Modulation of retinal blood flow by kinin B_1 receptor in Streptozotocin-diabetic rats

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ARTICLE INFO

ABSTRACT

The vasoactive kinin B_1 receptor (B_1R) is overexpressed in the retina of diabetic rats in response to hyperglycemia and oxidative stress. The aim of the present study was to determine whether B_1R could contribute to the early retinal blood flow changes occurring in diabetes. Male Wistar rats were rendered diabetic with a single i.p. injection of Streptozotocin (STZ) and studied 4 days or 6 weeks after diabetes induction. The presence of B_1R in the retina was confirmed by Western blot. The impact of oral administration of the B_1R selective antagonist SSR240612 (10 mg/kg) was measured on alteration of retinal perfusion in awake diabetic rats by quantitative autoradiography. Data showed that B_1R was upregulated in the STZ-diabetic retina at 4 days and 6 weeks. Retinal blood flow was not altered in 4-day diabetic rats compared with age-matched controls but was significantly decreased following SSR240612 treatment. In 6-week diabetic rats, retinal blood flow was markedly reduced compared to control rats and SSR240612 did not further decrease the blood flow. These results suggest that B_1R is upregulated in STZ-diabetic retina and has a protective compensatory role on retinal microcirculation at 4 days but not at 6 weeks following diabetes induction.

1. Introduction

The kallikrein-kinin system is involved in micro- and macro-vascular complications associated with diabetic nephropathy, cardio-myopathy and polyneuropathy (Buleon et al., 2008; Couture and Girolami, 2004; Dias et al., 2010; Gabra et al., 2005; Westermann et al., 2009). Through the activation of two G-protein coupled receptors, named B_1 (B_1R) and B_2 (B_2R) (Regoli and Barabe, 1980; Regoli et al., 1998), these inflammatory peptides mediate enhanced capillary permeability, edema, leukocytes infiltration, vasodilation and regulation of local blood flow (Couture et al., 2001; Marceau et al., 1998). The activation of either receptor induces release of nitric oxide (NO) and prostaglandins (PGs) from vascular endothelial cells which consequently promote vasodilation (Ahluwalia and Perretti, 1999; McLean et al., 1999). The widely distributed B_2R mediates the acute effects of bradykinin (BK) and kallidin (KD). In contrast, B_1R is virtually absent in physiological conditions and is highly inducible in

inflammatory, cardiovascular and neurological diseases (Bader et al., 2000; Leeb-Lundberg et al., 2005; Rodi et al., 2005). The preferential endogenous agonists of B_1R are the kininase I metabolites des-Arg^9-BK and des-Arg^{10}-KD (Marceau, 1995; Marceau et al., 1997).

Recent findings suggest that the kallikrein-kinin system could also contribute to the development of diabetic retinopathy (Phipps and Feener, 2008; Wilkinson-Berka and Fletcher, 2004). Diabetic retinopathy is characterized by progressive alterations of the retinal microvasculature leading to breakdown of the blood-retinal barrier, angiogenesis, retinal detachment and vision loss. Hyperglycemia initiates a series of pathological events in the retinal vessels including pericyte and endothelial cells loss, basement membrane thickening, microaneurysms and vascular leakage. Retinal hae-morrhages and capillary occlusion induce the formation of areas of vascular non-perfusion which trigger the proliferation of new blood vessels (Frank, 2004). Endothelial cell dysfunction and retinal blood flow alterations also contribute to the development of dia-betic retinopathy. Alterations of retinal blood flow are known to appear early in the progression of diabetic retinopathy in patients (Bursell et al., 1996; Clermont and Bursell, 2007; Grunwald et al., 1996; Kawagishi et al., 1995) and in animal models of diabetes (Alder et al., 1998; Clermont and Bursell, 2007; Cringle et al., 1993; Higashi et al., 1998; Sutera et al., 1992). Components of the kalli-krein-kinin system are known to be expressed in the human, rabbit

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and rat retina (Kuznetsova et al., 1991; Lim et al., 2008; Ma et al., 1996; Takeda et al., 1999). Particularly, B_1R is overexpressed in the retina of Streptozotocin (STZ)-induced diabetic rats as early as 4 days after diabetes induction through a mechanism involving oxidative stress (Abdouh et al., 2003, 2008). In STZ-diabetic rats, B_1R mediates vasodilation of retinal microvessels *in vitro* (Abdouh et al., 2003) and contributes to the breakdown of blood-retinal barrier *in vivo* (Abdouh et al., 2008; Lawson et al., 2005). These results suggest a role for B_1R in the development of vascular alterations in the diabetic retina.

Therefore, the objective of the present study was to determine whether B_1R is involved in retinal blood flow changes in the early stages of diabetes in the model of STZ-treated rat. The presence of B_1R in the retina of 4-day and 6-week STZ-diabetic rats was assessed by Western blot analysis. The ability of the B_1R antagonist SSR240612 (Gougat et al., 2004) to prevent retinal perfusion abnormality was measured by quantitative autoradiography. The impact of the antagonist was compared to cortical blood flow changes in the same animals.

2. Material and methods

2.1. STZ-diabetic rats

All experimental methods and animal care procedures were approved by the local institutional animal care committee at the University of Montreal, in accordance to the Canadian Council on Animal Care. Male Wistar rats ($n = 77$) weighting 200–250 g were purchased from Charles River (St-Constant, QC, Canada) and housed two per cage in a room under controlled temperature (23 °C), humidity (50%) and lighting (12-hour light/dark cycle) with food and water provided *ad libitum*. For the induction of diabetes, rats received a single i.p. injection of Streptozotocin (STZ, 65 mg/kg, Sigma-Aldrich, Oakville, ON, Canada). Age-matched controls were injected with vehicle (sterile saline 0.9%, pH. 7.4). Glucose concentrations were measured in blood samples obtained from the tail vein with a commercial blood glucose analyzer (Accusoft; Roche Diagnostics, Laval, QC, Canada) 48 h following STZ injection. Only STZ-treated rats with blood glucose concentration higher than 20 mmol/L were considered as diabetic. Rats showing a normal glycemia after STZ injection were discarded. Glycemia was measured once a week and just before experimentation to confirm that STZ-treated rats were diabetic.

2.2. Measurement of B_1R expression in the retina of control and diabetic rats by Western blot analysis

An anti-B_1R antibody was raised in rabbits (Biotechnology Research Institute, Montreal, QC, Canada; http://www.irb.cnrc.gc.ca) against a conserved amino acid sequence from the B_1R protein of mouse and rat aligned using ClustalW software (http://www.ebi.ac.uk/Tools/clustalw2). The used epitope contained 15 amino acids (VFAGRLLKTRVLGTL) localized in the C-terminal part of the B_1R protein. Care was taken to avoid sequence regions containing significant similarities to related mammalian proteins, including B_2R. Two negative controls were run: first, the pre-immune serum was tested and second, B_1R antibodies were pre-absorbed with the peptide which served for immunization. The specificity of B_1R antibodies was further determined in knock-out mice tissues and the results showed the absence of B_1R protein in knock-out mouse and very low expression in wild type mouse (Lin et al., 2010).

Control ($n = 8$) and diabetic ($n = 8$) rats were anaesthetised with pentobarbital (60 mg/kg, i.p.). The eyes were dissected out, the retinae were isolated, immediately frozen in isopentane (-55 °C)

and stored at -80 °C. The presence of B_1R in other ocular tissues was not assessed. Retinae were homogenised in Phosphate Buffer Saline (PBS) containing a cocktail of proteases inhibitors (Sigma-Aldrich, Canada). The homogenates were then centrifuged at 500 g for 5 min. Supernatants were recovered and protein concentration was determined by the BCA method (Thermo Scientific, USA), and Bovine Serum Albumin (BSA) was used as standard protein. Ten μg of total proteins were loaded in each well of 10% SDS-PAGE. The samples were electrophoresed and transferred onto nitrocellulose membranes. The efficiency of the overall procedure was monitored by Ponceau red staining. The membranes were blocked with a commercial blocking buffer from Thermo in PBS-T (Phosphate Buffer Saline -Tween 20, 0.1%), probed with the specific antibody at 1/1000 dilution followed by probing with an HRP (Horseradish Peroxidase)-linked goat anti-rabbit secondary antibody (Santa Cruz Biotech, CA, USA) at 1/25,000 dilution. All incubations with anti-bodies were performed in the commercial blocking buffer. Membranes were rinsed adequately between every step with PBS-T and revealed using Enhanced Chemiluminescence Detection System (ECL) (Super-Signal®, Thermo Scientific, Canada). Dynein was used as standard protein and revealed with mouse anti-dynein monoclonal antibody (Santa Cruz Biotech, CA, USA) at a 1:25,000 dilution. HRP-linked goat anti-mouse (Santa Cruz Biotech, CA, USA) was used as secondary antibody at a 1:25,000 dilution. A quanti-tative analysis of the protein was performed by densitometry using an MCID™ image analysis system (Imaging Research, St. Catharines, ON, Canada).

2.3. Measurement of retinal and cerebral blood flow in diabetic rats by quantitative autoradiography

Changes of retinal and cerebral blood flow were measured at 4 days and 6 weeks after the induction of diabetes by quantitative autoradiography using the diffusible radioactive tracer N-isopro-pyl-p-^{14}C-iodoamphetamine ($[^{14}C]$-IMP) as previously described (Deschenes et al., 2010; Pouliot et al., 2009; Vaucher et al., 1997). Polyurethane catheters were inserted into the femoral vein (Micro-Renathane, I.D. 0.040″, O.D. 0.020″, Braintree Scientific, Braintree, MA, USA) and artery (Tygon Micro Bore, I.D. 0.010″, O.D. 0.030″, Small Parts, Miramar, FL, USA) under 1.5% isoflurane (induction of anesthesia with 3% isoflurane for 5 min). During this procedure, body temperature was monitored with a rectal thermometer and maintained at 37 °C by a heating pad (FHC, Bowdoinham, ME). Before and immediately after the surgery, the topical anesthetic lidocaine hydrochloride 2% (AstraZeneca, Mississauga, ON, Canada) was applied on the skin incision to minimize pain. Rats were then installed in a hammock and left under minimal restraint over a 2-h period to recover from anesthesia. Body temperature was main-tained at 37 °C with a heating lamp and both blood pressure and heart rate were monitored from the tail using a non-invasive blood pressure cuff system (BP1000, Kent Scientific Corporation, Tor-rington, CT, USA) until the measurement of the retinal and cerebral blood flow. Blood chemistry was measured with a veterinarian clinical blood gases and electrolytes analyzer (i-STAT®, HESKA, Fort Collins, CO), from arterial blood samples collected via the arterial catheter.

For both duration of diabetes, rats were divided in 4 groups: control, control treated with the B_1R antagonist SSR240612, STZ-diabetic and STZ-diabetic treated with SSR240612. SSR240612 ((2R)-2-[((3R)-3-(1,3-benzodioxol-5-yl)-3-{[(6-methoxynaphthyl) sulphonyl] amino} propanoyl)amino]-3-(4-{[(2R,6S)-2,6 dimethylpiperidinyl]methyl} phenyl)-N-isopropyl-N-methylpropanamide fumarate) (Sanofi-Aventis R&D, Montpellier, France) is a highly selective ligand for B_1R (Gougat et al., 2004). It was given by gavage (10 mg/kg) 3 h before the autoradiography. The dose of 10 mg/kg was found effective to

block B_1R-mediated effects in several paradigms *in vivo* (Gougat et al., 2004; Dias et al., 2007, 2010). SSR240612 was dissolved in a solution of dimethylsulphoxide (0.5%), ethanol (5%) and Tween-80 (5%) and the solution was completed with distilled water (vehicle) and administered in a volume of 1 ml by 100 g of body weight to control and STZ-diabetic rats. Untreated control and STZ-diabetic rats received the vehicle by gavage.

[^{14}C]-IMP ((D,L)-N-Isopropyl-4- [^{14}C]-iodo(phenyl)amphetamine, 100 μCi/kg; PerkinElmer, Boston, MA, USA, custom synthesis) was dissolved in 600 μl of saline (injectable 0.9% NaCl solution) and infused in fully conscious rats over a 30 s period at a constant rate of 1.2 ml/min using an infusion pump (PHD 2000, Harvard Apparatus, Holliston, MA, USA) through the femoral vein. An average of 20−24 arterial blood samples (10 μl) were collected continuously from the beginning of [^{14}C]-IMP injection to the sacrifice of the animal (2 min) in order to evaluate the arterial contamination curve. Blood samples were digested in 300 μl of soluene (PerkinElmer, Boston, MA, USA) for 2 h at 37 °C. A total of 5 ml of scintillation fluid (Ultimagold, Perkin Elmer, Boston, MA, USA) was then added and the radioactivity was counted in a scintillation counter (LS6500, Beckman Coulter, Mississauga, ON, Canada). The rats were sacrificed by decapitation 2 min after the beginning of the infusion. The eyes and brain were quickly removed within 3 min. A small incision on the superior eyelid was made to indicate the orientation of the eyes and the eyes together with the attached superior eyelid were then harvested. The eyes were immediately excised, the lens removed and the eye cup immersed in a solution of 4% paraformaldehyde for post-fixation. After a one h fixation, retinae were removed from the eye cup, and dissected into four quadrants (superior, inferior, nasal and temporal). The vitreous body was carefully removed from the retinae using fine paint brush. Retinae were whole-mounted on a glass slide with the ganglion cells layer away from the slide. A small incision was made on the retina to indicate the nasal quadrant. The whole-mount retina was then exposed to an X-ray film (Hyperfilm, GE Healthcare Ltd, UK) for 4 days together with a set of [^{14}C]-standards (ARC, St. Louis, USA). The autoradiograms were analyzed using the computerized image analysis MCID Basic Software (v7.0, Interfocus Imaging, Linton, UK). For the CBF quantification at 4 days of diabetes, the brain was frozen in isopentane (−55 °C) and subsequently sliced in 20 μm thick coronal sections using a cryostat (−22 °C) for autoradiography. Slices were exposed on X-ray film for 8 days with [^{14}C]-standards (Amersham Biosciences, UK). For experiment at 6 weeks of diabetes, a piece of the frontal cerebral cortex was dissected, weighted and digested in soluene overnight at 37 °C for liquid scintillation counting. Previous study showed that the values measured by the two methods of sampling are equivalent (Pouliot et al., 2009).

2.4. Calculation of retinal and cerebral blood flow

Retinal and cerebral blood flow was evaluated using the principle of indicator-fractionation technique (Greenberg et al., 1999; Lear et al., 1982; Pouliot et al., 2009) using the equations Eq. (1) for digested cortex and autoradiographic analysis of CBF performed with 20 μm thick sections and Eq. (2) for autoradiographic analysis performed on whole-mount retinae.

$$F = \left[C_{IMP}(T) \bigg/ \int_0^T Ca(t) \right] \tag{1}$$

$$F = \left[C_{IMP}(T) \times 10^{-1} \bigg/ \int_0^T Ca(t) \right] \tag{2}$$

where F is the blood flow (ml/100 g/min), $C_{IMP}(T)$ is the radioactivity measured on the autoradiogram or digested tissue (μCi/g) at the time T (min) of sacrifice and $Ca(t)$ is the arterial concentration of radioactivity measured in the blood samples (μCi/ml). For autoradiographic measurements in the retina, $C_{IMP}(T)$ was read from circular regions of interest of 0.8 mm^2 (1 mm diameter) distributed at the 1, 2 and 3 mm isopters away from the center of the optic nerve head in all retinal quadrants (Pouliot et al., 2009). Since the common radioactive standards used for autoradiography are calibrated for 20 μm thick sections and the rat retina thickness is 205 ± 11 μm (Duong et al., 2008), the measured [^{14}C]-IMP concentration values from autoradiograms ($C_{IMP}(T)$) were uniformly corrected by a factor 10 (Pouliot et al., 2009). For the brain, 8 regions of interest (area adjusted to the region size, 2−9 mm^2) related to vision or in control areas were selected and analyzed at 3 coronal levels from Bregma: AP + 1.6 mm; cingulate, frontal cortex, striatum; AP −4.5 mm; parietal cortex, hippocampus, dorsolateral geniculate nucleus (DLG); AP −6.3 mm; occipital cortex, superior colliculus.

2.5. Statistical analysis

Student's *t*-test on unpaired samples was used for comparison of B_1R expression in the retina of control and STZ-diabetic rats. One-way ANOVA and Bonferroni post-hoc tests were performed for comparison between control group, control group treated with SSR240612, diabetic group and diabetic group treated with SSR240612 for each duration of diabetes separately (4 days and

Table 1
Effect of diabetes and B_1R antagonist SSR240612 on the physiological parameters monitored in the conscious rats after two hours of recovery from anaesthesia and prior to the injection of [^{14}C]-IMP.

	4 days				6 weeks			
	CTL (n = 12)	CTL + SSR (n = 6)	STZ (n = 8)	STZ + SSR (n = 8)	CTL (n = 7)	CTL + SSR (n = 6)	STZ (n = 7)	STZ + SSR (n = 7)
Body weight (g)	257 ± 17	260 ± 29	239 ± 26	255 ± 17	419 ± 31	472 ± 28	319 ± 71*	314 ± 65**
Glycemia (mmol/L)	6 ± 1	7 ± 1	26 ± 5***	29 ± 5***	7 ± 2	7 ± 2	31 ± 4***	31 ± 5***
Body temperature (°C)	37.5 ± 0.3	37.8 ± 0.3	37.2 ± 0.3	37.4 ± 0.3	37.9 ± 0.4	37.7 ± 0.4	37.5 ± 0.3	37.7 ± 0.3
Mean blood pressure (mmHg)	116 ± 13	120 ± 5	126 ± 10	126 ± 11	119 ± 15	117 ± 10	122 ± 9	127 ± 12
Heart Rate (Beats/min)	503 ± 52	475 ± 33	459 ± 56	497 ± 35	419 ± 52	528 ± 134	469 ± 130	473 ± 84
Arterial pH	7.45 ± 0.01	7.45 ± 0.02	7.45 ± 0.03	7.45 ± 0.02	7.47 ± 0.02	7.45 ± 0.01	7.42 ± 0.05	7.45 ± 0.04
Arterial pO$_2$ (mmHg)	86 ± 4	85 ± 3	85 ± 5	82 ± 2	86 ± 4	85 ± 6	87 ± 6	87 ± 4
Arterial pCO$_2$ (mmHg)	38 ± 2	38 ± 3	38 ± 1	39 ± 2	39 ± 1	40 ± 2	33 ± 5**	39 ± 2
Arterial HCO$_3^-$ (mmol/L)	26 ± 1	26 ± 1	26 ± 2	27 ± 1	28 ± 2	27 ± 1	21 ± 4**	27 ± 3
Hematocrit (% PCV)	40 ± 4	39 ± 2	41 ± 2	42 ± 1	44 ± 2	45 ± 3	50 ± 6*	48 ± 2

Values are means ± SD. The selective B_1R antagonist SSR240612 was administered in the CTL + SSR and STZ + SSR groups. The vehicle only was administered in CTL and STZ groups. pO$_2$, pCO$_2$, partial gas pressure of oxygen and carbon dioxide, respectively; HCO$_3^-$, bicarbonate; CTL, control group; PCV, packed cell volume STZ, diabetic group; SSR, SSR240612. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with control group.
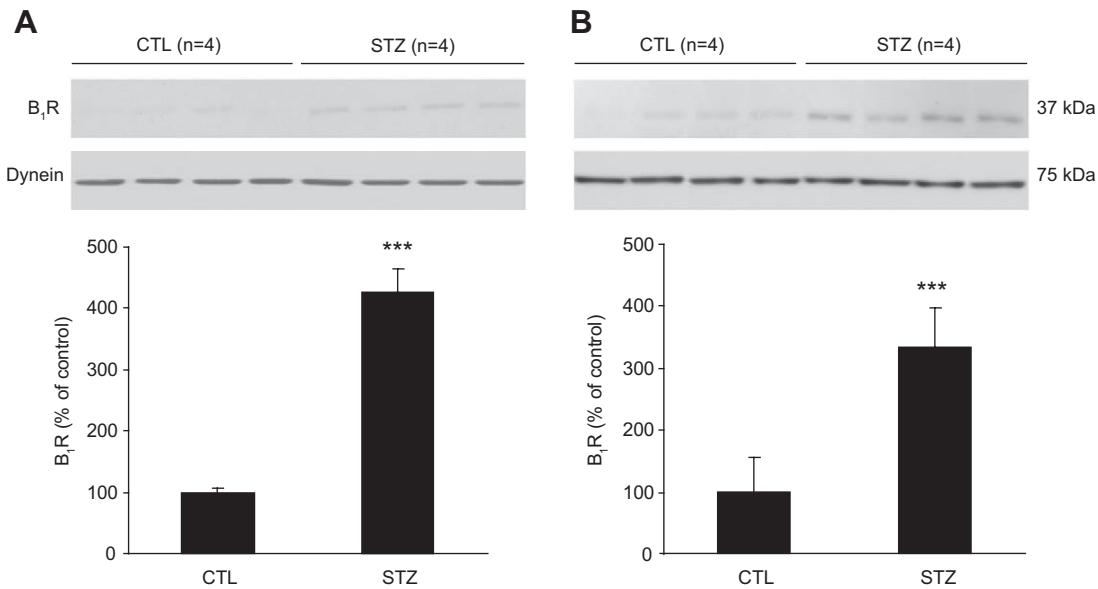
Fig. 1. Protein expression of B_1R in the retina of diabetic rats measured by Western blot. B_1R was upregulated in the retina at 4 days (A) and 6 weeks (B) after the induction of diabetes. ***$p < 0.001$, significantly different from control group.

6 weeks of diabetes) for (1) the physiological parameters, (2) the regional RBF in each isopter, (3) the CBF. A significance level of $p < 0.05$ was chosen.

3. Results

3.1. Physiological parameters

Glycemia was significantly increased in 4-day and 6-week diabetic rats compared to age-matched control rats ($p < 0.001$, Table 1). Treatment with SSR240612 had no effect on glycemia in both control and STZ-diabetic rats at both end points. All other physiological parameters were not changed in 4-day diabetic group treated or not with SSR240612 compared to the control group. Body temperature, blood pressure, heart rate, pH and pO_2 were not affected in 6-week diabetic rats treated or not with SSR240612 ($p > 0.05$, Table 1). However, body weight was significantly decreased in 6-week diabetic rats treated or not with SSR240612 compared to age-matched controls ($p < 0.01$, Table 1), which is commonly found in diabetic animals. pCO_2 and HCO_3^- were significantly decreased ($p < 0.01$) and hematocrit was significantly increased ($p = 0.02$), in 6-week diabetic rats not treated with SSR240612 compared to age-matched controls, which might indicate a slight acid-base unbalance which is often seen in diabetic animals. This dysregulation of acid-base balance was not seen in

SSR240612 treated animals ($p > 0.05$, Table 1), which needs further clarification.

3.2. Expression of B_1R in the retina of diabetic rats

The level of B_1R protein measured by Western blotting was significantly increased in the retina of 4-day (326%, $p = 0.000$) and 6-week (237%, $p = 0.001$) diabetic rats compared to age-matched controls (Fig. 1).

3.3. Effect of B_1R antagonist SSR240612 on retinal blood flow measured by quantitative and regional autoradiography

RBF was not affected by hyperglycemia in 4-day diabetic rats compared to age-matched control rats for all the retina subregions measured ($p > 0.05$, Table 2, Figs. 2 and 3). B_1R antagonist SSR240612 treatment had no effect on RBF in control animals ($p > 0.05$, Table 2, Figs. 2 and 3) – the slight non significant decrease of RBF may be attributed to a low level of B_1R expression in control animals – but significantly decreased RBF in 4-day diabetic rats compared to control rats at 2 mm isopter (21%, $p = 0.026$) and 3 mm isopter (26%, $p = 0.005$, Table 2, Figs. 2 and 3).

In contrast, RBF was significantly decreased in 6-week diabetic rats compared to control rats at 1 mm isopter (29%, $p = 0.003$), 2 mm isopter (29%, $p = 0.006$) and 3 mm isopter (39%, $p = 0.001$). In the optic nerve region, RBF was also decreased by 29% but this was

Table 2
Effect of diabetes and B_1R antagonist SSR240612 on retinal and cerebral blood flow.

	4 days				6 weeks			
	CTL	CTL + SSR	STZ	STZ + SSR	CTL	CTL + SSR	STZ	STZ + SSR
Retinal blood flow	($n = 12$)	($n = 6$)	($n = 8$)	($n = 8$)	($n = 7$)	($n = 6$)	($n = 7$)	($n = 7$)
Optic nerve	111 ± 18	102 ± 13	98 ± 25	86 ± 18	102 ± 19	90 ± 17	72 ± 29	81 ± 9
1 mm isopter	119 ± 18	114 ± 10	113 ± 25	97 ± 22	118 ± 13	102 ± 9	84 ± 25*	90 ± 7*
2 mm isopter	108 ± 15	106 ± 5	100 ± 18	85 ± 19*	108 ± 12	92 ± 9	77 ± 26*	83 ± 6*
3 mm isopter	87 ± 15	98 ± 4	80 ± 16	64 ± 13*	99 ± 13	82 ± 8	62 ± 25*	71 ± 7*
Cerebral blood flow	($n = 6$)	($n = 6$)	($n = 5$)	($n = 5$)	($n = 7$)	($n = 6$)	($n = 7$)	($n = 7$)
Frontal cortex	127 ± 12	105 ± 11	121 ± 15	105 ± 32	116 ± 26	87 ± 10	79 ± 24*	81 ± 18*

Values are means ± SD and are expressed in ml/100 g/min. The selective B_1R antagonist SSR240612 was administered in the CTL + SSR and STZ + SSR groups. The vehicle only was administered in CTL and STZ groups. CTL, control group; STZ, diabetic group; SSR, SSR240612. * $p < 0.05$ compared with control group.
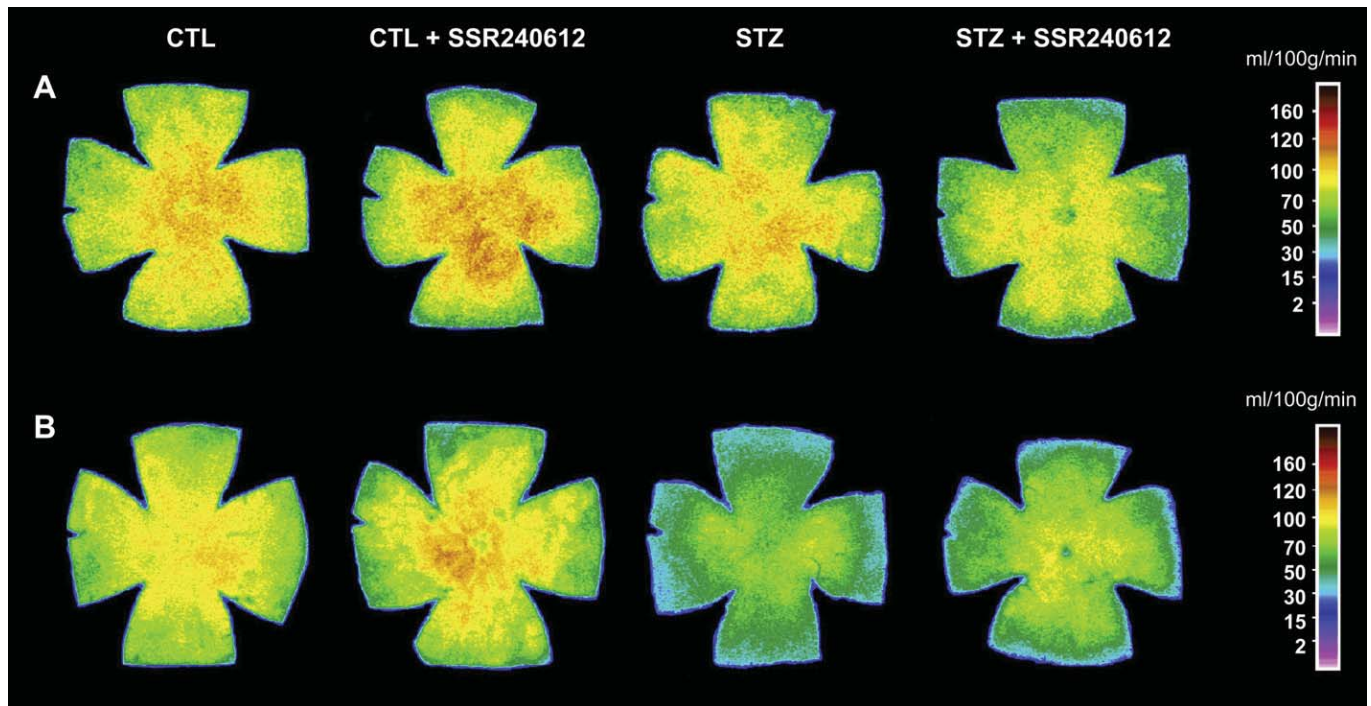
Fig. 2. Representative whole-mount retina autoradiograms displayed in pseudocolor from a control rat treated with vehicle, a control rat treated with the selective B_1R antagonist SSR240612, a STZ-diabetic rat treated with vehicle and a STZ-diabetic rat treated with SSR240612 at 4 days (A) and 6 weeks (B) after diabetes induction. The correspondence between pseudocolor and blood flow values (ml/100 g/min) is represented on the scale on the right side of the figure.

not statistically significant ($p = 0.171$). B_1R antagonist SSR240612 treatment significantly decreased RBF in 6-week diabetic rats compared to age-matched control rats at 1 mm isopter (24%, $p = 0.016$), 2 mm isopter (24%, $p = 0.034$) and 3 mm isopter (29%, $p = 0.012$). However, this decrease was not significant compared to 6-week diabetic rats not treated with SSR240612 (Table 2, Fig. 3).
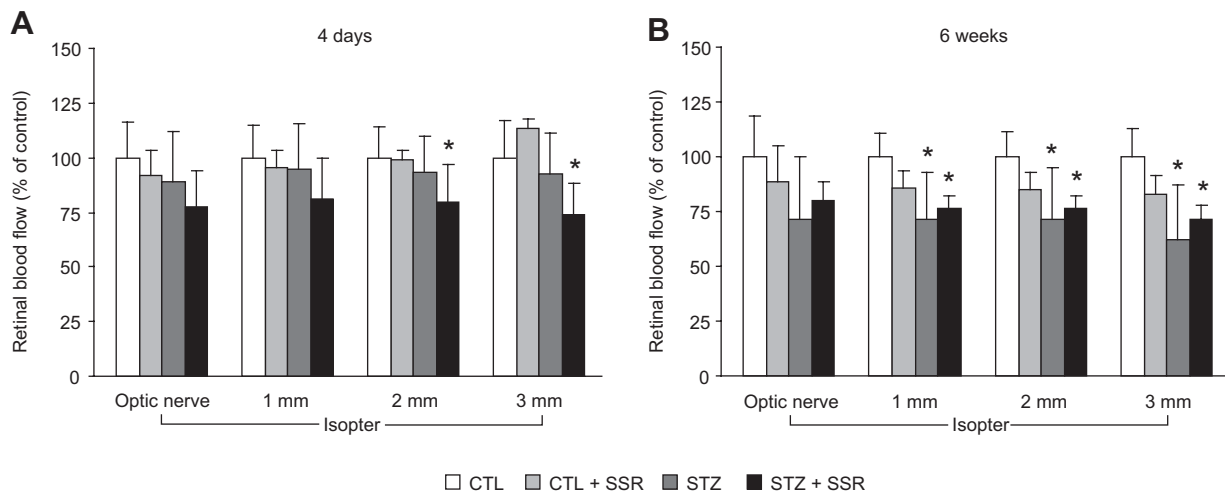
3.4. Effect of B1R antagonist SSR240612 on cerebral blood flow

Blood flow in frontal cortex was not altered in 4-day diabetic rats compared to controls and not changed by the B_1R antagonist treatment ($p > 0.05$, Table 2, Fig. 4). However, it was significantly reduced (32%) in comparison to age-matched controls in 6-week

diabetic rats treated ($p = 0.012$, Table 2, Fig. 4) or not ($p = 0.014$, Table 2, Fig. 4) with SSR240612, as shown in the retina of diabetic rats. Treatment with SSR240612 had no effect on CBF in 6-week control rats ($p > 0.05$). There was no regional variation since cerebral blood flow measured by autoradiography in cingulated cortex, striatum, parietal cortex, hippocampus, DLG, occipital cortex, and superior colliculus was not altered by diabetes and B_1R antagonist treatment (data not shown).

4. Discussion

The results clearly show that kinin B_1R is upregulated in the STZ-rat retina up to 6 weeks following diabetes induction. The B_1R

Fig. 3. Effect of the treatment with the selective B_1R antagonist SSR240612 on retinal blood flow. Percent change (versus control) of retinal blood flow were measured in diabetic rats treated with the selective B_1R antagonist SSR240612 (STZ + SSR) or vehicle (STZ) at 4 days (A) and 6 weeks (B) after diabetes induction and compared with aged matched sham animals (CTL and CTL + SSR). Regional blood flow was measured on each isopter by autoradiography. *$p < 0.05$, significantly different from control group.
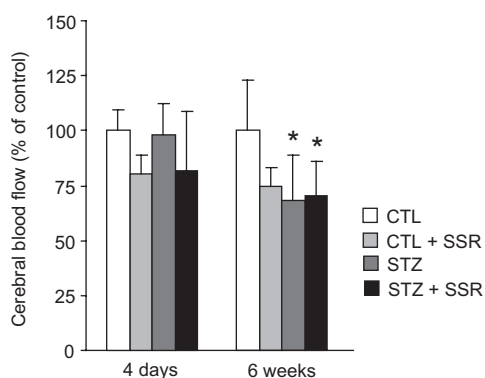
Fig. 4. Effect of the treatment with the selective B_1R antagonist SSR240612 on cerebral blood flow. Percent change (versus control) of cerebral blood flow was measured in control and diabetic rats treated with B_1R antagonist SSR240612 compared with CTL and STZ groups treated with vehicle only. The blood flow was measured in frontal cortex 4 days and 6 weeks after diabetes induction. $^*p < 0.05$, significantly different from control group.

blockade reduced the RBF at 4 days but not at 6 weeks. These results suggest that the B_1R is tonically activated by endogenous kinins and contributes to prevent diabetes-induced reduction of retinal blood flow 4 days following STZ injection. This positive effect of kinins on retinal blood flow was not maintained 6 weeks following STZ injection.

4.1. Retinal blood flow in diabetic rats

Our results demonstrated that retinal blood flow was not altered in 4-day diabetic rats but was significantly decreased in 6-week diabetic rats compared with age-matched controls. This is the first study evaluating RBF in the early days of diabetes onset. Previous studies using fluorescein angiography reported decreased RBF in STZ-diabetic rats at one (Bursell et al., 1992; Clermont et al., 1994; Takagi et al., 1996), 2 and 4 weeks (Higashi et al., 1998) of diabetes, although some studies using microsphere technique or hydrogen clearance reported increased RBF in STZ-diabetic rats from 3 to 6 weeks after diabetes induction (Cringle et al., 1993; Pugliese et al., 1990; Sutera et al., 1992; Tilton et al., 1989). Our findings suggest a reduction of blood flow in the diabetic rat retina. Moreover, our results show that the deficit of blood flow is not a primary event since it is not present at the fourth day of diabetes induction. In diabetic patients, previous studies on retinal blood flow have also provided diverse results. Decreased retinal blood flow in diabetic patients with absent or mild diabetic retinopathy was reported (Bursell et al., 1996; Feke et al., 1994; Feng et al., 2000) as well as a tendency to increased blood flow as retinopathy progresses (Clermont et al., 1997). Other studies also reported increased blood flow in diabetic patients with no or mild diabetic retinopathy (Grunwald et al., 1996; Pemp et al., 2010). However, most of the studies agree that impairment of retinal blood flow occurs in the early steps of diabetic retinopathy progression and even prior to the development of clinical signs of the disease.

4.2. Effect of the selective B_1R antagonist SSR240612 on retinal blood flow

The principal series of results show that the B_1R in the retina of STZ-diabetic rat could be involved in blood flow regulation during diabetes in the presence of appropriate endogenous ligands. Accordingly, B_1R appeared to maintain the blood flow in the diabetic retina at 4 days but not at 6 weeks. The B_1R protein expression within the retina early on after diabetes induction (4 days) corroborates previous findings on B_1R binding sites (Abdouh et al., 2003), but its presence at 6 weeks after diabetes onset is shown here for the first time. B_1R being indicative of inflammatory or oxidative processes, this data suggests that the retina undergoes a stress from the beginning of the hyperglycemia, which persists as long as hyperglycemia was maintained. The cumulative effect of this process would most likely damage the retina. Previous *in vitro* study showed selective and dose-dependent vasodilation of retinal vessels in isolated retina of STZ-diabetic rats in the presence of selective B_1R agonists; a response that involved intracellular calcium mobilization, release of nitric oxide and prostaglandins in endothelial cells (Abdouh et al., 2003). Thus, the B_1R seems to have a direct action on blood vessel endothelium. The effect of B_1R is most likely directly related to hyperglycemia caused by STZ since it has already been shown that retinal vessels from 4-day STZ-rats that failed to develop hyperglycemia do not dilate in response to B_1R agonist (Abdouh et al., 2003). As RBF was slightly but not significantly decreased in 4-day diabetic rats without SSR240612 treatment but was strongly decreased with SSR240612 treatment, this suggests that endogenous kinins may act on B_1R to induce vasodilation and maintain a normal blood flow in the diabetic retina. This suggests a protective role for B_1R on the retinal circulation at the very early stage of diabetes.

The positive effect of B_1R on retinal perfusion that was present in 4-day diabetic rats was not preserved 6 weeks following diabetes induction since B_1R blockade did not alter the blood flow level compared to diabetic rats without SSR240612 treatment. The decrease of blood flow in diabetic animals as well as the absence of a physiological response to the B_1R antagonist is indicative of either an absence of endogenous kinins that could activate B_1R or the presence of other vasoactive substances that could contribute to regulation of blood flow. The unbalance between vasoconstrictors and vasodilators in the diabetic retina is a major cause of RBF alteration. Vasoactive molecules such as Endothelin-1 (ET-1) and Angiotensin-II (Ang-II) are known to be expressed in the retina and contribute to retinal vasoconstriction and reduction of RBF in diabetic rats (Bursell et al., 1995; Horio et al., 2004). The modulation of RBF by vasoconstrictive molecules could therefore partially mask the vasodilatory effect of B_1R at 6 weeks of diabetes. On the other hand, the vasodilatory effect of B_1R could be altered because of an endothelial dysfunction and decreased NO-mediated response in 6-week diabetic rats, since B_1R is known to cause vasodilation of retinal vessels via an endothelium-dependent mechanism (Abdouh et al., 2003). In the retina and many vascular beds, endothelium-dependent vasodilation was found to be diminished in diabetes (De Vriese et al., 2000; Edgley et al., 2008; Oyama et al., 1986; Schmetterer et al., 1997; Tabit et al., 2010; Taylor et al., 1992).

4.3. Effect of the selective B_1R antagonist SSR240612 on cerebral blood flow in diabetic rats

Our results show that blood flow in frontal cortex was not altered 4 days after diabetes induction but was strongly reduced at 6 weeks. Previous studies have also reported decreased blood flow in cortex in diabetic rats 2 to 5 weeks post-STZ injection using n- $[^{14}C]$butanol (Harik and LaManna, 1988) and $[^{14}C]$-iodoantipyrine (Duckrow et al., 1987). A reduction of blood flow was also reported at 16 and 20 weeks following diabetes induction (Knudsen et al., 1991; Manschot et al., 2003). However, some studies reported normal (Granstam and Granstam, 2003) and increased blood flow (Sutera et al., 1992) in the brain of STZ-diabetic rats. In diabetic rats, changes of blood flow in frontal cortex were similar to changes of retinal blood flow since blood flow was not changed at 4 days but

significantly decreased at 6 weeks in both organs. This suggests that the diabetes has a tendency to induce a general deficit in blood flow to the same extent in the retina and frontal cortex. However, B_1R antagonist in 4-day diabetic rats induced a strong decrease of blood flow in the retina, whereas it had no effect on blood flow in frontal cortex. These results suggest that B_1R, which is overexpressed in the cortex of diabetic rats as early as 4 days after diabetes induction (Campos et al., 2005) does not exert the same protective effect on cortical blood flow as seen in the retina. This may reflect the lower level of endogenous kinins or B_1R in the brain compared to retina. This could be related to the stronger reactivity of the retina to oxidative stress compared to the brain (Grammas and Riden, 2003).

5. Conclusion

In summary, our results demonstrated that B_1R is overexpressed in the diabetic rat retina and exerts a protective effect to reduce RBF deficit in 4-day diabetic rats but is not involved in the RBF reduction present at 6 weeks of diabetes. Its involvement in other aspects of diabetic retina damage deserves further investigation.

Acknowledgements

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