallikrein inhibitor in STZ-diabetic rats (Clermont et al., 2011). The i.v. injection of R-954 was found to inhibit the enhanced vascular permeability in the retina of mice and rats treated with STZ 1 and 4 weeks earlier (Simard et al., 2002; Lawson et al., 2005). Five consecutive s.c. injections of R-954 also reduced the enhanced levels of NOX, kallikrein and vascular permeability and improved the reduced Na/K ATPase activity in diabetic rat retinas 4 and 12 weeks after STZ treatment (Catanzaro et al., 2012). For the first time, our study shows that eye drops application of R-954 is a feasible approach that has the advantage of being less invasive, avoiding the risks associated with intravitreal injections (viral and bacterial infection and cataracts) and the systemic effects of i.v. treatments, particularly if the goal is to develop a therapeutic treatment.

The adhesion of leukocytes to the wall of blood vessels was suggested as one of the factors involved in endothelial cell death and may also contribute to alterations in retinal perfusion (Joussen et al., 2002; Kern, 2007) and increased vascular permeability (Del Maschio et al., 1996). Evidence suggests that diabetic retinopathy is associated with increased levels of leukocytes in the retina and persistent leukostasis. Indeed, the number of neutrophils is significantly increased in the retinal blood vessels of diabetic patients and animals (McLeod et al., 1995; Miyamoto et al., 1998, 1999). Local production of kinins and up-regulation of B1 receptor expression in blood vessels contribute to the diapedesis of leukocytes and their migration into damaged tissues in response to the chemotactic attraction of cytokines (McLean et al., 2000).

The present study shows that treatment with R-954 reversed retinal leukostasis in STZ-diabetic retina. This finding highlights a primary role for B1 receptors in the cellular inflammation associated with DR.

**Co-localization of B1 receptors on endothelial cells in the retina of STZ-diabetic rats**

B1 receptor protein was mainly located in the retinal GCL, the INL, the ONL and the inner plexiform layer (IPL), representing the most vascularized retinal layers (except ONL) (Cuthbertson and Mandel, 1986). R-954 down-regulated the protein expression of B1 receptors in the vascularized retinal layers. This may occur by preventing the auto-induction of B1 receptors by its own agonist (Couture et al., 2014). Furthermore, the B1 receptor was shown on endothelial cells and astrocytic end feet of retinal blood vessels, supporting its action on endothelial function and retinal vascular permeability. The overexpression of B1 receptors in endothelial cells may also account for the alteration of retinal blood flow in the early phase of diabetes (Pouliot et al., 2011). The vasodilatation of isolated retinal microvessels was mediated by the release of NO and prosta-glandins derived from the COX-2 pathway in STZ-diabetic rats (Abdouh et al., 2003; Abdouh et al., 2008).

**Co-localization of B1 receptors on glial cells but not on microglia in the retina of STZ-diabetic rats**

Activated microglia are heavily involved in diabetes and retinal degeneration (Natoli et al., 2017). In our study, microglia were identified by Iba-1 immunolabelling and showed the characteristics of activated microglia with hypertrophied cell body in STZ-diabetic retina unlike microglia in control rats displaying small size cytoplasm, in agreement with previous studies (Zeng et al., 2000; Krady et al., 2005). At 2 weeks of diabetes, the majority of microglia were distributed in the inner retina, including GCL and the IPL, in both normal control and diabetic rats. At 12 weeks of diabetes, it was reported that the retinal microglia were more in the nerve fibre layer and GCL and less in the IPL; this is probably due to a redistribution associated with the reduction of the retinal thickness in the IPL (Chen et al., 2015). Importantly, our findings showed that treatment with R-838 and R-954 did not alter retinal microglia reactivity as evidenced by the intensity of Iba-1 labelling, mean diameter and density of microglia in the three main vascular layers at 2 weeks of diabetes. Microglial cells are activated from the beginning of neuroinflammation (Graeber et al., 2011; Natoli et al., 2017); subsequently, astrocytes and Müller cells are activated to further accentuate the inflammatory process (Sorrentino et al., 2016). The astrocytes are found mainly in the ganglion cell layer and the inner plexiform layer. These glial cells are in contact with the ganglion cells and surround the capillaries and contribute to the maintenance of the blood-retina barrier (Gardner et al., 1997; Wang et al., 2010). Recent studies suggest that astrocytes are strongly activated during the inflammatory process via the NF-κB signalling pathway and contribute to the up-regulation of inflammatory enzymes and mediators such as inducible NOS, TNF-α, IL-1β, IL-6 and IL-8 (Martorana et al., 2015; Zhong et al., 2016). The activation of NF-κB and the subsequent release of pro-inflammatory cytokines are involved in the induction of the B1 receptor (Couture et al., 2014) that makes a link with astrogliosis. Müller cells expressing B1 receptors also appeared to be activated since numerous GFAP hypertrophied cytoplasmic extension are seen throughout the tissue. The Müller cells also take part in the secretion of inflammatory mediators, which contribute to enhanced inflammation. They can be activated by microglia (Natoli et al., 2017).

Hence, data show that the distribution and co-localization of B1 receptor with retinal glial and Müller cells are strikingly correlated with the presence and distribution of retinal blood vessels. This provides a strategic role and mechanism for B1 receptors in microvascular inflammation and endothelial dysfunction in the diabetic retina. Further investigations are needed to determine the occurrence and plasticity of B1 receptor expression in other retinal cells (pericytes, photoreceptors and neurons) that can affect vision during diabetes.

**Conclusion**

This study shows that the B1 receptor is up-regulated in retinal blood vessels including glial, Müller cells and particularly on endothelial cells, supporting a direct action of this
receptor on blood vessels to enhance vascular permeability and leukostasis in STZ-diabetic retina. The B$_2$ receptor is also involved in the inflammatory cascade leading to its auto-induction and enhanced expression of B$_2$ receptors. In contrast, the B$_1$ receptor is not expressed on microglia in STZ-retina and its activation or inhibition failed to affect microgliosis. The functional overexpression of B$_1$ receptors in a late stage (6 months) of diabetes also suggests its involvement in the chronic phase of DR. Ocular application of the B$_1$ receptor antagonist R-954 by eye drops exerted anti-inflammatory effects in STZ-diabetic retinopathy, providing a promising and non-invasive therapeutic approach for the treatment of diabetic retinopathy.

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**Author contributions**

S.H., E.V. and R.C. designed the experiments and interpreted the results. S.H. and M.B. performed the experiments. S.H., F.H.G. and J.S. analysed the data. S.H. and F.H.G. drafted the paper. E.V. and R.C. co-supervised the work and wrote the final version of the paper. All authors approved the final manuscript.

**Conflict of interest**

The authors declare no conflicts of interest.

**Declaration of transparency and scientific rigour**

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

**References**


Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

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Figure S1 Tests of specificity on the polyclonal rabbit antiserum to rat B1 receptor. Upper panel: Representative images of immunofluorescent detection of B1 receptor in control rat retina (A, D), STZ-retina (B, E) and STZ-retina treated with siRNA 10 nmol (C, F), sections were counter-stained for DAPI (blue). In contrast to the abundant B1 receptor immunostaining in STZ-retina, B1 receptor immunostaining was absent in STZ-retina treated with siRNA. In control retina, B1 receptor showed weak immunostaining. Middle panel: Immunohistochemical labelling (revealed with DAB) in control rat retina (A″), STZ-retina (B″) and STZ retina treated with pre-immune rabbit serum (C″). The intensity of B1 receptor immunostaining was greatly increased in STZ-retina compared to control retina. Conversely, no B1 receptor labelling was detected in STZ-retina with the pre-immune rabbit serum. Bar scale: 75 μm. Bottom panel: Immunohistochemical labelling in wild-type mice retina (Wt; A‴) and B1 receptor/B2R-KO mice retina (B‴). B1 receptor staining in the GCL layer of Wt retina (arrows) was absent in B1 receptor/B2R-KO mice retina (B‴). This is consistent with the higher expression of B1 receptor in mice than in rats (Couture et al., 2014). Bar scale: 75 μm.