A primary role for kinin B1 receptor in inflammation, organ damage, and lethal thrombosis in a rat model of septic shock in diabetes

N Tidjane¹, A Hachem², Y Zaid², Y Merhi², L Gaboury³, J-P Girolami⁴, and R Couture¹

¹Department of Molecular and Integrative Physiology, Faculty of Medicine, Université de Montréal, Montréal, QC, Canada

²Laboratory of Thrombosis and Hemostasis, Research Centre, Montreal Heart Institute, Montréal, QC, Canada

³Department of Pathology and Cellular Biology, Faculty of Medicine, Université de Montréal, Montréal, QC, Canada

⁴Institute of Metabolic and Cardiovascular Diseases, INSERM, U 1048, Université Paul Sabatier, Toulouse, France

Abstract

Diabetes mellitus and septic shock increase the incidence of mortality by thrombosis. Although kinin B1 receptor (B1R) is involved in both pathologies, its role in platelet function and thrombosis remains unknown. This study investigates the expression, the inflammatory, and pro-thrombotic effects of B1R in a model of septic shock in diabetic rats. Sprague-Dawley rats were made diabetic with streptozotocin (STZ) (65 mg/kg, i.p.). Four days later, control and STZ-diabetic rats were injected with lipopolysaccharide (LPS) (2 mg/kg, i.p.) or the vehicle. B1R antagonist (SSR240612, 10 mg/kg by gavage) was given either acutely (12 and 24 h prior to endpoint analysis) or daily for up to 7 days. Moreover, a 7-day treatment was given either with cyclooxygenase (COX)-2 inhibitor (niflumic acid, 5 mg/kg, i.p.), non-selective COX-1 and COX-2 inhibitor (indomethacin, 10 mg/kg, i.p.), non-selective nitric oxide synthase (NOS) inhibitor (L-NAME, 50 mg/kg by gavage), iNOS inhibitor (1400W, 5 mg/kg, i.p.), or heparin (100 IU/kg, s.c.). The following endpoints were measured: edema and vascular permeability (Evans blue dye), B1R expression (qRT-PCR, western blot, flow cytometry), aggregation in platelet-rich plasma (optical aggregometry), and organ damage (histology). Rats treated with STZ, LPS, and STZ plus LPS showed significant increases in edema and vascular permeability (heart, kidney, lung, and liver) and increased expression of B1R in heart and kidney (mRNA) and platelets (protein). Lethal septic shock induced by LPS was enhanced in STZ-diabetic rats and was associated with lung and kidney damage, including platelet micro-aggregate formation. SSR240612 prevented all these abnormalities as well as STZ-induced hyperglycemia and LPS-induced hyperthermia. Similarly to

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Corresponding author: Réjean Couture, Department of Molecular and Integrative Physiology, Faculty of Medicine, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montréal, Québec H3C 3J7, Canada. rejean.couture@umontreal.ca.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

SSR240612, blockade of iNOS and COX-2 improved survival. Data provide the first evidence that kinin B1R plays a primary role in lethal thrombosis in a rat model of septic shock in diabetes. Pharmacological rescue was made possible with B1R antagonism or by inhibition of iNOS and COX-2, which may act as downstream mechanisms.

Keywords

diabetes mellitus; disseminated intravascular coagulation; inflammation; kinins; LPS; SSR240612; thrombosis

Disseminated intravascular coagulation (DIC) is an acquired syndrome secondary to excessive activation of coagulation leading to micro-vascular thrombosis, multiple organ failure, and death. In the most severe cases, excessive activation of fibrinolysis is observed. The normal anticoagulant and fibrinolytic systems are overwhelmed and coagulation activation cannot be contained. The pathophysiological phenomenon rapidly becomes systemic, increasing depletion of natural anticoagulants, and consumption of coagulation factors and platelets.¹

The biological and clinical expression of DIC is extremely polymorphic, particularly in diabetes mellitus and sepsis.² Diabetes mellitus is associated with a hypercoagulable and pro-thrombotic state³ that amplifies the systemic inflammatory response following infectious insults affecting multiple organs and systems in septic patients.² In addition, diabetes mellitus increases vascular permeability and blood viscosity, which reinforces thrombus formation.⁴ Moreover, diabetic patients display tortuous micro-vessels and large platelets; the extent of tortuosity is an important factor in shear-induced thrombosis in micro-vessels.⁵ Thrombosis accounts for 80% of deaths in patients with diabetes mellitus who have an increased risk of developing sepsis and infection, particularly in intensive care units.^{5,6}

Diabetes mellitus and septic shock increase the production of reactive oxygen species (ROS) and pro-inflammatory cytokines, involving particularly the kallikrein-kinin system.⁷ Kinin B1 receptor (B1R), an important component of this system, is upregulated by endotoxins and may represent a therapeutic target in sepsis, yet its contribution in the acute phase of lipopolysaccharide (LPS)-induced endotoxin shock remains controversial.^{8–12} Hypotension, renal failure, and mortality were increased by LPS in B1R knockout mice as a possible consequence of supra activation of kinin B2 receptor (B2R).⁸ Others showed that the hypotensive response to LPS treatment was markedly reduced in these mice during the first 35 min¹⁰ and completely abolished in double B1R-B2R knockout mice, yet both kinin receptors deficiency did not improve survival.¹¹ Moreover, transgenic rats with selective endothelial overexpression of B1R have an increased susceptibility to endotoxic shock.⁹ Conversely, B1R blockade reversed the beneficial effects of B2R blockade on survival in porcine endotoxin shock.¹² Thus whether or not B2R is salutary and B1R deleterious in septic shock remains uncertain as opposite data were obtained depending on the experimental model. In a preliminary study, we found that B1R blockade reversed the enhanced mortality to LPS-induced septic shock in streptozotocin (STZ)-diabetic rats. B1R is associated with pain neuropathy, nephropathy, retinopathy, and cardio-metabolic

complications in various models of type 1 and type 2 diabetes,⁷ but its role in platelet function and thrombosis in link with diabetes mellitus and septic shock remains poorly defined.

To address the role of kinin B1R in thrombus formation due to DIC encountered in diabetes mellitus with severe infection, we developed a unique model of sepsis in diabetes by combining STZ and LPS treatments in rats. It was hypothesized that kinin B1R expression is enhanced in cardiovascular tissues/platelets and its endogenous activation causes angioedema increasing blood viscosity and also the formation of microthrombi and organ damage, thereby increasing the risk of mortality. As mediators generated by cyclooxygenase-1 and -2 (COX-1 and COX-2) and inducible nitric oxide synthase (iNOS) are involved in septic shock and in B1R-induced inflammation, inhibitors of these enzymatic pathways were included in the study design.

Materials and methods

Experimental animals and care

All experimental methods and animal care procedures were approved by the Animal Care Committee of the Université de Montréal (protocols 09-030, 11-141, and 12-198), in accordance with the Canadian Council on Animal Care. Male Sprague-Dawley rats (200–225 g; Charles River, St-Constant, QC, Canada) were housed two per cage, under controlled conditions of temperature (23°C) and humidity (50%), on a 12 h light-dark cycle and allowed free access to normal chow diet (Charles River Rodent) and tap water.

Animal model of septic shock in STZ-diabetic rats

Rats were injected under a low light condition with freshly prepared STZ (65 mg/kg, i.p.; Zanosar, McKesson, QC, Canada). Age-matched controls were injected with vehicle (sterile saline 0.9%, pH 7.0). LPS from *E. coli* (2 mg/kg, i.p. 0111:B4 from Sigma-Aldrich, ON, Canada) was administered 4 days after treatment with STZ or in control rats to provoke the septic shock. Blood glucose was measured with a commercial blood glucose-monitoring kit (Accusoft; Roche Diagnostics, QC, Canada) from a drop of blood obtained from the tail vein. Only STZ-treated rats whose blood glucose concentration was higher than 20 mM at day 4 were used and considered as diabetic.

Acute treatment with the kinin B1R antagonist SSR240612

The impact of B1R antagonism on glycemia, core temperature, edema, vascular permeability, and B1R mRNA expression was measured as follows: SSR240612 (10 mg/kg) was administered by gav-age at the same time as LPS and 12 h later in rats made diabetic with STZ 4 days earlier or in control rats. Rats were sacrificed with isoflurane 12 h after the second treatment with SSR240612. In other words, SSR240612 was given 24 h and 12 h prior to sacrifice as defined in figure legends.

Measure of core temperature

Core temperature (°C) was measured with a flexible and lubricated digital thermometer inserted into the rectum (2.5 cm) for 10 s in unanesthetized rats. Readings were taken before (time 0) and at various intervals (3, 4, 6, 8, 12, and 24 h) post-LPS treatment.

Edema measurement

Heart, kidney, lung, and liver edema were measured by subtracting the value of dry weight tissue from that of wet weight tissue at sacrifice. The difference reflecting the amount of water (in grams) retained in tissues was translated into volume (mL) where 1 g corresponds to 1 mL of water. These rats were not used to evaluate vascular permeability.

Vascular permeability measurement

The increased in vascular permeability was measured by quantifying the Evans blue dye (Sigma-Aldrich, ON, Canada) bound to albumin in various tissues (heart, kidney, lung, and liver). Rats were anesthetized with isoflurane to insert a catheter PE-10 into a femoral vein through which 1000 IU of heparin sodium was injected. After 1–2 days recovery from vascular surgery, rats received intravenously Evans blue dye (35 mg/ kg) 20 min before decapitation under isoflurane. Then organs were collected, weighted, and placed in 8 mL formamide for 48 h at 60°C. After centrifugation, the optical density of the solution was measured by spectrophotometry at 620 nm. Data were expressed as µg of Evans blue/g of wet weight tissue.

Real-time quantitative PCR

After sacrifice, isolated heart and kidney cortex were put in RNA^{later} stabilization reagent (QIAGEN, CA, USA). Total RNA was extracted from about 10 mg of tissue according to the manufacturer's instructions. First-strand cDNA synthesized from 400 ng total RNA with random hexamer primers was used as template for each reaction with the QuantiTect Rev Transcription Kit (QIAGEN). qRT-PCR was performed in SYBR Green Master mix (QIAGEN) with 300 nM of each primer and signal detected using a Mx3000p device (Stratagene, CA, USA). For standardization and quantification, rat 18S was amplified simultaneously. The primer pairs were designed by Vector NTI software (Table 1). PCR conditions were: 95°C for 15 min, followed by 46 amplification cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The cycle threshold (Ct) value represents the cycle number at which a fluorescent signal rises statistically above background. The relative quantification of gene expression was analyzed by the 2^{-Ct} method.

Platelet isolation for flow cytometry and western blot

Treated rats were anesthetized with isoflurane and blood was collected by left ventricular puncture into syringes containing one-twentieth blood volume (0.2 mL) of heparin as anticoagulant. Blood was diluted (1:1) with modified Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO₃, 2 mM MgCl₂, 2 mM CaCl₂, 1 mg/mL BSA, 1 mg/mL dextrose, pH 7.4), containing 0.1 µg/mL PGE₁ and centrifuged at 160 g for 8 min. Platelet-rich plasma (PRP) was then centrifuged at 1000 g for 5 min in the presence of 0.1 µg/mL PGE₁ and the

platelet pellet was then resuspended in modified Tyrode's buffer to a final concentration of $250 \times 10^6/\text{mL}$.¹³

Platelet isolation for aggregometry

Rats were anesthetized with pentobarbital (65 mg/kg, i.p.) and a catheter was inserted into the right femoral artery. Arterial blood was collected into 3.8% sodium citrate at a ratio of 1:9 (1 part anticoagulant to 9 parts blood). Whole blood samples were processed within 1 h of collection. PRP was prepared by centrifugation at 150 g for 15 min. Platelet-poor plasma (PPP) was prepared by further centrifugation of the remaining plasma at 200 g for 15 min, and finally the PRP was transferred to a stoppered plastic tube. All procedures were performed at room temperature.

Western blot on platelets

For protein quantification, platelets were homogenized in Laemmli buffer and lysis buffer. In brief, protein extracts were assayed, loaded (15–30 μg) in 10% SDS-polyacrylamide gels, separated by electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated (1 h) in a blocking buffer containing 5% non-fat milk in Tris-buffered saline (TBS) + Tween 20 (TBS-T, 0.1%), then blotted overnight at 4°C with either rabbit anti-rat B1R (1:1000), anti-rat B2R (1:500), anti-CD45 (H-230) (SC2-5590, Santa Cruz Biotechnology, 1:500), or anti-dynein (SC-13524, Santa Cruz Biotechnology, 1:25,000). Following washing steps, membranes were labeled with horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit # 611-703-127, Rockland, 1:25,000) for 1 h, washed, and bound peroxidase activity was then detected by enhanced chemiluminescence (PerkinElmer Life Sciences, MA, USA). Band intensities were assessed by densitometry quantification. Detection of BK receptor proteins was made with selective anti-B1R and -B2R antibodies raised in rabbits (Biotechnology Research Institute, Montréal, QC, Canada) against a conserved amino acid sequence from B1R and B2R proteins of mouse and rat. The epitopes used contained 15 amino acids localized in the C-terminal region of B1R (VFAGRLKTRVLGTL) and 15 amino acids localized in the second extracellular domain of B2R (TIANNFDWVFGEVLC). Care was taken to avoid sequences with similarity to related mammalian proteins, including the opposite receptor. One negative control was run for each antibody using the pre-immune serum. Specificity of our anti-B1R and -B2R antibodies was further determined using mouse kidney extracts from wild-type (WT), B1R and B2R knockout (KO) mice.^{14,15}

Flow cytometry

B1R and B2R expression in isolated platelets was measured by flow cytometry. Briefly, platelets ($250 \times 10^6/\text{mL}$) were fixed with paraformaldehyde for 30 min, washed, and permeabilized with PBS containing 0.01% Triton X-100 and 1% BSA for 10 min. Platelets were then washed and incubated with saturating concentrations of our primary rabbit anti-rat B1R antibody, rabbit anti-rat B2R antibody, or their isotype-matched control IgG for 30 min. Platelets were then fixed for 30 min with paraformaldehyde, washed, and resuspended in PBS. Platelets were incubated with secondary goat anti-rabbit anti-IgG-FITC (SC-2012, Santa Cruz Biotechnology) for 30 min, fixed, and analyzed (5000 events) on an EPICS XL Coulter (Beckman Coulter) after gating for their characteristic forward and side scatter

properties. Platelet gating was confirmed by an anti-rat CD61-Phycoerythrin (PE) conjugated antibody (SC-73, Santa Cruz Biotechnology). Thrombin (0.1 U/mL) was also added to induce full activation of platelets.

Pharmacological treatments to prevent endotoxin-induced septic shock

LPS (2 mg/kg, i.p.) dissolved in saline was administered only once either to control rats (n = 8) or 4 days after STZ (65 mg/kg, i.p., n = 56). Two cohorts composed of STZ plus LPS rats were divided into two groups: the first group (n = 38) was treated either with heparin (100 IU/kg, s.c.) (Organon Canada Ltd, ON, Canada), L-NAME (50 mg/kg, by gavage) (Sigma Aldrich, ON, Canada) prepared in saline, or indomethacin (10 mg/kg, i.p.) (Sigma-Aldrich, ON, Canada) dissolved in ethanol (70%) or with their vehicle. The second cohort (n = 18) received either 1400W (5 mg/kg, i.p.) (Enzo Life Science, NY, USA) dissolved in saline, niflumic acid (5 mg/kg, i.p.) (Sigma-Aldrich, ON, Canada) dissolved in saline with 2.5% NaOH (6 mol/L), or SSR240612 (10 mg/kg, by gavage) dissolved in dimethyl sulfoxide (DMSO, 0.5% v/v), ethanol (5% v/v), and Tween-80 (5% v/v). These treatments with inhibitors or antagonists were given 1 h before LPS administration and daily for up to 7 days post LPS to prevent the lethal septic shock. In all cases, STZ was given 4 days prior to LPS and to all pharmacological treatments with inhibitors. In pilot experiments, the administration of LPS in STZ-diabetic rats caused mortality within 24–48 h. To avoid uncontrolled mortality and suffering, rats were closely monitored three times/day and rectal temperature at 3 h intervals (first 48 h). Rats were humanely sacrificed with isoflurane when they reached the following ethic endpoints (rectal temperature greater than 39°C, respiratory distress, severe diarrhea, tachycardia, loss of locomotor activity and rearing, apathy). Survival rats were those which did not reach lethal septic shock based on these endpoints.

Platelet aggregation testing in platelet-rich plasma

Platelet aggregation was induced by adding 0.1 M of ADP (Sigma Chemical Co) in PRP and monitored with an optical aggregometer (Chronolog Corp., PA, USA). Platelet aggregation was monitored and analyzed with Aggro-link software (Chronolog Corp) after stabilization of aggregation traces.

Platelet aggregation was first compared between control, 4-day STZ-diabetic rats, LPS, and STZ-LPS treated rats. Then the mechanism of platelet aggregation was studied by measuring the impact of the following pharmacological treatments given at the same time than LPS (2 mg/kg, i.p.): SSR240612 (10 mg/kg, by gavage), des-Arg⁹-BK (2 mg/kg, s.c.), 1400W (5 mg/kg, i.p.), or heparin (100 IU/kg, s.c). Rats were sacrificed 3 h later and platelets collected.

Histological injury evaluation

After sacrifice, kidney and lung were fixed in 1% formalin and blocked in paraffin. Samples were then cut in coronal sections of 2 µm, routinely stained with hematoxylin and eosin and visualized on a microscope (Olympus, BX 61, digital camera DP71). Microscopic evaluations were done blindly and supervised by a pathologist.

Drugs

The selective non-peptide B1R antagonist SSR240612 ((2R)-2-(((3R)-3-(1,3-benzodioxol-5-yl)-3-[[[(6-methoxy-2-naphthyl)sulfonyl]amino]propanoyl]amino)-3-(4-[[[(2R,6S)-2,6-dimethylpiperidinyl]methyl]phenyl]-N-isopropyl-N-methylpropanamide,fumarate) was generously obtained from Sanofi-Aventis R&D (Montpellier, France). All other chemicals and reagents used were purchased from standard commercial suppliers and were of analytical grade.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 5.00, GraphPad Software Inc.). Data are expressed as means $\pm$ SEM of values obtained from n rats. Statistical significance was determined with Student's t -test for unpaired samples or with one-way analysis of variance (ANOVA) or two-way ANOVA (core temperature) followed by the Bonferroni or Dunnett post-test for multiple comparisons. The non-parametric Kruskal-Wallis post-test was used for histological data. Only values of $P < 0.05$ were considered statistically significant.

Results

Acute effect of B1R antagonist on blood glucose and core temperature

Blood glucose levels were significantly increased in STZ rats treated or not with LPS compared with control rats. Treatment with LPS alone did not significantly affect blood glucose levels. Hyperglycemia in STZ and STZ-LPS rats was significantly reduced and almost normalized by acute treatment with SSR240612 (Figure 1a).

Rats treated with STZ had resting core temperature similar to control (37°C). LPS produced significant increases of core temperature from 3 h to 24 h post-injection and the pyretic response induced by LPS was somewhat similar in control and STZ-treated rats. Acute treatment with SSR240612 abolished the increase of body temperature in LPS and LPS-STZ treated rats. The same treatment with SSR240612 had no significant effect in control or STZ-treated rats (Figure 1b).

Acute effect of B1R antagonist on edema and vascular permeability

In 4-day STZ rats, significant increases in edema (Figure 2) and vascular permeability (Figure 3) were measured in all tested organs, including heart, kidney, lung, and liver compared with matched control rats. Treatment with LPS also caused significant increases of edema and vascular permeability in the same organs. These responses were somewhat similar in STZ-diabetic rats treated with LPS. Acute treatment with SSR240612 significantly reduced both edema and vascular permeability in most organs from rats treated with STZ, LPS, or both STZ plus LPS.

Acute effect of B1R antagonist on B1R mRNA expression

B1 receptor mRNA levels were significantly increased in kidney cortex and in the heart of STZ, LPS, and particularly in STZ-LPS treated rats. The increases of B1R mRNA levels were greater in the kidney than in the heart for all treated groups. Acute treatment with the

B1R antagonist, SSR240612, prevented the enhanced B1R mRNA levels in both organs to control values in all treated groups (Figure 4).

Flow cytometry and western blot analysis of B1R and B2R in platelets

Flow cytometry technique using permeabilized platelets revealed that both kinin receptors are present in control platelets (Figure 5a). Moreover, data highlighted significant increases in the expression of B1R and B2R in platelets of LPS-treated rats, yet these changes were no longer significant in thrombin-activated platelets. Internalization of kinin receptors or their reduced accessibility to antibodies following platelet aggregation by thrombin may account for their reduced detection by flow cytometry. In contrast, platelets isolated from STZ-treated rats did not show significant changes of B1R and B2R protein expression in the absence or presence of thrombin when compared to control (Figure 5a). Likewise, western blot analysis confirmed the presence of B1R protein expression in control platelets and a marked increase of B1R in platelets isolated from LPS-treated rats with no significant change in platelets of STZ-treated rats (Figure 5b). The enhancing effect of LPS on the expression of B1R was even blunted in STZ-diabetic rats (Figure 5b). Western blot analysis confirmed the presence of B2R protein expression in platelets of control rats with no significant increase in platelets of STZ-diabetic rats (Figure 6).

Prolonged effects of B1R antagonist and inhibitors of inflammatory mediators on rat survival

While all rats treated with STZ survived, the septic shock induced by LPS caused high mortality, particularly in STZ-diabetic rats where 0% rats have survived beyond 24–48 h (Figure 7a). Lethality-induced by LPS in STZ-diabetic rats was not affected by the B1R agonist des-Arg⁹-BK (2 mg/ kg. s.c.) (data not shown) but was significantly prevented by the B1R antagonist when administered 1 h prior to LPS as 90% of the rats had survived after 7 days of daily treatment with the B1R antagonist. The contribution of other mediators of inflammation was assessed on the lethality induced by LPS in STZ-diabetic rats. Treatment with niflumic acid or 1400W completely prevented the lethal effect of LPS in STZ-diabetic rats as 100% of the rats have survived after 7 days, supporting a role for COX-2 and iNOS mediators. In contrast, L-NAME (a non-selective NOS inhibitor) and indomethacin (a non-selective COX-1/COX-2 inhibitor) did not significantly improve survival. The anticoagulant heparin increased significantly survival to 75% during the 7-day period (Figure 7a).

Impact of treatments on platelet-rich plasma aggregation

Platelet aggregation induced by ADP *ex vivo* was decreased in platelet-rich plasma isolated from STZ but did not reach statistical significance when compared to control. In contrast, it was completely disrupted in platelets isolated from LPS-treated rats, suggesting that platelet aggregation occurred previously *in vivo* in this group. The *ex vivo* platelet aggregation was also completely disrupted in platelet-rich plasma isolated from LPS-STZ treated rats. A 3 h *in vivo* treatment with SSR240612 induced significant greater platelet aggregation *ex vivo*, suggesting prevention of *in vivo* platelet aggregation in this group. Platelet aggregation induced by LPS in STZ-diabetic rats was also significantly improved *ex vivo* following 3 h *in vivo* treatment either with 1400W or heparin, yet this effect was less striking than with SSR240612 (Figure 7b). Treatment with the B1R agonist des-Arg⁹-BK had no significant

effect on the complete disruption of platelet aggregation in LPS-STZ treated rats (Figure 7b).

Effect of B1R antagonist on early stage of thrombus formation and histological injury

Early histological lesions were observed in the lung and kidney of LPS and particularly of LPS-STZ-diabetic rats. The lung displayed a significant vascular congestion, pulmonary infarction, interstitial edema and intra-alveolar hemorrhage and multiple thrombi (Figures 8a and 8a'). The kidney showed edema of proximal tubules, cortical and glomerular hemorrhage, glomerular shrinkage and necrosis, hyalin and fibrin deposits (Figure 8b and b'). No pathological changes were seen in control groups (Figure 8). Several histological abnormalities seen in LPS-STZ-treated rats were significantly reduced after the 1-week treatment with the B1R antagonist SSR240612 (10 mg/kg/day), particularly in the kidney.

Discussion

This study provides the first experimental evidence that kinin B1R is a key player in thrombogenesis induced by the septic shock during the course of diabetes and that its antagonism offers a protective effect. Lethality associated with the LPS treatment was significantly enhanced in STZ-diabetic rats and this phenomenon appears to be related to B1R-induced multiple organ damage as a consequence of DIC. This is documented by the reduction with SSR240612 of histological inflammatory injuries in peripheral organs, together with the inhibition of platelet aggregation leading to almost complete rat survival. Our data also suggest that NO from iNOS and prostaglandins generated by COX-2 are key mediators in this process as specific inhibitors of iNOS (1400W) and COX-2 (niflumic acid) caused complete survival. B1R inhibition was, however, more effective than 1400W or the anticoagulant heparin in preventing platelet aggregation in STZ-LPS rats.

The increased expression of B1R in platelets from LPS-treated rats may predispose platelets to activation and thrombus formation *in vivo*. In addition, B1R activation may also operate through the release of inflammatory mediators from iNOS and COX-2. Indeed, B1R can cause post-translational activation of iNOS via $G_{\alpha i}$ and the ERK/MAP kinase pathway¹⁶ in addition to inducing the upregulation of iNOS in STZ-diabetic vasculature.¹⁷ The excessive production of NO from iNOS can react rapidly with superoxide anion to cause the formation of the highly reactive peroxynitrite (ONOO⁻), which is involved in the oxidation and nitration of fibrinogen (a key protein in the coagulation cascade) and plasminogen (the main protein of fibrinolysis process).¹⁸ In agreement with this, mutant mice lacking iNOS were resistant to LPS-induced mortality.¹⁹ In contrast, the blockade of all isoforms of NOS with L-NAME did not protect from the lethal septic shock, suggesting that eNOS may counteract the negative effect of iNOS. Other studies also suggest the regulation of COX-2 by B1R. For instance, B1R is involved in the upregulation of COX-2 and B1R causes COX-2 activation through G_o/G_i G-protein in blood vessels of STZ-diabetic rats.¹⁷ Selective inhibition of COX-2 with nimesulide also protected from arterial thrombosis in LPS-treated dogs, yet selective COX-1 inhibition or non-selective COX inhibition had no influence.²⁰ In our study, indomethacin also failed to protect against the lethal effect of LPS, in agreement with the cytoprotective effect of prostaglandins (PGE₂ and PGI₂) generated by constitutively

expressed COX-1. The upregulation of COX-2 by LPS, STZ, and B1R is believed to generate greater amount of inflammatory prostaglandins, which can account for the protective effect of COX-2 inhibitor in septic shock.

Diabetes and LPS-induced increased vascular permeability and edema may also reinforce thrombus formation by increasing blood viscosity. In most tissues, we failed however to demonstrate important differences in the increased vascular permeability and edema between STZ, LPS, and STZ-LPS treated rats, suggesting that the lethal effect induced by LPS in STZ-diabetic rats is not primarily due to this phenomenon. The inhibition of increased vascular permeability and edema by SSR240612 is consistent with previous studies using other B1R antagonists in selected peripheral tissues of STZ-diabetic rats.^{17,21} Our study, however, is one of the first showing inhibition of LPS-induced vascular permeability and edema by a B1R antagonist as most studies have documented the effects of exogenous B1R agonists on plasma extravasation and the pharmacological characterization of the response with kinin receptors antagonists in LPS-treated animals.

The inhibition of kinin B1R with SSR240612 reversed hyperglycemia in STZ-diabetic rats. The anti-diabetic effect of B1R antagonism is consistent with the reduction of glycemia in B1R knockout mice⁸ and was ascribed to an inhibition of insulinitis in STZ-diabetic rats.²² On the other hand, the inhibition of LPS-induced fever by SSR240612 could be partly due to a central mechanism as intracerebroventricular administration of a B1R antagonist inhibited the late phase (4–6 h) of the LPS response.²³ A peripheral mechanism is also likely as SSR240612 inhibited fever induced by intraperitoneally injected B1R agonist, a pyretic response which occurs through a vagal sensory mechanism involving prostaglandins (via COX-2) and NO.²⁴

In addition to blocking B1R, a treatment with two doses of SSR240612 (24 h and 12 h prior to sacrifice) was found to suppress B1R enhanced expression in the kidney and heart of diabetic and LPS treated rats. The latter downregulation of B1R expression in peripheral organs is likely to contribute to the beneficial effects of the antagonist in this study. The suppression of B1R mRNA and protein expression by one-week treatment with SSR240612 was previously shown in a rat model of insulin resistance²⁵ and is believed to derive from different possible mechanisms: (1) Inhibition of homologous upregulation of B1R expression; indeed direct binding of the B1R agonist enhanced expression of B1R (both protein and mRNA) by activating the NF- κ B pathway.²⁶ In turn, the subsequent release and expression of pro-inflammatory cytokines and the formation of ROS aggravate B1R expression;⁷ (2) The infiltration of macrophages and CD4⁺ T cells in tissues can account for the enhanced expression of B1R as these immune cells express B1R in STZ-diabetic rats (Tidjane and Couture, personal communication). By inhibiting the infiltration of immune cells bearing the B1R, SSR240612 contributes directly to the decrease of B1R expression; (3) Given that B1R activates NADPH oxidase to increase the formation of ROS²⁵ directly involved in the induction of B1R,⁷ the inhibition of the pro-oxidative effect of B1R with SSR240612 should also result in the suppression of B1R expression.

In conclusion, kinin B1R was found upregulated in peripheral organs and platelets in a model of septic shock induced by LPS in STZ-diabetic rat. This model reproduces several

hallmarks of septic complications encountered at intensive care units with diabetic patients who are at higher risk.^{1–4,6} Treatment with the kinin B1 receptor antagonist, SSR240612, provided a protective effect against organ damage by interfering with multiple target functions, including the suppression of B1R-induced inflammation and platelet aggregation. This further highlights a detrimental role for B1R in septic shock. Hence, kinin B1R appears a valuable target to design anti-inflammatory and anti-thrombotic drugs in the treatment of the septic shock in co-morbidity with diabetes mellitus.

Acknowledgments

The authors are thankful to the excellent expertise of Mr. Jacques Sénécal (Physiology of Université de Montréal) and Mrs Julie Hinsinger (Pathology of Université de Montréal), and to Drs Pedro D'Orléans-Juste and Julie Labonté (Pharmacology, Université de Sherbrooke) for technical assistance on the aggregometry.

Funding

This work was supported by grants from the Canadian Institutes of Health Research (MOP-119329 to RC) and FRQS-INSERM to RC and J-PG. A studentship from GRUM-Sanofi/Aventis was awarded to NT.

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Figure 1.

Changes on (a) blood glucose levels (mmol/L) in overnight fasted rats, and (b) rectal temperature (°C) in control, 4-day STZ-diabetic rats, LPS-treated rats (2 mg/kg, i.p.) or STZ + LPS-treated rats in the presence (+) of SSR240612 (10 mg/kg by gavage, 24 h and 12 h earlier) or its vehicle (-). Data represent means $\pm$ SEM of eight rats in each group. In (a) *** $P < 0.001$ vs. control; ### $P < 0.001$ vs. STZ-vehicle or STZ + LPS-vehicle. In (b) *** $P < 0.001$ vs. control; ### $P < 0.001$ vs. LPS-vehicle or STZ + LPS-vehicle.

Figure 2.

Edema (mL) induced in heart, kidney, lung, and liver of control, 4-day STZ-diabetic rats, LPS-treated rats (2 mg/kg, i.p.), or STZ + LPS-treated rats in the presence (+) of SSR240612 (10 mg/kg by gavage, 24 h and 12 h earlier) or its vehicle (-). Data represent means $\pm$ SEM of eight rats in each group. ++, ## $P < 0.01$; ***, +++, ###, ††† $P < 0.001$ when compared with control (*), STZ-vehicle (+), LPS-vehicle (†), or STZ + LPS-vehicle (#).

Figure 3.

Changes of vascular permeability (µg/g wet weight tissue) in heart, kidney, lung, and liver of control, 4-day STZ-diabetic rats, LPS-treated rats (2 mg/kg, i.p.) or STZ + LPS-treated rats in the presence (+) of SSR240612 (10 mg/kg by gavage, 24 h and 12 h earlier) or its vehicle (-). Data represent means $\pm$ SEM of eight rats in each group. *, +, † $P < 0.05$; ++, ##, †† $P < 0.01$; ***, ### $P < 0.001$ when compared with control (*), STZ-vehicle (+), LPS-vehicle (†), or STZ + LPS-vehicle (#).

Figure 4.

B1R mRNA levels in kidney cortex and heart of control, 4-day STZ-diabetic rats, LPS-treated rats (2 mg/kg, i.p.) or STZ + LPS-treated rats in the presence (+) of SSR240612 (10 mg/kg by gavage, 24 h and 12 h earlier) or its vehicle (–). Data are means $\pm$ SEM obtained from five rats in each group. *, # $P < 0.05$; ## $P < 0.01$; ***, ###, ††† $P < 0.001$ when compared with control (*) or between SSR240612 (+) and its vehicle (–) in each set of columns (#). The difference between LPS and STZ plus LPS is indicated by (†††).

Figure 5.

(a) B1R and B2R protein expression measured by flow cytometry on non-activated and thrombin-activated permeabilized platelets ($250 \times 10^6/\text{mL}$) isolated from control, LPS (2 mg/kg, i.p.), and 4-day STZ-diabetic rats. (b) Western blot analysis of B1R expression on platelets ($250 \times 10^6/\text{mL}$) isolated from control, LPS (2 mg/kg, i.p.), and 4-day STZ-diabetic rats treated or not with LPS. Data are means $\pm$ SEM obtained from four rats in each group.

** $P < 0.01$; *** $P < 0.001$ when compared with control.

Figure 6.

Western blot analysis of B2R protein expression with two concentrations (3 and 6×10^6 /mL) of platelets isolated from control and 4-day STZ-diabetic rats. Data are means $\pm$ SEM obtained from three rats in each group. No statistical significance was found between control and STZ-treated rats.

Figure 7.

(a) Percentage of rats which survived 7 days after treatment with either LPS (2 mg/kg, i.p.) or LPS in 4-day STZ-diabetic rats treated or not with several inhibitors (n = number of rats per group). Data represent means $\pm$ SEM. *** $P < 0.001$ when compared with STZ + LPS (group 2). (b) Platelet aggregation (%) assessed by optical aggregometry in platelets isolated from control, LPS (2 mg/kg, i.p.), 4-day STZ-diabetic rats treated or not with LPS in the absence (-) and presence (+) of SSR240612 (10 mg/kg by gavage) or after treatment either with des-Arg⁹-BK (2 mg/kg, s.c.), 1400W (5 mg/kg, i.p.), or heparin (100 IU/kg, s.c.). Treatments were given at the same time than LPS and rats were sacrificed 3 h later. Data are means $\pm$ SEM obtained from eight rats in each group. ***, +++, ### $P < 0.001$ when compared with control (# or +) or STZ + LPS without (-) SSR240612 (*).

Figure 8.

Qualitative representation of histological lesions observed in the lung (a, a') and kidney (b, b') of control, LPS (2 mg/kg. i.p.), and 4-day STZ-diabetic rats treated with LPS receiving or not SSR240612 (10 mg/kg) daily for up to 7 days. Data are means $\pm$ SEM obtained from four sections per rat in four rats for each group. ** $P < 0.01$; *** $P < 0.001$ when compared with control and ## $P < 0.01$ or ### $P < 0.001$ when compared with LPS + STZ.

Table 1

PCR primer pairs used.

Gene	Sequence	Position	GenBank accession no.
18S Forward	5'-TCA ACT TTC GAT GGT AGT CGC CGT-3'	363–386	X01117
18S Reverse	5'-TCC TTG GAT GTG GTA GCC GTT TCT-3'	470–447	
B1R Forward	5'-GCA GCG CTT AAC CAT AGC GGA AAT-3'	367–391	NM_030851
B1R Reverse	5'-CCA GTT GAA ACG GTT CCC GAT GTT-3'	478–454	