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Absence of ligand-induced regulation of kinin receptor expression in the rabbit

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1 The induction of B_1 receptors (B_1Rs) and desensitization or down-regulation of B_2 receptors (B_2Rs) as a consequence of the production of endogenous kinins has been termed the autoregulation hypothesis. The latter was investigated using two models based on the rabbit: kinin stimulation of cultured vascular smooth muscle cells (SMCs) and *in vivo* contact system activation (dextran sulphate intravenous injection, 2 mg kg^{-1}, 5 h).

2 Rabbit aortic SMCs express a baseline population of B_1Rs that was up-regulated upon interleukin-1β treatment ([^{3}H]-Lys-des-Arg^9-BK binding or mRNA concentration evaluated by RT–PCR; 4 or 3 h, respectively). Treatment with B_1R or B_2R agonists failed to alter B_1R expression under the same conditions.

3 Despite consuming endogenous kininogen (assessed using the kinetics of immunoreactive kinin formation in the plasma exposed to glass beads *ex vivo*) and producing hypotension mediated by B_2Rs in anaesthetized rabbits, dextran sulphate treatment failed to induce B_1Rs in conscious animals (RT–PCR in several organs, aortic contractility). By contrast, lipopolysaccharide (LPS, 50 μg kg^{-1}, 5 h) was an effective B_1R inducer (kidney, duodenum, aorta) but did not reduce kininogen reserve.

4 We tested the alternate hypothesis that endogenous kinin participate in LPS induction of B_1Rs. Kinin receptor antagonists (icatibant combined to B-9858, 50 μg kg^{-1} of each) failed to prevent or reduce the effect of LPS on B_1R expression. Dextran sulphate or LPS treatments did not persistently down-regulate vascular B_2Rs (jugular vein contractility assessed *ex vivo*).

5 The kinin receptor autoregulation hypothesis is not applicable to primary cell cultures derived from a tissue known to express B_1Rs in a regulated manner (aorta). The activation of the endogenous kallikrein-kinin system is ineffective to induce B_1Rs *in vivo* in an experimental time frame sufficient for B_1R induction by LPS.

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Abbreviations: ACE, angiotensin converting enzyme; B_1R, B_1 receptor; B_2R, B_2 receptor; BK, bradykinin; FBS, foetal bovine serum; IL, interleukin; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; RT–PCR, reverse transcriptase-polymerase chain reaction; Sar, sarcosine; SMC, smooth muscle cell

Introduction

The kinins (peptides related to bradykinin, BK) are known to activate two types of G protein coupled receptors, termed B_1 and B_2 (Marceau *et al*., 1998). Lys-des-Arg^9-BK (des-Arg^{10}-kallidin) is the optimal agonist sequence of the human and rabbit B_1 receptors (B_1Rs), whereas BK and Lys-BK in low concentrations stimulate B_2 receptors (B_2Rs). While the latter are preformed in a wide variety of cells, a large body of evidence shows that the B_1Rs are generally not expressed in normal physiological conditions, but rapidly induced following some types of injury; the cytokine network, mitogen-activated protein (MAP) kinases and specific transcription factors have been implicated in this phenomenon (Deblois *et al*., 1988; Larrivée *et al*., 1998; Marceau *et al*., 1998; Ni *et al*., 1998; Yang *et al*., 1998).

In several systems pertaining to persistent inflammation, there is a temporal shift of kinin effect mediation from preformed B_2Rs to induced B_1Rs over several hours or days (e.g., inflammatory hyperalgesia and intestinal inflammation models in rodents) (Kachur *et al*., 1996; Perkins *et al*., 1993). This also applies to the oedema induced by exogenous kinin injection into the rat paw (Campos *et al*., 1995). In the latter system, daily injection of the B_2R agonist [Tyr^8]BK induces oedema of decreasing amplitude; however, the B_1R agonist des-Arg^9-BK, initially inert, becomes a powerful inflammatory substance by the end of the protocol. These and other observations suggest a pattern of kinin receptor 'autoregulation' by the agonists. Agonist-induced temporary desensitization of the B_2R, involving receptor phosphorylation and endocytosis, is well documented (Blaukat *et al*., 1996; Faussner *et al*., 1998; Pizard *et al*., 1999), but B_2R down-

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regulation is not defined at the molecular level (protein, mRNA). As for B_1R induction, the agonist Lys-des-Arg^9-BK has been shown to induce B_1Rs (mRNA, protein) in the human cell line IMR-90 (Schanstra *et al*., 1998). This cell line expresses a certain baseline population of B_1R, which may not be representative of normal tissues *in vivo*; NF-κB binding to some DNA sequences reproduced from the B_1R gene promoter was activated by the B_1R agonist in these cells. Further, the MAP kinase pathways that were found to determine post-isolation B_1R induction in rabbit vascular tissue (Larrivée *et al*., 1998) may be also activated upon kinin receptor stimulation in HEK 293 cells expressing either recombinant B_1 or B_2Rs (Naraba *et al*., 1998). In these cells, either B_1R or B_2R agonists stimulate the nuclear translocation of the transcription factor AP-1, an effect which is blocked by PD98059, the MEK-1 inhibitor. We and others have produced complementary evidence for the role of an AP-1 site in the B_1R gene promoter (Yang *et al*., 1998; Angers *et al*., 2000). Ultimately, the full model of autoregulation has been illustrated by Phagoo *et al*. (1999) based on IMR-90 cells: incubation with BK (100 nM) suppressed 89% of the surface B_2Rs in a few minutes, but upregulated B_1Rs (2–3 fold) in a few hours. Lys-des-Arg^9-BK did not influence the population of B_2Rs, but also upregulated B_1Rs. The agonists increased the expression of IL-1β in the cells, and the natural IL-1 receptor antagonist, IRA, decreased the induction of B_1Rs by BK (Phagoo *et al*., 1999). Therefore, this 'autoregulation' model integrates several known or suspected regulatory events (autologous desensitization of B_2R, involvement of cytokine, MAP kinases and transcription factors in B_1R gene transcriptional control).

We have investigated whether intense activation of the endogenous kallikrein-kinin system using dextran sulphate (Kaplan *et al*., 1998) could result in the definite pattern of kinin receptor regulation described above (desensitization or down-regulation of B_2Rs, up-regulation of B_1Rs) in the live rabbit. Complementary testing of the autoregulation hypothesis has been performed using primary cultures of rabbit vascular SMCs. In addition, kinin participation in LPS induction of B_1Rs has been tested as a secondary objective, as LPS treatment may activate the kallikrein-kinin system in rabbits (Erdös & Miwa, 1968).

Methods

Drugs

Dextran sulphate (500 kDa), amastatin, phosphoramidon, captopril and enalapril maleate were purchased from Sigma (St. Louis, MO, U.S.A.). LPS, extracted from *Escherichia coli* serotype O111:B4, was produced by Difco (Detroit, MI, U.S.A.). Several BK-related peptides were used: BK itself (B_2R agonist; Bachem Bioscience Inc., King of Prussia, PA, U.S.A.), Lys-des-Arg^9-BK (a B_1R agonist; Peninsula Laboratories, Belmont, CA, U.S.A.), Sar-[D-Phe^8]des-Arg^9-BK (a metabolically stable B_1R agonist; Drapeau *et al*., 1993; gift from Prof D. Regoli, Sherbrooke, Canada), the B_1R antagonist B-9858 (Lys-Lys-[Hyp^3, Igl^5, D-Igl^7, Oic^8]des-Arg^9-BK; Larrivée *et al*., 2000) and the B_2R antagonist icatibant (Hoe 140; D-Arg[Hyp^3, Thi^5, D-Tic^7, Oic^8]-BK; both icatibant and B-9858 were gifts from Laboratoires Fournier S.C.A., Daix, France).

B_1R expression in cultured rabbit aortic SMCs

Rabbit aortic SMCs were cultured as previously described (Levesque *et al*., 1993); the identity of these cells was confirmed using immunohistochemistry for the marker α-actin (monoclonal antibody from Sigma). Cells were used at passages 3–6, at a stage where the B_1R basal expression is relatively low and its hormonal induction (epidermal growth factor treatment) is high (Schneck *et al*., 1994). Separate protocols dealt with the effect of kinin receptor ligands or IL-1 on B_1R expression; both mRNA and radioligand were assessed in these experiments which were based on confluent cells cultured in 6- or 12-well plates, respectively. In order to reduce the basal B_1R expression level, the FBS containing medium was replaced by serum-free medium 199 for 24 h; then various drugs (kinin analogue, captopril or IL-1β) were added to the serum-free medium and the total RNA was extracted after 3 h according to Chomczynski & Sacchi (1987). These cells were also the basis of a binding assay to rabbit B_1Rs (12-well plates; approximately 60 μg of total protein per well). The assay was conducted as described (Levesque *et al*., 1995a), except for the identity of the ligand which was [^{3}H]Lys-des-Arg^9-BK ([^{3}H]des-Arg^{10}-kallidin, NEN Biosciences, 64 Ci mmol^{-1}). The cells were treated as outlined above for 3 h with a kinin analogue, captopril or IL-1β; then, the medium containing stimulants was replaced by serum-free medium 199 for one additional hour of incubation at 37°C before performing the binding assay to avoid binding interference from some of the kinin analogues used in pretreatments. There is evidence that a 1 h washout at 37°C is sufficient for a full dissociation of the antagonist Ac-Lys-[Leu^8]des-Arg^9-BK from the rabbit recombinant B_1Rs (Larrivée *et al*., 2000), and it is assumed that this will apply to the moderate affinity ligands used in the present experiments. The radioactivity bound to adherent intact cells after incubation with a radioligand concentration (4 nM) sufficient to reveal the B_{max} (Schneck *et al*., 1994) was determined; a cold competing peptide (1 μM Lys-des-Arg^9-BK) was added to some cell wells to determine the non specific binding (amounting to 15–40% of the total binding).

Treatment of animals used as sources of tissues

Groups of male New Zealand White rabbits, weighing 1.5–2.2 kg were used as a source of tissues for all experiments. In order to potentiate endogenous kinins produced by the treatments, the ACE inhibitor enalapril maleate (2 mg kg^{-1} per day in the drinking water) was given to all animals for 5 days prior to acute treatments. Each rabbit was weighed before the acute treatments, which consisted in the intravenous injection of the following drugs: dextran sulphate (2 mg kg^{-1}) or LPS (50 μg kg^{-1}). The first substance is a contact system activator which has previously been used to produce hypotension mediated *via* both B_1 and B_2Rs in the LPS-treated pigs, thus presumably by producing endogenous kinins (Schmid *et al*., 1998); the second one was used as a control inducer of B_1Rs (Marceau *et al*., 1999). Control animals received the saline vehicle (500 μl kg^{-1}). Additional groups of animals were treated intravenously with a

combination of kinin receptor antagonists: icatibant (50 μg kg^{-1}, a B_2R antagonist) and B-9858 (50 μg kg^{-1}, a B_1R antagonist). These drugs provide long lasting receptor blockade at these dose levels in the rabbit (Gobeil *et al*., 1999). The intravenous injection was followed 5 min later by an intravenous injection of LPS or saline, as described above. Five hours after the last injection, all rabbits were consecutively killed by CO_2/O_2 asphyxiation and several organs (heart, abdominal aorta, kidney, duodenum and psoas muscle) were quickly removed, frozen in liquid nitrogen and kept at $-80°C$ until RNA isolation (methods described previously) (Marceau *et al*., 1999). The thoracic aorta and the jugular veins were immediately used in the contractility studies (two separate tissues of each type were used per animal), for their functional response to the B_1 or B_2R agonists, Sar-[D-Phe^8]des-Arg^9-BK or BK, respectively (the responses were assessed within 1 h of tissue equilibration and expressed as a per cent of an internal contraction standard, the maximal responses to phenylephrine or histamine, respectively; Marceau *et al*., 1999).

Semiquantitative duplex RT–PCR

The RT–PCR experiments were conducted using the Ready-To-Go™ RT–PCR Beads (Amersham Pharmacia Biotech) as indicated by the manufacturer. The general conditions (primers used, PCR conditions and Southern analysis of the RT–PCR) were previously reported (Marceau *et al*., 1999). Briefly, 2 μg of total RNA sample, 250 ng of sense and antisense primers for the amplification of rabbit B_1R or B_2R fragments, 25 ng of sense and antisense primers for the amplification of a rabbit GAPDH fragment (used as an internal standard) and 250 ng of an oligo $(dT)_{15}$ were added to each tube of Ready-To-Go™ RT–PCR beads. The tubes were incubated for 30 min at 42°C for the RT reaction. The samples were then submitted to a PCR followed by a Southern analysis as described previously (Marceau *et al*., 1999).

Kininogen reserve in anaesthetized rabbits submitted to the acute treatments

Separate male rabbits were used for these experiments. The animals also received oral enalapril maleate treatment (2 mg kg^{-1} per day) for 5 days prior to the experiment. This chronic treatment allows complete inhibition of plasma ACE activity and potentiates the conversion of BK into des-Arg^9-BK which constitutes a minor metabolic pathway in absence of ACE inhibition (Décarie *et al*., 1996). The rabbits were anaesthetized using sodium pentobarbitone (approximately 50 mg kg^{-1} i.v., individually adjusted). A throat incision made under lidocaine 2% local anaesthesia was followed by trachea intubation for ventilatory assistance and cannulation of the left common carotid artery for blood sampling and drug injection (Raymond *et al*., 1995). After 10 min of equilibration, 5 ml of arterial blood was sampled in a polyethylene tube containing buffered sodium citrate (final concentration 13 mM) for the measurement of blood kininogen reserve. These sampling procedures have been previously validated and do not result in significant kininogen consumption *in vitro* (Adam *et al*., 1985a, b, c). The intravenous injection of test drugs used to induce B_1Rs, dextran sulphate (2 mg kg^{-1}) or LPS (50 μg kg^{-1}) was then performed, and the blood sampling was repeated after 90 min in order to document acute treatment-induced kininogen consumption. Euthanasia was then applied under the form of a lethal dose of sodium pentobarbitone. Blood samples were centrifuged, and the plasma frozen for later testing.

Rabbit plasma samples were exposed to acid-washed glass beads at 37°C to activate the contact system and several aliquots were removed at different intervals of time between 1–60 min to determine the kinetic release of BK from high molecular weight kininogen and the formation of des-Arg^9-BK. The concentration of immunoreactive BK and des-Arg^9-BK was determined using two separate enzyme immunoassays after extraction from plasma (Décarie *et al*., 1994; Raymond *et al*., 1995).

Hypotensive effect of dextran sulphate in anaesthetized rabbits

Other groups of male rabbits, orally dosed with enalapril maleate, then anaesthetized and surgically prepared as described above, were used to assess the effect of dextran sulphate on mean arterial blood pressure measured using a pressure transducer (EM 751, Elcomatic Ltd., Glasgow, U.K.) connected to the left carotid artery catheter. Upon blood pressure stabilization, dextran sulphate (2 mg kg^{-1}) was injected in an ear vein to closely reproduce the treatment applied to conscious animals, and the blood pressure was monitored for the next 30 min period (limited by the need to re-administer the anaesthetic drug). The animals were then sacrificed with an overdose of pentobarbitone. To analyse the mechanism of dextran sulphate-induced haemodynamic changes, a separate group of anaesthetized rabbits submitted to the blood pressure recording were initially treated with icatibant (50 mg kg^{-1}, i.v.) followed 5 min later by dextran sulphate (2 mg kg^{-1}, i.v.).

Statistical analysis

Statistical analysis was performed using the Kruskal–Wallis test followed by Mann–Whitney test or ANOVA followed by Dunnett's test using the InStat 2.0 computer program (GraphPad Software, San Diego, CA, U.S.A.).

Results

Kinin B_1R expression in cultured aortic SMCs

Cultured rabbit aortic SMCs express a regulated population of B_1Rs mediating such effects as phosphoinositide hydrolysis, prostaglandin release and DNA synthesis (Levesque *et al*., 1993; 1995a, b). Confluent cells maintained for 24 h in serum-free medium 199 expressed a relatively small population of B_1Rs (Figure 1A). As reported previously (Levesque *et al*., 1995a), IL-1β treatment (5 ng ml^{-1}, applied for 3 h and rinsed for an additional hour of incubation) increased the expression of the B_1R, as assessed with a specific radioligand. A treatment with the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK (100 nM) did not influence [^{3}H]Lys-des-Arg^9-BK binding (Figure 1A). The employed agonist concentration was maximally effective to increase DNA

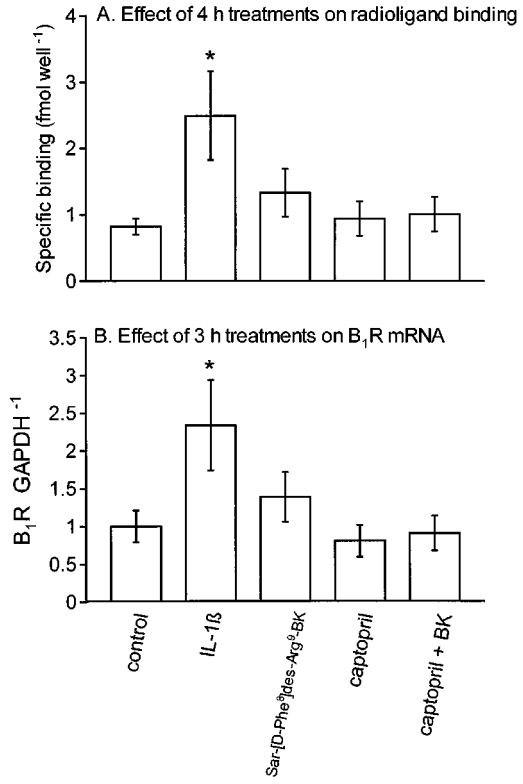

Figure 1 Investigation of the autoregulation hypothesis in cultured SMCs derived from the rabbit aorta. (A) Effect of 3 h drug treatments followed by 1 h drug washout incubation period on the specific binding of [^{3}H]Lys-des-Arg^9-BK (4 nM) to rabbit aortic SMCs maintained in serum-free medium for 24 h. Cells were treated with saline vehicle (2 μl per well), human recombinant IL-1β (5 ng ml^{-1}), the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK (100 nM), captopril (1 μM) alone or combined with the B_2R agonist BK (100 nM). Results are the means$\pm$s.e.mean of four determinations based on cell lines derived from different animals. (B) Effect of 3 h drug treatments on B_1R mRNA concentration in rabbit aortic SMCs maintained in serum-free medium for 24 h. Values (B_1R GAPDH^{-1} ratio, derived from RT–PCR) are arbitrary scanning units normalized to saline group = 1 and are the mean$\pm$s.e.mean of six determinations. The treatments were the same as in A. In both A and B, one-way analysis of variance showed that the experimental groups were different between them ($P<0.05$). Dunnett's test was applied to compare the effect of each treatment with the control values (*$P<0.05$).

synthesis in these cells (Levesque *et al*., 1995b). Rabbit aortic SMCs possess a certain population of B_2Rs mediating phosphoinositide metabolism (E_{max} of about 20% relative to B_1R agonist E_{max}; Schneck *et al*., 1994); however, in other assays, there is no functional response to B_2R agonists in these cells (Levesque *et al*., 1993; 1995b). The effect of B_2R stimulation was checked in the present experiments to exclude any effect on B_1R regulation. BK (100 nM) also failed to alter the B_1R radioligand binding (Figure 1A). In these experiments, BK was combined with captopril (1 μM) in order to improve peptide stability in the cultured cell system (Bachvarov *et al*., 2001); captopril alone had no effect on the B_1R ligand binding. The same set of treatments was applied for 3 h to rabbit aortic SMCs for the evaluation of B_1R mRNA concentration (Figure 1B). Again, only IL-1β upregulated B_1R mRNA, as kinin receptor agonists failed to increase B_1R mRNA concentration above the control value.

Multiplex RT–PCR analysis of kinin receptor mRNA in organs from treated rabbits

The B_1 and B_2R mRNA expression levels in several rabbit organs were determined by a semi-quantitative RT–PCR approach and the results are presented in Table 1. A baseline mRNA expression was detected in each organ for both receptors (normalized to 1 in Table 1). The dextran sulphate treatment failed to significantly induce the expression of B_1R mRNA over the baseline. The 5 h LPS treatment significantly induced B_1R expression over the baseline in the kidney, duodenum and aorta (values did not reach statistical significance for the two other organs, but the trends were similar). Animals pretreated with the combination of kinin receptor antagonists 5 min before LPS administration exhibited B_1R mRNA expression levels similar to those of rabbits treated with LPS alone, whereas the antagonists given alone were ineffective in this respect. The short-term treatments applied (5 h) did not change significantly B_2R mRNA concentrations in the five tested organs, although considerable value dispersion was observed in some groups (Table 1).

Vascular contractility mediated by B_1 and B_2Rs as a function of treatments applied in vivo *to rabbits*

Sigmoidal concentration-effect curves for each tested agonist (Figures 2 and 3) were characterized by the half-maximal effective concentration (EC_{50}) and the maximal absolute contraction amplitude (E_{max}, per cent of internal standard) (Table 2). The aortic ring responses to the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK were recorded within the first hour of tissue isolation. LPS treatment induced a definite state of responsiveness to this peptide when compared to saline-treated controls, even in animals pretreated with the combination of kinin antagonists (E_{max} values were not significantly different, $P=0.08$). Dextran sulphate or the mixture of kinin receptor antagonists were ineffective to induce responsiveness to the B_1R agonist 5 h following the treatments (Figure 2, Table 2).

The rabbit jugular vein stimulated with the B_2R agonist BK revealed a similar state of sensitivity and maximal response in groups treated with saline, dextran sulphate or LPS (Figure 3, Table 2). The groups pretreated with the kinin antagonist combination and further treated with saline or LPS exhibited a trend towards reduced maximal effects of BK (significant only in the group treated with the antagonists alone; Figure 3, Table 2).

Kininogen reserve in rabbits submitted to the acute treatments

The kinetics of the appearance of immunoreactive BK, followed by its partial conversion into des-Arg^9-BK, has been studied as an index of kininogen reserve in plasma exposed *ex vivo* to glass beads. Plasma samples were obtained from ACE-inhibitor treated rabbits in order to improve peptide stability and the conversion rate of BK into des-Arg^9-BK (Décarie *et al*., 1996). As compared to pre-treatment values, acute dextran sulphate treatment reduced the capacity of the plasma to produce BK, but not to convert a part of BK into des-Arg^9-BK (Figure 4). By contrast, LPS did not

Table 1 Variations in B_1R and B_2R mRNA expression levels in selected rabbit organs as a function of various treatments[†] applied *in vivo*

	Kidney		*Duodenum*		*Aorta*		*Heart*		*Striated muscle*	
	B_1R[‡]	B_2R	B_1R	B_2R	B_1R	B_2R	B_1R	B_2R	B_1R	B_2R
Saline	1±0.30	1±0.46	1±0.25	1±0.18	1±0.15	1±0.17	1±0.29	1±0.27	1±0.15	1±0.22
Dextran sulphate	1.34±0.21	1.16±0.13	0.73±0.07	1.26±0.29	2.32±1.05	0.75±0.11	2.67±1.05	2.8±1.01	1.70±0.56	0.78±0.32
LPS	5.50±0.55*	3.09±1.2	2.60±0.16*	2.04±0.93	11.29±1.11*	1.41±0.61	20.02±9.59	1.74±0.29	9.44±4.45	0.79±0.28
Icatibant+ B-9858/saline	1.51±0.51	0.88±0.26	0.76±0.10	5.62±4.77	1.70±0.27	6.16±5.29	6.51±5.78	1.39±0.28	1.23±0.45	1.32±0.33
Icatibant + B-9858/LPS	8.35±1.78*	3.26±1.12	2.21±0.26*	1.66±0.37	8.16±2.84	1.69±0.52	14.09±4.73	2.16±0.39	10.83±5.17	0.96±0.12
Kruskal-Wallis test[#]	$P=0.007$	$P=0.03$	$P=0.009$	$P=0.77$	$P=0.04$	$P=0.73$	$P=0.24$	$P=0.08$	$P=0.07$	$P=0.60$

[†]Treatments: saline vehicle (0.5 ml kg^{-1}), dextran sulphate (2 mg kg^{-1}), LPS (50 μg kg^{-1}) or the combination of icatibant (50 μg kg^{-1}) and B-9858 (50 μg kg^{-1}) followed by either saline or LPS were intravenously injected 5 h before sacrifice. Animal weight at the time of sacrifice: saline group: 1.70±0.4 kg; dextran sulphate: 1.63±0.04 kg; LPS: 1.65±0.09 kg; icatibant+B-9858/saline group: 1.66±0.03 kg; icatibant+B-9858/LPS group: 1.57±0.05 kg ($n=4$ in each group; no significant difference with the saline controls by the Mann–Whitney test). [‡]Values (B_1R/GAPDH or B_2R/GAPDH ratio, derived from RT–PCR) are arbitrary scanning units normalized to saline group=1. Values are means±s.e.mean of four determinations derived from four rabbits in each group. [#]The Kruskal-Wallis test was performed on column of values to determine the effect of drug treatments. Then, the Mann–Whitney test was applied in significantly heterogenous sets of values to compare each drug value with the saline vehicle controls (*$P<0.05$).

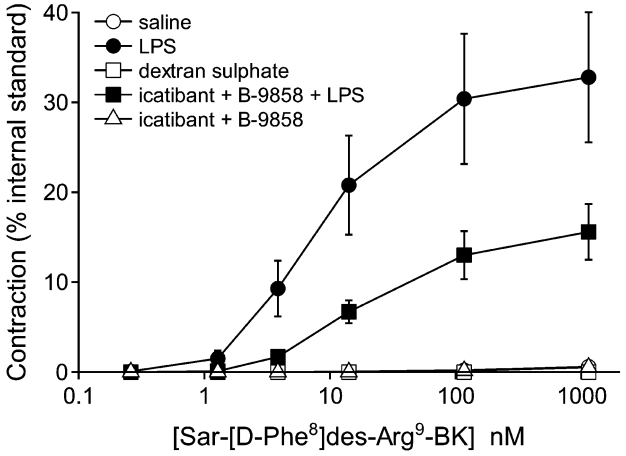

Figure 2 Initial responsiveness of aortic rings derived from control (saline vehicle i.v.), dextran sulphate-treated, LPS-treated or kinin antagonist-treated rabbits to an agonist of the B_1 receptor. Kinin antagonist treatment was combined with either saline or LPS injection. Treatments were in the form of intravenous injections 5 h prior to sacrifice. The cumulative concentration-effect curve of the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK was established after a short *in vitro* incubation (45 min) to minimize the influence of isolation and tissue incubation on the responses. Values (means±s.e.mean of eight determinations from four animals in each group) are expressed as a per cent of an internal contractile standard, the maximal effect of phenylephrine, established in each tissue. See Table 2 for statistical analysis.

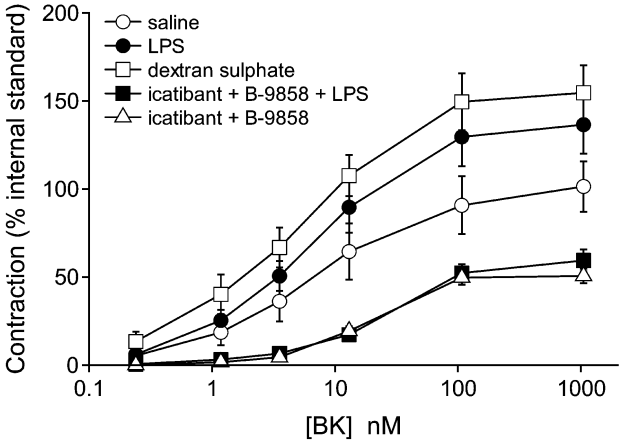

Figure 3 Initial responsiveness of external jugular vein strips derived from control (saline vehicle i.v.), dextran sulphate-treated, LPS-treated or kinin antagonist-treated rabbits to an agonist of the B_2 receptor. Kinin antagonist treatment was combined with either saline or LPS injection. Treatments were in the form of intravenous injections 5 h prior to sacrifice. The cumulative concentration-effect curve of the B_2R agonist BK was established after a short *in vitro* incubation (60 min). Values (means±s.e.mean of eight determinations from four animals in each group) are expressed as a per cent of an internal contractile standard established in each tissue, the maximal effect of histamine. See Table 2 for statistical analysis.

produce a significant consumption of the contact system components, as evidenced by an intact capacity to form immunoreactive kinins 90 min post-treatment.

Hypotensive effect of dextran sulphate in anaesthetized rabbits

Anaesthetized rabbits treated with dextran sulphate exhibited a slow developing, but sustained hypotensive response over the 30 min observation period (Figure 5). Animals that were pretreated with the B_2R antagonist icatibant initially responded to dextran sulphate by a decreased mean blood pressure, but this haemodynamic parameter returned towards baseline values within a few minutes (Figure 5). Thus, the blood pressure changes were significantly different between the two groups at time points 25 and 30 min.

Discussion

Rabbit aortic SMCs express a cytokine-regulated and functionally coupled population of B_1Rs (Levesque *et al*., 1993; 1995a, b; Schneck *et al*., 1994; Galizzi *et al*., 1994) and also a certain population of B_2Rs coupled to phospholipase C

Table 2 Effects of *in vitro* treatments on the contractile response of rabbit isolated blood vessels to kinins

Treatment[†]	*Rabbit aorta: effect of Sar-[D-Phe^8]des-Arg^9-BK*		*Rabbit jugular vein: effect of BK*	
	E_{max}[‡]	EC_{50}[‡]	E_{max}	EC_{50}
Saline	0.6±0.4	–[#]	101±14	18.6±9.6
Dextran sulphate	0	–	155±16	5.2±0.9
LPS	32.8±7.2**	14.2±3.4	137±16	8.5±2.7
Icatibant+B-9858/saline	0.5±0.2	–	51±4*	19.4±2.1
Icatibant+B-9858/LPS	15.6±3.8**	21.4±3.0	59±6	28.7±2.4
Kruskal-Wallis test[+]	$P<10^{-4}$	–	$P<10^{-4}$	$P=0.001$

[†]Treatments and animal groups are the same as in Table 1. [‡]Sigmoidal concentration-response curves were characterized by a maximal effect (E_{max}) and a half-maximal concentration (EC_{50}). E_{max} values are expressed as a per cent of the maximal contractile response obtained with the reference agonist for each tissue (phenylephrine for the aorta, histamine for the jugular vein). EC_{50} values are expressed as nM concentrations. Values are means±s.e.mean of eight determinations derived from four rabbits in each group. [#]No accurate EC_{50} value can be derived from very small contractions. [+]The Kruskal–Wallis test was performed on column of values to determine the effect of drug treatments. Then, the Mann–Whitney test was applied in significantly heterogenous sets of values to compare each drug value with the saline vehicle controls (*$P<0.01$, **$P<0.001$).

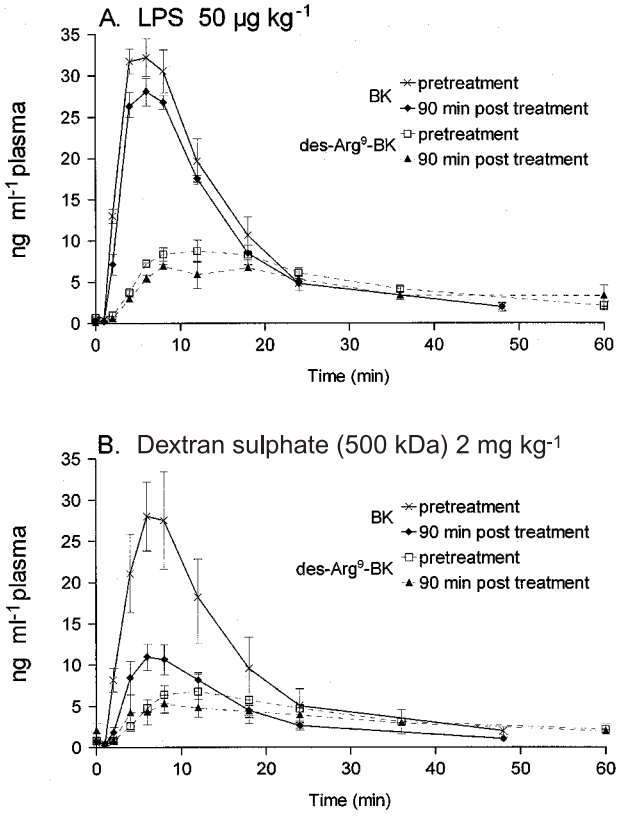

Figure 4 Consumption of the contact system components in rabbit plasma by *in vivo* treatments as assessed by *in vitro* formation of immunoreactive BK and des-Arg^9-BK. Blood has been sampled in anaesthetized animals before and 90 min after injection of either LPS or dextran sulphate at the indicated doses. Then, the contact system was maximally activated using glass beads, and the plasma was periodically sampled for the measurement of immunoreactive kinins (see text). Values are the means±s.e.mean of three determinations in each group.

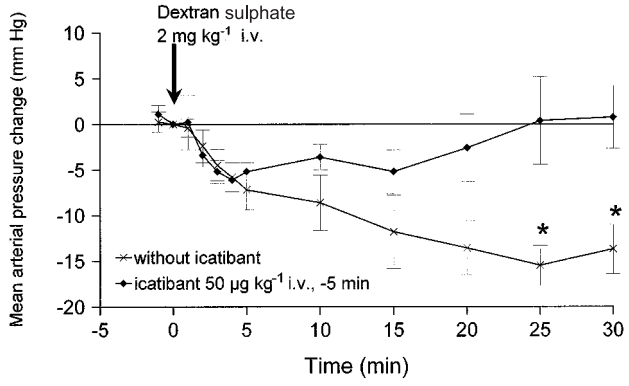

Figure 5 Change of mean blood pressure in anaesthetized rabbits treated with dextran sulphate. Values are the means±s.e.mean of seven (control) or five (icatibant) determinations in each group. A group of animals had been treated with icatibant (50 μg kg^{-1}, i.v.) 5 min before dextran sulphate injection. *Values significantly different between groups ($P<0.05$, Mann–Whitney) at the considered time point.

activity (Schneck *et al*., 1994). These primary cultures are suitable to study the kinin receptor autoregulation hypothesis, as the stimulation of at least the B_1Rs could regulate B_1R expression. Our results are negative in this respect (Figure 1) in a time frame sufficient for a positive effect of IL-1β. The discrepancy between the cellular system, the IMR-90 cells (see Introduction), where the kinin receptor autoregulation has been documented and the present *in vitro* system, in which kinin receptor stimulation failed to up-regulate B_1Rs, may be attributed to the biology of the cultured cell models. Either IMR-90 or SMCs exhibit a proliferative phenotype, as they grow in artificial medium supplemented with FBS rich in growth factors. However, IMR-90 cells themselves are a source of autocrine IL-1β, the IL-1 isoform usually produced by phagocytic leukocytes. Kinin-induced IL-1-dependent induction of B_1Rs in IMR-90 cells (Phagoo *et al*., 1999) is not a specific phenomenon, as agonists for other G protein coupled receptors are active in this respect in IMR-90 cells (e.g., IL-8; Bastian *et al*., 1998). Therefore, a form of 'immunological priming' may facilitate B_1R expression in IMR-90 cells, a phenomenon that may not be widely applicable (most cells expressing B_1Rs may not produce IL-1β *in vivo*). Indeed, the lack of effect of dextran sulphate administered *in vivo* on B_1R expression in several organs does not generally support the coupling of cytokine synthesis with kinin receptor stimulation.

The rabbit has been previously used as a model organism to test whether endogenous levels of kinins can regulate the

expression of either type of kinin receptors: chronic blockade of either B_1 or B_2Rs did not alter significantly receptor expression (Marceau *et al*., 1999). Further, angiotensin converting enzyme (ACE) inhibition also failed to induce B_1Rs (Marceau *et al*., 1999), although this intervention is known to somewhat increase blood immunoreactive kinins in this species (Raymond *et al*., 1995). In the present experiments, we have attempted to simulate a higher, pathological level of endogenous kinins by activating *in vivo* the contact system using high-molecular dextran sulphate, a model previously described in the pig (Schmid *et al*., 1998). This treatment was effective in the rabbit, as evidenced by the consumption of contact system components necessary to *ex vivo* BK formation (Figure 4B) and the B_2R-mediated slow-developing hypotension produced at least initially by this treatment; such a slow developing hypotensive effect was also evident in the pig and lasted less than 1 h (Schmid *et al*., 1998). Thus, dextran sulphate generated pharmacologically relevant concentration levels of endogenous kinin *in vivo* over an extended period. Dextran sulphate treatment did not induce B_1R expression within an experimental time frame sufficient for B_1R induction by LPS, thus failing to support the autoregulation hypothesis. The *ex vivo* experimental system that we used to assess kininogen reserve exhibits several distinctive features: it involves a maximal and rapid contact system activation, stronger that the ones that can be reasonably applied *in vivo* (Kaplan *et al*., 1998); the conversion of BK into des-Arg^9-BK is relatively inefficient, as the relevant reaction is only one of several BK metabolic pathways in plasma (Décarie *et al*., 1996); des-Arg^9-BK itself is slowly metabolized (Décarie *et al*., 1996), which explains the fact that it can accumulate to some extent (Figure 4). Could a higher intensity of *in vivo* B_2R stimulation or a more persistent one be more effective to induce B_1Rs? This possibility is supported by a system where exogenous [Tyr^8]-BK is injected daily into rat tissues (Campos *et al*., 1995), but the latter system does not rely on endogenous formation of kinins. On the other hand, high concentrations of exogenous kinins, representing the maximal and persistent stimulation of kinin receptors in aortic SMCs, fail to upregulate B_1Rs in primary cultures derived from a tissue known to express these receptors in a regulated manner (Figure 1).

The functional response to BK in the jugular vein from dextran sulphate-treated rabbits was not depressed, suggesting that autologous B_2R desensitization observed in cellular systems (see Introduction) is rapidly and completely reversible. Supporting this view, experimental systems pertaining to the vascular function in the rat suggest that a specific time window ($\approx$15 min) of desensitization follows the administration of a B_2R agonist *in vivo*, followed by complete resensitization (renal vasodilation, blood brain barrier opening) (Bartus *et al*., 1996; Praddaude *et al*., 1995). Further, our recent results, based on a rabbit B_2R-green fluorescent protein conjugate stably expressed in an heterologous cell line, suggest that agonist-induced internalization is followed by complete recycling to the membrane in 1 h, with no significant down-regulation at the protein level (Bachvarov *et al*., 2001). This time frame is compatible with the possible *ex vivo* resensitization of the jugular vein in dextran sulphate-treated rabbits during the equilibration period allowed before BK stimulation, thus explaining the intact response of the preparation.

Our experimental data indicate that LPS was the only consistent treatment to induce B_1R expression (functional response in aorta, mRNA induction in several organs) in the live rabbit. Low B_1R mRNA levels measured in all tissues from control animals using the non-linear technique RT–PCR may not be associated with significant receptor populations, as suggested by the lack of functional response in control aortas (Table 2). LPS effects *in vivo* are particularly complex (Karima *et al*., 1999). However, at the low and sublethal dose used in the present experiments, the kallikrein-kinin system does not appear to mediate LPS effect to an important extent for the considered end points. Firstly, the kininogen reserve is not reduced to a significant extent in anaesthetized rabbits 90 min after LPS administration (Figure 4B); only considerably higher, lethal doses of LPS can deplete kininogen in rabbits (Erdös & Miwa, 1968). Secondly, combined blockade of B_1 and B_2Rs applied just before LPS administration exerted no modulatory (inhibitory) effect on B_1R induction by LPS (rabbit aorta contractility, mRNA measurements in various organs). The two kinin receptor antagonists used in the present experiments, B-9858 and icatibant, are peptide drugs that work well in the rabbit, as either one exerts a prolonged action *in vivo* and *in vitro* (Gobeil *et al*., 1999; Houle *et al*., 2000; Larrivée *et al*., 2000). The depressed response to BK in jugular veins from animals treated with the kinin receptor antagonists (Figure 3) is likely to be determined by the practically irreversible effect of icatibant given *in vivo* several hours before the experiments (added to a 1 h washout period *ex vivo* applied for tissue equilibration). Similarly, *in vivo* treatment with both B-9858 and icatibant had a tendency to reduce the response to the B_1R agonist Sar-[D-Phe^8]des-Arg^9-BK in aortic rings from LPS-treated rabbits, relative to those from animals treated with LPS alone (Figure 2), a fact probably explained by the insurmountable and slowly reversible effect of B-9858 on the rabbit B_1R (Larrivée *et al*., 2000). Treatment with combined receptor antagonists did not influence B_1R mRNA expression induced by LPS in various organs, supporting that the endogenous kinins do not participate in this effect of LPS. However, LPS may recruit other endogenous mediators that modulate B_1R expression *in vivo*.

The present experiments support the exquisitely sensitive regulation of B_1Rs by IL-1 *in vitro* and LPS *in vivo*; this has obvious implications for infectious disease physiopathology and immunopathology. Induction of vascular B_1Rs following LPS treatment has been shown to occur in the mouse, rat, pig and a non-human primate (reviewed by Marceau *et al*., 1998; McLean *et al*., 2000; see also Schanstra *et al*., 2000; Deblois & Horlick, 2001). High sensitivity to endotoxin and the optimal stimulation of B_1R by the sequence Lys-des-Arg^9-BK are features of the rabbit model shared with primate species, as opposed to rodent models. B_1R induction also occurs in complex pathological models, such as chemical inflammation of the urinary bladder (Bélichard *et al*., 1999), myocardial infarction (Tschöpe *et al*., 2000), or ischaemia-induced angiogenesis in the rat (Emanueli *et al*., 2001) or myocardial ischaemia-reperfusion in rabbits (Mazenot *et al*., 2001); the formation of both inflammatory cytokines and endogenous kinins is plausible in all these situations. The relatively simple experimental models presented here may help to clarify that endogenous kinins are not likely to be the major regulators of kinin receptor populations. The cytokine network is activated in a complex manner by LPS in the rabbit, and the relative

importance of individual cytokines (IL-1, tumor necrosis factor-α, IL-6 etc.) (Dinarello, 1991), and of downstream effectors (MAP kinases, transcription factors) remains to be established for *in vivo* B_1R induction. The occurrence of ligand-mediated B_2R down-regulation in chronic inflammation remains uncertain. Persistent desensitization of the B_2R may occur *in vivo* if endogenous kinins are continuously produced locally, perhaps accounting for some experimental systems where inflammation is long lasting (Kachur *et al*., 1996; Perkins *et al*., 1993). In addition, long term transcriptional suppression of the B_2R mRNA may also be mediated by inflammatory cytokines and/or other mediators from infiltrating leukocytes; there is also preliminary evidence that limited proteolysis of the B_2R by extracellular proteases results in its rapid degradation by cells (Bachvarov *et al*., 2001).

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