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RESEARCH PAPER

Retinal plasma extravasation in streptozotocin-diabetic rats mediated by kinin B_1 and B_2 receptors

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Background and purpose: We investigated whether or not kinin receptors play a role in diabetic blood–retinal barrier breakdown, which is a leading cause of vision loss.
Experimental approach: Blood–retinal barrier breakdown was quantified using Evans blue, and expression of kinin B_1 receptor mRNA was measured using quantitative reverse transcrition-PCR. Diabetic rats (streptozotocin (STZ), 65 mg kg^{-1}) received a single intraocular injection of bradykinin (BK) or des-Arg^9-BK, alone, or in combination with antagonists for B_1 (des-Arg^{10}-Hoe140, R-715) and/or B_2 (Hoe140) receptors, given intraocularly or intravenously (i.v.).
Key results: In control rats, BK (0.1–10 nmol) dose-dependently increased plasma extravasation, which was inhibited by Hoe140 (0.2 nmol), whereas des-Arg^9-BK (0.1 and 1 nmol) was without effect. B_1 receptor mRNA was markedly increased in retinas of diabetic rats, and this was prevented by *N*-acetyl-L-cysteine (1 g kg^{-1} day^{-1} for 7 days). Plasma extravasation in retinas of STZ-diabetic rats was higher than in controls and enhanced by des-Arg^9-BK. Response to des-Arg^9-BK was inhibited by intraocular or i.v. injection of B_1 receptor antagonists. Diabetes-induced plasma extravasation was inhibited only by a combination of des-Arg^{10}-Hoe140 and Hoe 140 (100 nmol kg^{-1}, i.v. 15 min earlier) or by R-715 (1 μmol kg^{-1}, i.v.) injected daily for 7 days.
Conclusions and implications: Kinin B_1 receptors are upregulated in retinas of STZ-diabetic rats through a mechanism involving oxidative stress. Both kinin B_1 and B_2 receptors contribute to increased plasma extravasation in diabetic retinopathy. Chronic inhibition of both kinin receptors, possibly with antioxidant adjuvants, may be a novel therapeutic strategy for diabetic retinopathy.
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Keywords: bradykinin; B_1 receptors; B_2 receptors; retina; plasma extravasation; diabetes

Abbreviations: BK, bradykinin; des-Arg^9-BK, des-Arg^9-bradykinin; QRT-PCR, quantitative reverse transcription-PCR; STZ, streptozotocin

Introduction

Diabetic retinopathy is a leading cause of blindness among the adult population of developed countries (Javitt *et al.*, 1994) and represents a major economic burden to society. The greatest source of vision loss in diabetes is macular oedema (Moss *et al.*, 1998), a pathological condition that is a direct consequence of the breakdown of the blood–retinal barrier. However, an effective pharmacological treatment for this diabetic complication currently does not exist.

Kinins and their active C-terminal metabolites, des-Arg-kinins, are potent vascular permeability-inducing factors (Emanueli *et al.*, 1998; Wille *et al.*, 2001; Hayashi *et al.*, 2002) and may play a role in the blood–retinal breakdown in diabetes. Kinins are a family of structurally related 9- to 11-amino-acid peptides including bradykinin (BK), kallidin (Lys-BK), T-kinin (Ile-Ser-BK; exclusively in rats) and des-Arg-kinins, which are active metabolites (des-Arg^9-BK and Lys-des-Arg^9-BK). Kinins exert a variety of biological effects through the activation of two transmembrane G-protein-coupled receptors designated B_1 and B_2 (Regoli and Barabé, 1980; Regoli *et al.*, 1998). B_2 receptors are constitutively expressed, whereas B_1 receptors are generally not expressed or under-expressed under physiological conditions. Induction or upregulation of B_1 receptors is known to occur following tissue injury, inflammation, diabetes, and following treatment with bacterial endotoxins, certain cytokines or growth factors (Marceau *et al.*, 1998). Kinins have been shown to cause vasodilatation, regulation of local blood flow, stimulation of cell proliferation, production of pain and inflammatory responses (Marceau *et al.*, 1998; Couture *et al.*, 2001).

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Components of the kallikrein-kinin system are known to be expressed in the retina (Kuznetsova *et al*., 1991; Ma *et al*., 1996; Takeda *et al*., 1999). Non-ocular *in vivo* studies have shown that kinin receptors are involved in the pathogenesis of diabetes (Zuccollo *et al*., 1996, 1999). More recently, we showed increased kinin B_1-receptor-binding sites in the retina of rats with diabetes induced by streptozotocin (STZ), and we demonstrated that the kallikrein-kinin system is functional within an isolated retina (Abdouh *et al*., 2003). Kinin B_2-receptor stimulation evokes intracellular calcium mobilization in cultured retinal capillary endothelial cells (Hasséssian *et al*., 2001) and vasodilation in the isolated retinal microcirculation (Abdouh *et al*., 2003). Kinin B_1-receptor-mediated vasodilation of retinal vessels could only be observed in the STZ-diabetic rat (Abdouh *et al*., 2003). However, until now, neither the role of kinin receptor subtypes in the breakdown of the blood–retinal barrier nor the mechanism involved in the induction of B_1 receptors in the retina of diabetic rats has been fully elucidated.

This is the first study to provide mRNA evidence for an upregulation of kinin B_1 receptors in the retina of diabetic rats through a mechanism involving oxidative stress, as revealed by treatment with *N*-acetyl-L-cysteine (NAC), a potent antioxidant, and the first study to demonstrate that both B_1 and B_2 receptors participate in the breakdown of the blood–retinal barrier in diabetes. The results suggest that kinin receptors may serve as therapeutic targets to treat certain retinopathies involving oedema.

Methods

STZ-diabetic rat model

All animal procedures complied with the guiding standards for the care and use of animal experimentation as stated by the Association for Research in Vision and Ophthalmology, the Canadian Council on Animal Care and approved by the Animal Care Committee of the Guy-Bernier Research Centre. Male Wistar rats weighing 225–250 g were purchased from Charles River (St-Constant, QC, Canada) and housed, four per wire-bottomed cage, in rooms under controlled temperature (23–25 °C), humidity (50%) and lighting (12-h light–dark cycle) with food and tap water available *ad libitum*. They were used 3–5 days after their arrival and injected under low light with freshly prepared STZ (65 mg kg^{-1} i.p.; Sigma-Aldrich, Oakville, ON, Canada). Age-matched controls were injected with sodium citrate buffer (0.05 M, pH. 4.5) vehicle. Glucose concentrations were measured, with a commercial blood glucose-monitoring kit (Accusoft; Roche Diagnostics, Laval, QC, Canada), in blood samples obtained from the tail vein, in non-fasting animals, before STZ injection, and after STZ injection, just before experimentation. Only STZ-treated rats whose blood glucose concentration was higher than 20 mM were considered as diabetic.

Evans blue extravasation

Evans blue dye is a widely used nonradioactive intravascular tracer. When injected intravenously, the Evans blue dye rapidly binds to plasma albumin and thus remains within the vasculature. If plasma extravasation occurs, Evans blue leaks out into the surrounding tissues. Therefore, the quantity of Evans blue that has leaked into tissues can be used as a marker for plasma extravasation. Rats were anaesthetized with ketamine (50 mg kg^{-1}) and xylazine hydrochloride (100 mg kg^{-1}), before the right jugular vein was cannulated with a PE-50 polyethylene catheter (Beckton Dickinson, Sparks, MD, USA). Evans blue was injected through the jugular vein over 10 s at a dose of 45 mg kg^{-1} and the dye was allowed to circulate. Immediately after Evans blue infusion, the rats turned visibly blue, confirming the uptake and distribution of the dye. Both eyes were enucleated and Evans blue dye was extracted by incubating each retina in 1 ml formamide (Sigma) for 18 h at 70–75 °C. Afterward, the absorbance was measured with a spectrophotometer (model Du-640; Beckman, Fullerton, CA, USA) at 620 nm. All experiments involving diabetic rats were performed on the seventh day following STZ treatment.

Measurement of kinin-induced blood–retinal barrier breakdown

After induction of generalized anaesthesia as outlined above, pupils were dilated with 0.5% tropicamide (Dioptic Laboratories, ON, Canada). The vitreous of both eyes was injected with BK (0.1–10 nmol) or des-Arg^9-BK (0.001–1 nmol) in 5 µl volume using a 10-µl, 27-gauge Hamilton syringe (Hamilton Company, Reno, NV, USA). Agonists were injected 10 min after injecting Evans blue dye and the rats killed 20 min after receptor stimulation. Conversely, B_1 and B_2 receptor antagonists were injected in the vitreous or i.v. through the tail vein before the injection of des-Arg^9-BK and BK, respectively. For the 7-day treatment with antagonists, rats received a single injection of the antagonist everyday for 7 consecutive days. On the seventh day, rats were injected with Evans blue and subjected or not to agonist treatment. Afterward, they were killed. For the 24-h treatment with antagonists, rats received a single injection of antagonist. Twenty-four hours later, the rats were injected with Evans blue and killed. Afterward, both eyes were enucleated and the absorbance measured as described above.

SYBR Green-based quantitative reverse transcription-PCR

Twelve rats were treated with STZ (65 mg kg^{-1} i.p.) or the vehicle (controls, $n = 5$). Five rats treated with STZ received, by gavage every morning, a treatment with NAC (1 g kg^{-1} d^{-1}) for 7 days. None of these rats received agonists or antagonists or Evans blue dye. At the term of the 7-day treatment, rats were anaesthetized with CO_2 inhalation and then decapitated. Retinas were isolated and approximately 10 mg of tissue was put in RNA*later* stabilization reagent (QIAGEN, Valencia, CA, USA). Total RNAs were extracted from tissue according to the manufacturer's protocol. 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 QPCR (quantitative PCR) was performed as described (Aoki *et al*., 2002). Mx3000p (Stratagene, La Jolla, CA, USA) was used for signal

detection and PCR was performed in SYBR Green master mix (QIAGEN) with 300 nM of each primer. The following primer pairs were designed by Vector NTI and used: 5′-GCAGCGCTT AACCATAGCGGAAAT-3′ (forward, 367–391) and 5′-CCAGTT GAAACGGTTCCCGATGTT-3′ (reverse, 478–454) for amplification of rat B_1 receptor (GenBank accession no. NM_030851); 5′-TCAACTTTCGATGGTAGTCGCCGT-3′ (forward, 363–386) and 5′-TCCTTGGATGTGGTAGCCGTTTCT-3′ (reverse, 470–447) for amplification of rat 18s (GenBank accession no. X01117). 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 C_t (cycle threshold) value represents the cycle number at which a fluorescent signal rises statistically above background (Wada *et al*., 2000). The relative quantification of gene expression was analysed by the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

Statistical analysis

Data are expressed as mean ± s.e.mean and *n* represents the number of rats used in each experiment. Results were analysed using Student's *t*-test for unpaired samples. ANOVA followed by Dunnett's test was used for multiple comparisons with one control group. Only *P*-values <0.05 were considered to be statistically significant.

Materials

All reagents were purchased from Sigma-Aldrich. BK and des-Arg^9-BK were from Bachem Bioscience Inc. (King of Prussia, PA, USA), Hoe 140 (D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK) (B_2 receptor antagonist; Hock *et al*., 1991) and des-Arg^{10}-Hoe 140 (des-Arg^9-D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-BK) (B_1 receptor antagonist; Wirth *et al*., 1991a, b) were from Peninsula Laboratories Inc. (Belmont, CA, USA). R-715 (AcLys [D-βNal^7,Ile^8]des-Arg^9-BK) (B_1 receptor antagonist; Regoli *et al*., 1998) was a generous gift from Dr Domenico Regoli (Department of Pharmacology, Sherbrooke University, QC, Canada). Evans blue and all ligands were dissolved in 0.9% NaCl, which had no significant effect on baseline plasma extravasation.

Results

Effect of BK and des-Arg^9-BK on Evans blue extravasation in healthy retinas

In the retina of healthy control rats, BK induced a dose-dependent increase in Evans blue extravasation ($P<0.05$, $n=5$). At the highest dose injected, this represents a 100% increase when compared with non-injected retina (control) or NaCl (0.9%)-injected retina (Figures 1a and b). In contrast, des-Arg^9-BK was without effect on Evans blue extravasation ($P>0.05$, $n=4$) (Figure 1a). When an eye was injected with only saline (0.9% NaCl), or treated with tropicamide (0.5%), no leakage was seen, demonstrating that neither the surgical procedure nor the experimental protocol affected the integrity of the vessel (Figure 1a). Intraocular pretreatment, with the B_2 receptor antagonist Hoe140, markedly reduced ($P<0.05$, $n=5$) the BK-induced increase in plasma extravasation in the retina (Figure 1b). Hoe140 alone had no direct effect on retinal vessel leakage (Figure 4).

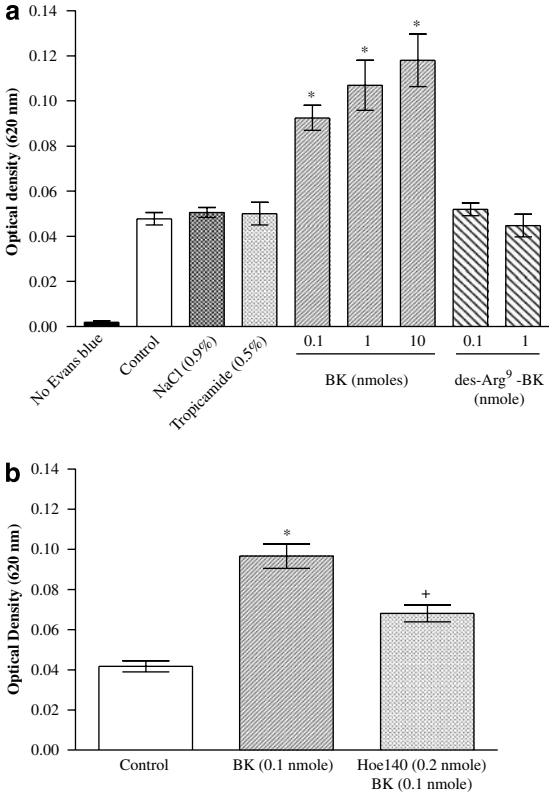


Figure 1 Bradykinin (BK) induces plasma extravasation in the retinas of healthy rats. (**a**) Dose–response effect of BK or des-Arg^9-BK on plasma extravasation after intravitreous injection. (**b**) B_2 receptors mediate BK-induced retinal plasma extravasation. The intravitreous injection of Hoe140 was followed 10 min later by the intravitreous injection of BK. Responses are expressed as optical density measured at 620 nm wavelength ($n=4$ or 5). Statistical significance are set at $P<0.05$, where * shows differences between rats treated with and without agonist and + shows differences between rats treated with and without antagonist.

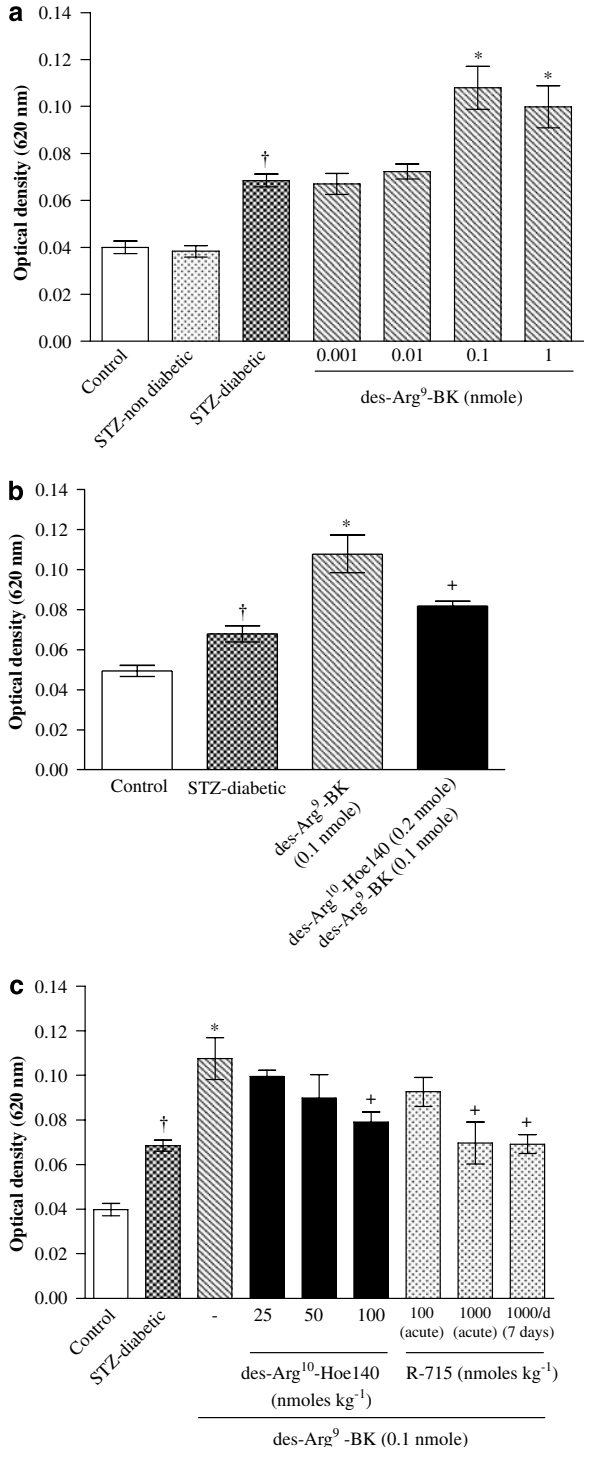
Effect of des-Arg^9-BK on Evans blue extravasation in retinas of STZ-induced diabetic rats

Evans blue extravasation was quantified in the retinas of 7-day STZ-diabetic rats. Diabetes (rats with blood glucose concentration of 28 ± 4 mM) induced a nearly twofold increase in Evans blue extravasation ($P<0.05$, $n=5$; Figures 2a–c). In contrast, no increase in Evans blue extravasation ($P>0.05$, $n=5$) was seen in rats treated with STZ but which did not develop diabetes (blood glucose concentration of 6 ± 2 mM; Figure 2a). In the retina of STZ-diabetic rats, des-Arg^9-BK induced a significant increase in Evans blue extravasation ($P<0.05$, $n=3$–5) when compared with STZ-diabetic rats not injected with des-Arg^9-BK (Figures 2a–c). Intraocular pretreatment with the B_1 receptor antagonist des-Arg^{10}-Hoe140 significantly reduced ($P<0.05$, $n=4$) the des-Arg^9-BK-induced increase in plasma extravasation in the retina (Figure 2b). In addition, Evans blue extravasation induced by intraocular injection with des-Arg^9-BK, in

STZ-diabetic rats, was dose-dependently inhibited ($P<0.05$, $n=4$–5) by i.v. administration of either des-Arg^{10}-Hoe140 or R-715 (Figure 2c).

Effect of NAC on kinin B_1 receptor expression in the retina of STZ-diabetic rats

Kinin B_1 receptor mRNA was underexpressed in the retina of control rats (Figure 3). Treatment with STZ (65 mg kg^{-1} day^{-1}), 7 days earlier, increased B_1 receptor mRNA in the retina by 12.7-fold. This marked increase in B_1 receptor expression in STZ-diabetic rats was significantly prevented by treatment during 7 days with NAC (1 g kg^{-1} day^{-1}) (Figure 3). Treatment with NAC affected neither the hyperglycaemia nor the loss of body weight observed in STZ-diabetic rats. Although the antioxidant did not alter food intake, it reduced slightly, but significantly, the higher volume of water intake in STZ-diabetic rats (Table 1).



Effect of kinin receptor antagonists on diabetes-induced Evans blue extravasation in rat retina

Intraocular injection of Hoe140 (0.2 nmol) or des-Arg^{10}-Hoe140 (0.2 nmol) alone did not affect ($P>0.05$, $n=5$) Evans blue extravasation in the retina of STZ-diabetic rat (Figure 4a). In addition, i.v. administration of either Hoe140 (25–500 nmol kg^{-1} for 15 min), or des-Arg^{10}-Hoe140 (25–500 nmol kg^{-1} for 15 min), had no effect ($P>0.05$, $n=4$) on retinal vessel dye leakage (Figures 4b and c). A longer period of treatment with either Hoe140 or des-Arg^{10}-Hoe140 (500 nmol kg^{-1} for 24 h) was still without effect ($P>0.05$, $n=4$; Figures 4b and c). In contrast, the metabolically more stable and long-acting B_1 receptor antagonist (R-715) given i.v. (1 μmol kg^{-1} day^{-1}) for 7 days significantly reduced ($P<0.05$, $n=4$) diabetes-induced Evans blue extravasation in the retina (Figure 4c). Furthermore, combined acute i.v. administration of kinin receptor antagonists Hoe140 and des-Arg^{10}-Hoe140 significantly decreased ($P<0.05$, $n=5$) Evans blue extravasation in the retina, at does which not reach significant inhibition when either antagonist was administered individually (Figure 4d). This inhibition in the presence of both antagonists was over by 24 h after injection.

Discussion

This is the first *in vivo* study to provide evidence for a direct role of both kinin B_1 and B_2 receptors in diabetes-induced blood–retinal barrier breakdown. Clearly, both receptor subtypes must be targeted when forming a strategy for the development of a kinin receptor-based treatment for retinal oedema. Ocular tissues have been shown to possess components of the kallikrein-kinin system (Kuznetsova *et al*., 1991;

Figure 2 Des-Arg^9-BK induces retinal plasma extravasation in streptozotocin (STZ)-diabetic rats. (**a**) Plasma extravasation was quantified in the retina of STZ-diabetic rat and STZ-nondiabetic rat, and after an intravitreous injection of increasing doses of des-Arg^9-BK in STZ-diabetic rats. (**b**) Retinal plasma extravasation was quantified after an intravitreous injection of des-Arg^{10}-Hoe140 followed 10 min later by the intravitreous injection of des-Arg^9-BK. (**c**) Retinal plasma extravasation was quantified in intravitreous-injected des-Arg^9-BK STZ-diabetic rat before and after an i.v. injection of des-Arg^{10}-Hoe140 or R-715. Responses are expressed as optical density measured at 620 nm ($n=3$–5). Statistical significance are set at $P<0.05$, where † shows differences between rats with and without STZ-diabetes, * shows differences between diabetic rats treated with and without agonist and + shows differences between diabetic rats treated with and without antagonist.

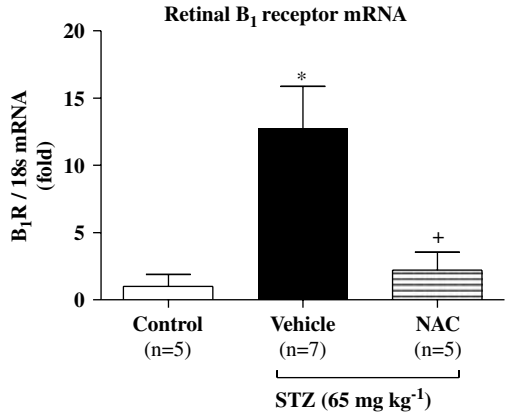


Figure 3 Effect of treatment with *N*-acetyl-L-cysteine (NAC) for 7 days on B_1 kinin receptor mRNA overexpression induced by streptozotocin (STZ) in the retina. Control rats received the vehicle only. NAC (1 g kg^{-1} day^{-1}) was given by gavage 3 h after STZ and daily until the end of the experiment. Data are means ± s.e.m. of 5–7 rats per group. Statistical comparison with controls (*) and STZ-diabetic rats (+) is indicated at $P<0.05$.

Table 1 Systemic effects of *N*-acetyl-L-cysteine (1 g kg^{-1} day^{-1}) for 7 days in STZ-treated rats

Parameters	*Control rats (n = 5)*	*STZ (n = 7)*	*STZ + NAC (n = 8)*
Plasma glucose (mmol l^{-1})	5.6 ± 0.3	29.8 ± 2.2***	28.0 ± 1.8***
Water intake (ml)	38.7 ± 1.4	100.3 ± 3.9***	87.1 ± 3.7***†
Food intake (g)	28.1 ± 0.6	29.2 ± 0.3	25.7 ± 0.6
Body weight (g)	330.8 ± 4.6	293.7 ± 7.1**	279.8 ± 6.2***

Values represent the mean ± s.e.mean of *n* rats.
Statistical comparison with control rats (*) and STZ (†) is indicated by $^{\dagger}P<0.05$, **$P<0.01$ and ***$P<0.001$.

Ma *et al*., 1996; Takeda *et al*., 1999), and we have demonstrated a haemodynamic potential of kinins in healthy as well as STZ-diabetic retina (Hasséssian *et al*., 2001; Abdouh *et al*., 2003). Our present results show that in healthy control rats, BK produces a B_2-receptor-mediated plasma extravasation in the retina. Furthermore, our data show that the control rat retina does not express functional B_1 receptors, as des-Arg^9-BK failed to induce Evans blue leakage from retinal vessels. In an earlier *in vitro* study on retinal capillary endothelial cells, we could initiate a Ca^{2+} response following stimulation of B_2 receptors, but no such response was evoked by B_1 receptor stimulation (Hasséssian *et al*., 2001). This is consistent with our finding that des-Arg^9-BK was not able to vasodilate naïve rat retinal vessels *in vitro* (Abdouh *et al*., 2003). Moreover, by using real-time quantitative reverse transcription-PCR in the present study, and a sensitive autoradiographic technique for kinin receptor-binding sites in an earlier study (Abdouh *et al*., 2003), we have shown minimal expression of B_1 receptors in the retina of control rats.

Until now, there were reports by Ma *et al*. (1996), which found B_1 receptors in the retina of healthy humans, and Lawson *et al*. (2005b), which demonstrated that inhibition of B_1 receptors in retinas of diabetic rats inhibits plasma extravasation. From the sum of these reports, it is not clear whether the B_1 receptors are induced in the retina of a diabetic rat or are constitutively expressed, as would be suggested if one extrapolates from human to rat, based on the conclusions of Ma *et al*. (1996). Our data clearly demonstrate that this extrapolation is not possible. Here, we provide molecular and functional evidence in rat for a marked upregulation of B_1 receptors during diabetes. In contrast, by using reverse transcription-PCR analyses and *in situ* hybridization, Ma *et al*. (1996) found that endothelial cells of human retinal blood vessels express mRNA for both B_1 and B_2 receptors. However, the authors did not report the clinical and pathological status of the donor eyes, which were collected 6 h post-mortem. It is unknown whether these tissues were obtained from healthy human or if the B_1 receptor was induced after death. Hence, it is not possible for us to comment on any potential species difference, between rat and man, with respect to induction of kinin receptor subtypes in the retinal circulation. However, our finding that the B_1 receptor is induced in rat, whereas it may be constitutively expressed in retinas of man, does not preclude using the rat as a model to study B_1-receptor-mediated plasma extravasation in diabetic retinopathy.

Generally, the B_1 receptor is only minimally expressed under physiological conditions, and can easily be induced by infectious diseases (Marceau *et al*., 1998), or oxidative stress (Couture and Girolami, 2004; Lungu *et al*., 2007). Because the potent antioxidant NAC prevented the upregulation of B_1 receptor mRNA in the retina of STZ-diabetic rats, it is suggested that the induction of B_1 receptors in the retina of diabetic animals is mediated by oxidative stress. It is worth noting that the inhibitory effect of NAC on B_1 receptor expression is not correlated with the prevention of diabetes or hyperglycaemia. NAC was also found (R Couture, unpublished observations) to reverse the upregulation of B_1 receptor mRNA in the spinal cord, and renal cortex, in glucose-fed rats (an animal model of insulin resistance), which display increased production of vascular superoxide anion. NAC acts as a free-radical scavenger and a cysteine donor, thus sustaining intracellular glutathione levels (an endogenous antioxidant), and permitting mitochondrial oxidative metabolism, by protecting the cytochrome oxidase complex I from nitric oxide-mediated damage (Moncada, 2000). Hence, using an antioxidant adjuvant, to kinin receptor antagonists, may be of benefit in treating kinin-mediated plasma extravasation in the retina.

Our finding that STZ-induced diabetes evokes strong plasma extravasation within the retina is consistent with pharmacological results obtained in other tissues (D'Orléans-Juste *et al*., 1997), and with the numerous functional manifestations of B_1 receptor overexpression in STZ-diabetic rats (Cloutier and Couture, 2000; Couture *et al*., 2001; Mage *et al*., 2002; Vianna *et al*., 2003; Ongali *et al*., 2004; Campos *et al*., 2005). Our observation is directly related to STZ-induced hyperglycaemia in rats, and not due to a direct effect of STZ on retinal vessels, as STZ-injected rats that did not develop hyperglycaemia also did not show increased retinal extravasation of Evans blue. Furthermore, our ability to inhibit using an intraocular route with the rapidly

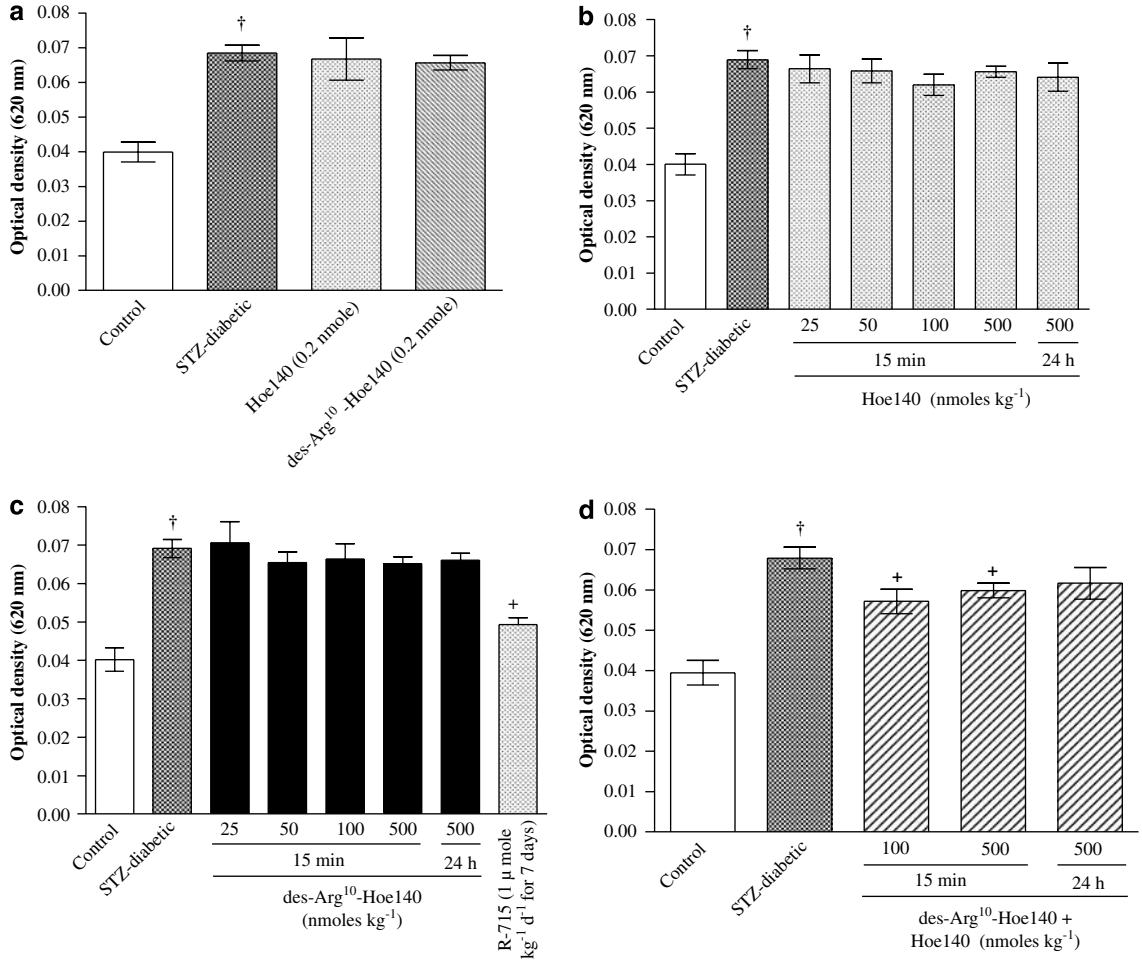


Figure 4 Kinin receptors induce diabetes-evoked retinal plasma extravasation. (**a**) Retinal plasma extravasation was quantified in streptozotocin (STZ)-diabetic rat retina before and after an intravitreous injection of Hoe140 or des-Arg^{10}-Hoe140. (**b** and **c**) Retinal plasma extravasation was quantified in STZ-diabetic rat retina before and after i.v. injection of Hoe140 (**b**) or des-Arg^{10}-Hoe140 or R-715 (**c**). (**d**) Retinal plasma extravasation was quantified in STZ-diabetic rat retina before and after i.v. injection of a combination of Hoe140 and des-Arg^{10}-Hoe140. Responses are expressed as optical density measured at 620 nm ($n=3$–5). Statistical significance are set at $P<0.05$, where † shows differences between STZ-diabetic rats versus healthy control rats, + shows differences between rats treated with and without antagonist.

metabolized B_1 receptor antagonist des-Arg^{10}-Hoe140 is consistent with a direct local intraocular effect of these antagonists, as opposed to an effect leading to a systemic drop in glucose levels.

Although diabetes-induced retinal plasma extravasation was not blocked by intraocular or acute i.v. treatment, with either B_1 or B_2 receptor antagonists given individually, significant reduction occurred when both antagonists were given together 15 min earlier. Our data show that the inhibitory effect of des-Arg^{10}-Hoe 140 and Hoe 140 was lost 24 h after treatment. As des-Arg^{10}-Hoe 140 is the derivative of the selective B_2 receptor antagonist Hoe 140, which was found to be active for more than 4 h *in vivo* (Wirth *et al.*, 1991a, b), it is expected that the inhibition of B_1 receptors by acute i.v. injection of des-Arg^{10}-Hoe 140 (15 min) will not be different from that achieved by R-715, which displays similar affinity for the B_1 receptor (Regoli *et al.*, 1998). Thus, it is unlikely that R-715 administered alone would have the same inhibitory effect on retinal plasma extravasation as des-Arg^{10}-Hoe 140 and Hoe 140 injected together. We propose that both receptors are involved, and that inhibition of only one of the two kinin receptor subtypes allows endogenous kinins to exert their proinflammatory effect via the receptor subtype that is not pharmacologically blocked. However, results obtained with the daily administration of the relatively more stable, metabolically resistant B_1 receptor antagonist, R-715, for the 7-day period, revealed the significant contribution of B_1 receptors to the enhanced vascular plasma leakage in diabetic rat retina. Earlier, studies have demonstrated that kinins induce increased macromolecule extravasation *in vivo* (Gao *et al.*, 1993; Jaffa *et al.*, 1995). More recently, it has been reported that increased vascular permeability in several target tissues, other than the retina, of STZ-diabetic rats as well as mice, is inhibited by acute treatment (300 μg kg^{-1} i.v. or 2 mg kg^{-1} s.c.) with the B_1 receptor antagonist R-954 (Ac-Orn-[Oic^2,αMePhe^5,D-βNal^7, Ile^8]des-Arg^9-BK) (Simard *et al.*, 2002; Lawson *et al.*, 2005a). A preliminary report also showed that the elevated retinal vascular permeability in STZ-diabetic rats after 1 and 4 weeks is normalized 2 h after subcutaneous treatment with

2 mg kg^{-1} R-954 (Lawson *et al*., 2005b). Discrepancy between the latter study and our inability to inhibit plasma extravasation with acute treatment using B_1 receptor antagonists is probably related to the doses given. We used doses in the nanomolar range compared with earlier studies, which used micromolar and milligram per kilogram doses of R-954 (Simard *et al*., 2002; Lawson *et al*., 2005b). Nevertheless, our data with R-715 are the first to show that chronic inhibition of B_1 receptors is a viable therapeutic approach to inhibiting retinal plasma extravasation. Importantly, we demonstrate that during chronic inhibition with B_1 receptor antagonists, there is no compensation by the B_2 receptor, as was observed during acute inhibition of the B_1 receptor. A nontransient, continued role for B_1 receptors in the development of diabetic retinopathy is consistent with observations that this receptor is less vulnerable to desensitization and cellular internalization (Couture *et al*., 2001).

Although ACE inhibitors prevent degradation of kinins *in vivo*, benefits of these drugs in diabetic retinopathy have been attributed thus far to the inhibition of the direct and indirect influence of the renin-angiotensin system on endothelial function (Neroev *et al*., 2006). Beyond their antihypertensive effect, ACE inhibitors exert positive effects on the overexpression of retinal vascular endothelial growth factor, and glucose accumulation within retinal cells (Zhang *et al*., 2003; Xie and Zhao, 2004; Zheng *et al*., 2007). Interestingly, it has recently been found that a treatment of 4 weeks with ramipril or NAC blocks both vascular oxidative stress (overproduction of superoxide anion) and the induction of B_1 receptor expression in target tissues of type 2 diabetic rats (Couture and Girolami, 2004; R Couture, personal observations). This suggests that the inhibition of oxidative stress and B_1 receptor expression may contribute to the efficacy of ACE inhibitors in retinopathy, and, thus, inhibition of kinin breakdown during ACE inhibitor therapy is unlikely to lead to the activation of B_1 receptors. Hence, in diabetic retinopathy, when ACE inhibitors are used, one should preferably block B_2 kinin receptors. The potential of this approach deserves further investigation.

In summary, the present data show that in early stages of diabetes, plasma extravasation takes place in the retina of the rat, and that this phenomenon is directly related to diabetes-induced activation of the kallikrein-kinin system. These results provide evidence for the involvement of both kinin B_1 and B_2 receptors in the pathology associated with the early stages of diabetic retinopathy. Our data show that the best scheme for a therapeutic intervention targeting kinin receptors would be to inhibit both B_1 and B_2 kinin receptors chronically and, possibly, to use antioxidants and/or ACE inhibitors as adjuvant therapy.

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Conflict of interest

The authors state no conflict of interest.

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