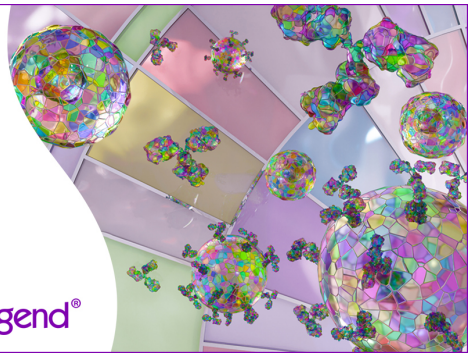

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Kinin B_1 Receptor Up-Regulation after Lipopolysaccharide Administration: Role of Proinflammatory Cytokines and Neutrophil Influx[1]

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Several studies have now clearly established the ability of LPS to induce bradykinin B_1 receptor up-regulation in vivo and the functional relevance of this up-regulation for the pathophysiological effects of LPS. Using an in vivo system in which LPS is injected locally into the rat paw, we have examined the potential contribution of proinflammatory cytokines, NF-κB activation, and neutrophil influx for the functional and molecular up-regulation of the bradykinin B_1 receptor. Treatment with LPS resulted in a rapid and sustained functional up-regulation of B_1 receptors in the rat paw that correlated with the increase in B_1 receptor mRNA levels. B_1 receptor up-regulation is preceded by the rapid activation of the transcription factor NF-κB and the production of proinflammatory cytokines, including TNF-α and IL-1β. More importantly, blockade of NF-κB translocation, TNF-α, or IL-1β prevented the functional and molecular up-regulation of B_1 receptors. Injection of LPS also induced the influx of neutrophils that followed the peak of cytokine production and associated with the persistent activation of NF-κB and functional B_1 receptor up-regulation. Blockade of neutrophil influx with platelet-activating factor receptor antagonists or cell adhesion molecule blockers prevented B_1 receptor up-regulation. Thus, by acting in cooperation and in a coordinated, timely manner, TNF-α, IL-1β, neutrophils, and the transcription factor NF-κB are major and essential players in the ability of LPS to induce B_1 receptor expression in vivo. ***The Journal of Immunology*****, 2004, 172: 1839–1847.**

Uncontrolled Gram-negative bacteria infection is a leading cause of the systemic inflammatory response syndrome (SIRS)[3] and has high lethality rates (1). Experimentally, one can mimic the SIRS to Gram-negative bacteria by the injection of LPS derived from the outer cell wall of these bacteria (2, 3). Responses to the injection of high doses of LPS include neutropenia, hypotension, the production of many proinflammatory cytokines, such as IL-1β and TNF-α, acute phase reactant proteins, and a marked lethality. In addition, the injection of lower amounts of LPS in tissue induces the expression of several genes, including chemokines, cytokines, and cell adhesion molecules, which lead to the recruitment of leukocytes, especially neutrophils, and edema formation (4–8).

The bradykinin B_1 receptor is among the many genes induced by the injection of LPS. Several studies have now clearly established the ability of LPS to induce bradykinin B_1 receptor up-regulation either in vitro or in vivo (9–13). Experimental evidence demonstrated that NF-κB is involved in the inducible expression of B_1 receptor after inflammatory stimuli (14–16). NF-κB is a well-characterized transcription factor, activated by several stimuli, especially bacterial products and cytokines. Its most common form is a heterodimer composed of two subunits, p50 and p65, that is normally found inactive in cytoplasm, bound to an inhibitory protein, I-κB. Under stimulation, specific kinases phosphorylate I-κB (IKK), causing its rapid degradation. Then, NF-κB translocates to the nucleus and activates a series of target genes, including cytokines, inflammatory enzymes, and receptors (17–19). Drugs interfering with NF-κB activity can prevent the phosphorylation of I-κB (such as pyrrolidine dithiocarbamate (PDTC), *N*-acetyl-L-cysteine, and salycilates), causing the up-regulation of the I-κB gene or masking the *trans*-activating domain for NF-κB (glucocorticoids) or inhibiting proteasomes (N^{α}-tosyl-L-chloromethylketone (TLCK)) (20, 21).

The systemic injection of LPS has been shown to induce up-regulation of the bradykinin B_1 receptor in vascular smooth muscle in rabbits (22–24), rats (25, 26), pigs (27), mice (28), and nonhuman primates (29). LPS was also capable of inducing the up-regulation of bradykinin B_1 receptors in diverse tissues such as the nephrons (30) and heart (31). More importantly, there is now good evidence to suggest that blockade of the bradykinin B_1 receptor is

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[3] Abbreviations used in this paper: SIRS, systemic inflammatory response syndrome; BK, bradykinin; i.d., intradermal; IRA, IL-1 receptor antagonist; MPO, myeloperoxidase; PAF, platelet-activating factor; PDTC, pyrrolidinedithiocarbamate; TLCK, N^{α}-tosyl-L-chloromethylketone; TFIID, transcription factor IID.

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beneficial during endotoxic shock (28, 29, 32). For example, we have recently shown that the initial LPS-induced hypotension is severely blunted in bradykinin B_1 receptor-deficient mice (29). In contrast, transgenic overexpression of bradykinin B_1 receptors in tissues rendered mice more susceptible to LPS-induced shock (33). This evidence shows the importance of understanding the mechanisms by which LPS induces the up-regulation of bradykinin B_1 receptors in vivo.

Using an in vivo system in which we inject LPS locally into the rat paw, we have examined the potential contribution of proinflammatory cytokines, NF-κB activation, and neutrophil influx for the functional and molecular up-regulation of the bradykinin B_1 receptor. In addition, we aimed at understanding how these factors may interact to coordinate the expression of the receptor and, hence, play an important role in LPS-mediated pathophysiological events in vivo.

Materials and Methods

Measurement of rat paw edema

Nonfasted male Wistar rats (140–180 g) kept in controlled temperature (22 ± 2°C) under a 12-h light, 12-h dark cycle (lights on at 6:00 a.m.) were used. The experiments were conducted according to the procedures described previously (34). The animals received a 0.1 ml intradermal (i.d.) injection in one hind paw (right paw) of PBS (composition, 137 mmol/liter NaCl, 2.7 mmol/liter KCl, and 10 mmol/liter phosphate buffer) containing des-Arg^9-bradykinin (des-Arg^9-BK; 100 nmol/paw). Another group of animals was pretreated with the angiotensin-converting enzyme inhibitor, captopril (5 mg/kg, s.c.), and received Tyr^8-BK (3 nmol/paw) in the right paw. The contralateral paw (left paw) received 0.1 ml of PBS and was used as the control. Edema was measured with a plethysmometer (Ugo Basile, Comerio, VA, Italy) at several time points (10, 20, 30, 60, and 120 min) after injection of des-Arg^9-BK or Tyr^8-BK. Edema is expressed in milliliters as the difference between the right and left paws. In most experiments animals were treated with LPS (1 μg/paw, 1–72 h before experiments) at the same site of injection of kinins. In all experiments the i.d. injections were performed under slight anesthesia with 2,2,2-tribromoethanol (0.125 g/kg). The reported experiments were conducted in accordance with current guidelines for the care of laboratory animals and ethical guidelines for investigations of experiments in conscious animals (35).

Mechanisms underlying B_1-receptor-mediated paw edema in rats pretreated with LPS

In a separate series of experiments, to confirm the involvement of kinin B_1 receptors in des-Arg^9-BK-induced rat paw edema, animals pretreated with LPS (1 μg/paw, 12 h previously) received an i.d. injection of the selective B_1 receptor agonist, des-Arg^9-BK (100 nmol/paw), coinjected with selective B_1 des-Arg^9-[Leu^8]-BK (100 nmol/paw) or R715 (60 nmol/paw) receptor antagonists. The possible involvement of protein synthesis was evaluated in another group of animals pretreated with the anti-inflammatory steroid dexamethasone (0.5 mg/kg) or with the protein synthesis inhibitor cycloheximide (1.5 mg/kg), both administered s.c. 6 h before LPS injection. To analyze the participation of NF-κB in the development of B_1 receptor-mediated paw edema in rats that had received LPS, the animals were pretreated with the NF-κB inhibitor PDTC (100 mg/kg i.p.) or TLCK (2 mg/kg i.p.) 30 min before LPS injection.

To assess the contribution of secondary cytokine synthesis, animals received an i.d. injection of the anti-murine Ab anti-IL-1β or anti-TNF-α (50 ng/paw) coinjected with LPS 12 h before challenge with the B_1 agonist des-Arg^9-BK. Other animals received LPS in association with the IL-1 receptor antagonist (IRA; 100 μg/paw) or with the anti-inflammatory cytokine, IL-10 (10 ng/paw), 12 h before des-Arg^9-BK injection.

The participation of adhesion molecules was assessed in a separate group of animals pretreated with the nonspecific selectin inhibitor fucoidan (10 mg/kg i.v., 15 min) or with the monoclonal (integrin β_2-chain) anti-CD18 Ab (WT3; 1 mg/kg i.v., 15 min) before LPS injection. To examine the implication of platelet-activating factor (PAF) receptor activation, animals locally treated with LPS received a coinjection of the PAF receptor antagonist, WEB2086 (15 μg/paw), and rat paw edema caused by des-Arg^9-BK was evaluated as described above.

All doses of inhibitors were chosen based on pilot experiments or in accordance with previous studies (16, 36–38).

Expression of B_1 receptor mRNA in rat paw

The methodology used was similar to that previously described by Campos et al. (37). Rats were treated with LPS (1 μg/paw) and were sacrificed at various intervals (1–48 h). In another series of experiments, to assess the role of NF-κB in B_1 receptor mRNA expression, animals were pretreated with PDTC (100 mg/kg i.p.) 30 min before LPS injection (1 μg/paw, 6 h). PBS-treated paws were used as a control. After sacrifice, the s.c. tissue of the paws was removed and frozen under liquid nitrogen. The samples were then homogenized, and total RNA was extracted using the TRIzol reagent (Life Technologies, Gaithersburg, MD). One microgram of total RNA was reverse transcribed using oligo(dT) as the primer (25 μg/ml) and 200 U of reverse transcriptase (Life Technologies) in 20 μl of PCR buffer containing 0.5 mM dNTP, 10 mM DTT, 2.5 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris-HCl, pH 8.4. The samples were incubated for 50 min at 42°C, heated for 15 min at 70°C, and cooled in ice. After treatment with 2 U of RNase H (20 min, 37°C), cDNA amplification of a specific sequence of rat B_1 receptor and β-actin was performed by PCR using the following primers: for B_1 receptor: sense, TGAAGCTGTGAGCTCTTTG; and antisense, GC CAGTTGAAACGGTTCCC; and for rat β-actin: sense, GTTCCGATGC CCCGAGGATCT; and antisense, GCATTTGCGGTGCACGATGGA. β-Actin cDNA was used for standardization of the amount of RNA. Five microliters of RT aliquots were mixed in a 20 mM Tris-HCl buffer (pH 8.4) containing 1.5 mM $MgCl_2$, 300 μM dNTP, 2 μg/ml of each primer, and 50 U/ml of *Taq* polymerase (Life Technologies) in a final volume of 100 μl. The cycling protocol used was the following: 4 min at 94°C, 36 cycles of 35 s at 94°C, 45 s at 60°C, 45 s at 72°C, and finally, 7 min at 72°C. Aliquots of 25 μl were analyzed on a 20% Tris/borate/EDTA polyacrylamide gel and stained by ethidium bromide. The size of the products is 450 bp for B_1 receptor and 600 bp for β-actin.

Gel mobility shift analysis of nuclear extracts binding to NF-κB consensus oligonucleotide

Paw tissues were obtained from LPS-treated rats (1 μg/paw, 1–48 h before experiments) and from rats pretreated with the NF-κB inhibitor PDTC (100 mg/kg i.p., 30 min) before the LPS injection (1 μg/paw, 6–12 h before experiments). PBS-treated paws were used as a control. Tissues were frozen under liquid nitrogen, and nuclear extracts were prepared as described by D'Acquisto et al. (39) with some modifications. Tissues were first suspended in 3 ml of ice-cold hypotonic lysis (10 mM HEPES, 1.50 mM $MgCl_2$, 10 mM KCl, 0.5 mM PMSF, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, and 0.5 mM DTT) and were then homogenized in a Polytron (Brinkmann Instruments, Westbury, NY) for 1 min. The homogenate was divided into three aliquots of 1 ml, chilled on ice for 15 min, and then vigorously shaken for 15 min in the presence of 25 μl of 10% Nonidet P-40. The nuclear fraction was precipitated by centrifugation at 1,500 × *g* for 5 min. The nuclear pellet was resuspended in 600 μl of high salt extraction buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, and 0.5 mM DTT), incubated under continuous shaking at 4°C for 30 min, and then centrifuged for 15 min at 13,000 × *g*. The supernatant was aliquoted and stored at −70°C. The protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA).

For EMSAs, NF-κB double-stranded consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was end-labeled with [γ-^{32}P]ATP (DuPont-NEN, Boston, MA) in the presence of T_4 polynucleotide kinase (10 U) for 10 min at 37°C. Unincorporated nucleotides were removed by passing the reaction mixture over a Sephadex G-25 spin column (Amersham Pharmacia Biotech, Arlington Heights, IL). In a total volume of 20 μl, nuclear extracts (10 μg) were incubated with gel shift binding buffer (10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 4% glycerol, and 1 μg of poly(dI-dC)) for 20 min at room temperature. Each sample was further incubated for 30 min at room temperature with 25,000 cpm of ^{32}P-labeled NF-κB consensus oligonucleotide. Protein-DNA complexes were resolved by nondenaturing 6% acrylamide/bis-acrylamide (37.5/1) in 0.25× Tris-borate/EDTA buffer at 150 V for 2 h. The gel was vacuum-dried and analyzed using a BAS 2000 phosphorimager system (FUJIX, Dusseldorf, Germany). For competition studies, NF-κB or transcription factor IID (TFIID) (5′-5′-GCAGAG CATATAAGGTGAGGTAGGA-3′) unlabeled double-stranded oligonucleotide was included in molar excess over the amount of radiolabeled probe to detect specific and nonspecific DNA/protein interactions, respectively.

Expression of TNF-α mRNA in rat paw

The methodology used was similar to that described by Flohé et al. (40). The animals were treated with LPS (1 μg/paw, 1–48 h before experiments) and had the s.c. tissue of the right hind paw removed. PBS-treated paws were used as a control. PCR was performed using 1 U of *Taq* polymerase (Roche, Mannheim, Germany), 0.2 μmol of dNTP, and 200 pmol of each primer in a total volume of 100 μl in a GeneAmp 2400 thermocycler (PerkinElmer, Norwalk, CT). The primers used were ATGAGCACA GAAAGCATGATCC (sense) and GAAGATGATCTGAGTGTG (antisense). All primers were exon/intron overspanning or included one intron to prevent amplification of chromosomal DNA. Semiquantitative PCR was performed using different amounts of cDNA (0.6–5 μl) and a minimum of cycles ($n = 33$) to obtain a PCR product, so that the amount of the amplificate reflects the quantity of the cDNA and mRNA present in the preparation. Each PCR cycle consisted of 30 s at 95°C, 30 s at 50°C, and 60 s at 72°C. The amplificate was analyzed after separation by agarose gel electrophoresis and ethidium bromide staining. The product amount was quantified by Fluor-Imager analysis (Molecular Dynamics, Krefeld, Germany). The mRNA was measured in two to four dilutions of starting cDNA. The specificity of the PCR was exemplary-proven for the TNF-α product by hybridization with a 3′-fluorescein-11-dUTP-labeled TNF-α oligonucleotide probe (CTCGAGTGACAAGCCCGTAG). After transfer onto a positively charged nylon membrane, an ECL 3′-oligolabeling and detection system (Amersham Pharmacia Biotech, Little Chalfont, U.K.) revealed the TNF-α-specific PCR product. All measured PCR products were normalized for the amount of cDNA of GAPDH in each sample and are expressed as relative units per GAPDH present in each sample.

Measurement of IL-1β and TNF-α levels in the rat paw

The measurements of tissue IL-1β and TNF-α levels were similar to those described by Francischi et al. (41). The animals were treated with LPS (1 μg/paw, 1–48 h prior experiments) and had the s.c. tissue of the right hind paw removed and placed on a PBS solution containing 0.05% Tween 20, 0.1 mM PMSF, 0.1 mM benzamethonium chloride, 10 mM EDTA, and 20 potassium iodide-aprotinin A. PBS-treated paws were used as a control. The tissue was homogenized, centrifuged at 3000 × *g* for 10 min, and stored at −70°C until further analysis. TNF-α levels were evaluated using a standard sandwich ELISA technique as previously described (42).

Neutrophil myeloperoxidase (MPO) assay

Neutrophil recruitment to the rat paw was measured by means of tissue MPO activity, determined according to the method described previously (43). Animals received an i.d. injection of LPS (1 μg/paw, 1–48 h) in the right paw and were sacrificed. PBS-treated paws were used as a control. At the time of sacrifice, the s.c. tissue of the paws was removed, homogenized at 5% (w/v) in EDTA/NaCl buffer (pH 4.7) and centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was resuspended in 0.5% hexadecyltrimethyl ammonium bromide buffer (pH 5.4), and the samples were frozen and thawed three times in liquid nitrogen. Upon thawing, the samples were recentrifuged (10,000 rpm, 15 min, 4°C), and 25 μl of the supernatant was used for the MPO assay. The enzymatic reaction was assessed with 1.6 mM tetramethylbenzidine, 80 mM $NaPO_4$, and 0.3 mM hydrogen peroxide. The absorbance was measured at 690 nm, and the results are expressed as OD per milligram of tissue.

Drugs and reagents

The following drugs were used: *Escherichia coli* LPS (serotype 0111:B4), PBS tablets, captopril, Tyr^8-bradykinin, 2,2,2 tribromoethanol, dexamethasone, cycloheximide, TLCK, PDTC, fucoidan, EDTA, hexadecyltrimethyl ammonium bromide, tetramethylbenzidine, DTT, PMSF, HEPES, benzamethonium chloride, aprotinin A, leupeptin, pepstatin A, and soybean trypsin inhibitor (all from Sigma-Aldrich, St. Louis, MO); glycerol (Invitrogen, Carlsbad, CA); Polyidet P-40 (Polyscience, Warrington, PA); des-Arg^9-bradykinin and des-Arg^9-Leu^8-bradykinin (Bachem Bioscience, King of Prussia, PA); $NaPO_4$, hydrogen peroxide, $MgCl_2$, KCl, Tris-HCl, NaCl, and Tween 20 (all from Merck, Haar, Germany); WEB2086 (gift from Roche, Mannheim, Germany); and anti-CD18 mAb (gift from Dr. P. Hellewell, University of Sheffield, Sheffield, U.K.). R-715 (AcLys[D-βNal^7,Ile^8]des-Arg^9-BK) was donated by Dr. D. Regoli (University of Sherbrooke, Sherbrooke, Canada). Anti-murine neutralizing Abs anti-IL-1β (lot B01D3), anti-TNF-α (lot CT101), the recombinant rat cytokine IL-10 (lot ARE150101), and human recombinant IRA were obtained from R&D Systems (Minneapolis, MN). The stock solutions of des-Arg^9-bradykinin, des-Arg^9-Leu^8-bradykinin, and LPS were prepared in PBS. All were stocked in siliconized plastic tubes, maintained at −18°C, and diluted to the desired concentration just before use. The other drugs were prepared daily in 0.9% (w/v) NaCl solution, except for dexamethasone, which was diluted in 5% ethanol.

Data analysis

The results are presented as the mean ± SEM of four to six animals. The percentages of inhibition are reported as the mean ± SEM inhibition obtained in each individual experiment at the peak of the edema (20 min after injection of des-Arg^9-bradykinin) or 1–72 h after LPS injection depending on the experimental protocol. Statistical comparison of the data was performed by ANOVA, followed by Dunnett's test or by Student's unpaired *t* test. A value of $p < 0.05$ was considered significant.

Results

Modulation of kinin B_1 receptors after LPS treatment

As reported previously (34), i.d. injection of the selective kinin B_1 receptor agonist des-Arg^9-BK (100 nmol/paw) in naive animals produced a very weak increase in rat paw edema (0.134 ± 0.016 ml; $n = 5$). In contrast, i.d. injection of des-Arg^9-BK in rats that had been treated with an i.d. injection of LPS (1 μg/paw, 6–36 h previously) induced a significant increase in rat paw edema (Fig. 1*A*). Edema formation in response to des-Arg^9-BK administration peaked at 12 h (0.41 ± 0.03 ml; $n = 5$) and then decreased gradually at 24 and 36 h after LPS administration. At 72 h after LPS,

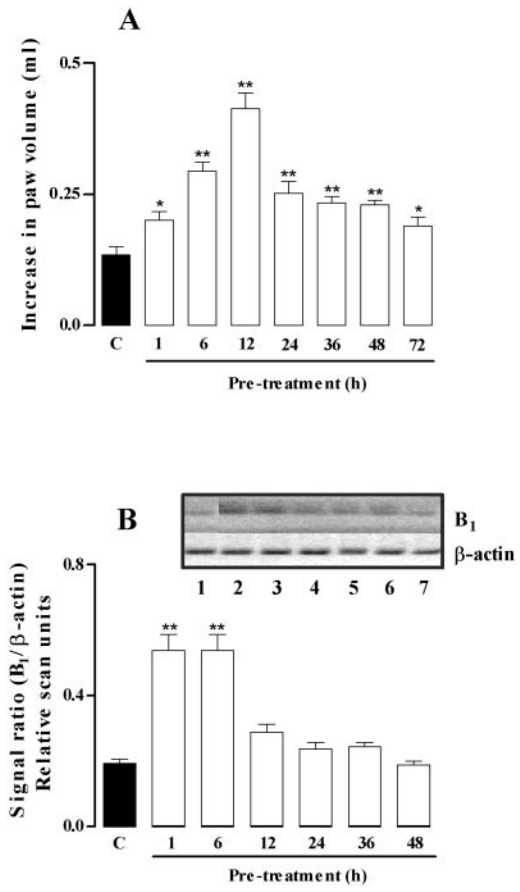

FIGURE 1. Time-dependent effect of LPS treatment on B_1 receptor upregulation in the rat paw. *A*, Increase in rat hind paw volume in response to intradermal injection of des-Arg^9-BK (100 nmol/paw) in PBS-pretreated (■) or LPS-pretreated (1 μg/paw, 1–72 h; □) animals. Edema was measured 20 min after intraplantar injection of des-Arg^9-BK. Each point represents the mean ± SEM of four or five animals. *B*, Effect of LPS (1 μg/paw, 1–48 h) injection on kinin mRNA B_1 receptor expression in the rat paw. *Bottom*, Graphic representation of B_1/β-actin signals ratio. *Lane 1*, PBS; *lane 2*, LPS, 1 h; *lane 3*, LPS, 6 h; *lane 4*, LPS, 12 h; *lane 5*, LPS, 24 h; *lane 6*, LPS, 36 h; *lane 7*, LPS, 48 h. Number of replicates = 4. ∗, $p < 0.05$; ∗∗, $p < 0.01$.

des-Arg^9-BK-induced edema responses were decreased, but remained significant compared with those in saline-treated animals (Fig. 1*A*). The time-dependent functional up-regulation of B_1 receptor-mediated paw edema after LPS treatment (1 μg/paw, 1–48 h) was accompanied by a similar increase in kinin B_1 receptor mRNA expression in the rat paw, as assessed by RT-PCR experiments (Fig. 1*B*). In contrast, injection of the selective B_2 agonist, Tyr^8-BK (3 nmol/paw), induced no further increase in paw volume in LPS-treated animals compared with their vehicle-treated controls (results not shown). As the edema formation induced by des-Arg^9-BK was maximal 12 h after LPS treatment, this time point was chosen for subsequent studies of functional bradykinin B_1 receptor up-regulation.

The edema induced by des-Arg^9-BK in rats pretreated with LPS (1 μg/paw, 12 h previously) was significantly inhibited by the coinjection of selective B_1 receptor antagonists, des-Arg^9-[Leu^8]-BK (100 nmol/paw) or R715 (60 nmol/paw), with inhibitions of 43 $\pm$ 6 and 50 $\pm$ 1%, respectively (Fig. 2). The latter results provide further evidence that des-Arg^9-BK was indeed acting on bradykinin B_1 receptors to induce an increase in paw volume.

Moreover, and in agreement with the need for the synthesis of novel bradykinin B_1 receptors, the LPS-induced responsiveness to des-Arg^9-BK was diminished by the systemic treatment of animals with the anti-inflammatory steroid dexamethasone (0.5 mg/kg s.c.) or with the protein synthesis inhibitor cycloheximide (1.5 mg/kg s.c.), both administrated 6 h before the LPS injection (46 $\pm$ 8 and 58 $\pm$ 8% inhibition; results not shown).

Participation of NF-κB in the up-regulation of B_1 receptors in animals pretreated with LPS

Gel mobility shift analysis was employed to assess the possible involvement of the transcriptional factor NF-κB after treatment with LPS. Local administration of LPS (1 μg/paw, 1–48 h previously) determined a marked increase in NF-κB/DNA binding. This response presented a biphasic profile, with a first peak between 1 and 12 h and a second one at 36–48 h. LPS-induced NF-κB activation in the rat paw (12 h) was completely prevented by systemic treatment with the NF-κB inhibitor PDTC (100 mg/kg i.p., 30 min before LPS; Fig. 3).

The relevance of NF-κB for B_1 receptor modulation by LPS was further confirmed by functional experiments. Thus, the results demonstrate that pretreatment (30 min before LPS) of animals with the NF-κB inhibitor PDTC (100 mg/kg i.p.) or TLCK (2 mg/kg i.p.) prevented the des-Arg^9-BK-induced increase in paw volume by 74 $\pm$ 1 and 50 $\pm$ 3%, respectively (Fig. 4, *A* and *B*). In addition to preventing functional B_1 receptor up-regulation, PDTC treatment prevented the LPS-induced increase in B_1 receptor mRNA (Fig. 4*C*). Overall, these results demonstrate a rapid, sustained, and functionally relevant (with regard to the B_1 receptor) NF-κB activation after LPS administration.

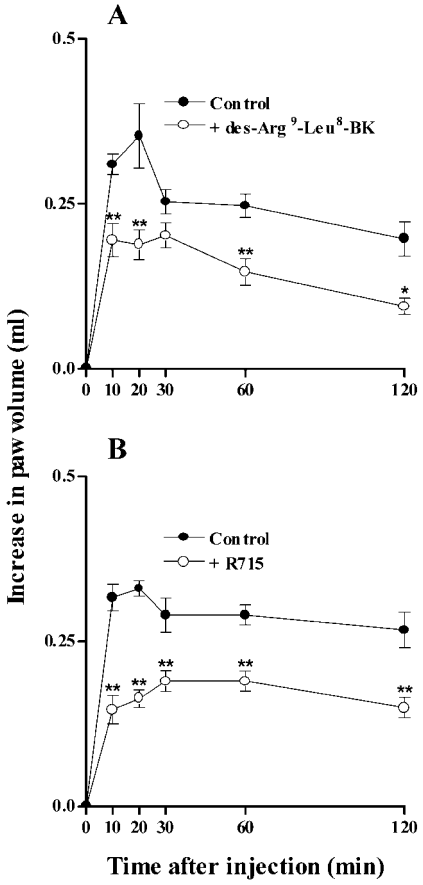

FIGURE 2. Effect of B_1 receptor antagonists on des-Arg^9-BK-induced paw edema in rats pretreated with LPS. Des-Arg^9-Leu^8-BK (100 ng/paw; *A*) or R715 (60 ng/paw; *B*) was coadministrated with des-Arg^9-BK (100 nmol/paw) 12 h after LPS treatment (1 μg/paw, 12 h). Values represent the differences between volume (in milliliters) of vehicle-injected (0.1 ml of PBS solution) and drug-injected paws. Each point represents the mean $\pm$ SEM of four or five animals. *, $p < 0.05$; **, $p < 0.01$.

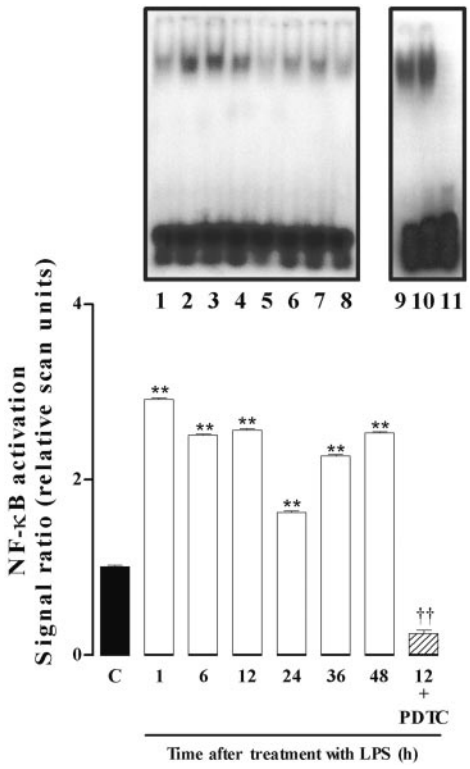

FIGURE 3. Time-dependent effect of LPS treatment on NF-κB activation. LPS (1 μg/paw) was administrated 1–48 h before experiments. *Bottom*, Graphic representation of relative scan units. *Lane 1*, PBS; *lane 2*, LPS, 1 h; *lane 3*, LPS, 6 h; *lane 4*, LPS, 12 h; *lane 5*, LPS, 24 h; *lane 6*, LPS, 36 h; *lane 7*, LPS, 48 h; *lane 8*, LPS, 12 h, plus PDTC. The competition experiments were performed using nuclear extracts from rats pretreated with LPS (i.d., 12 h) in the absence (*lane 9*) or in the presence of an excess of unlabeled NF-κB probe (1.75 pmol/μl; *lane 11*) or TFIID probe (1.75 pmol/μl; *lane 10*). Number of replicates = 3. ■, Response of PBS-treated animals. Significantly different from PBS-injected (**) or LPS-injected (††) values, $p < 0.01$.

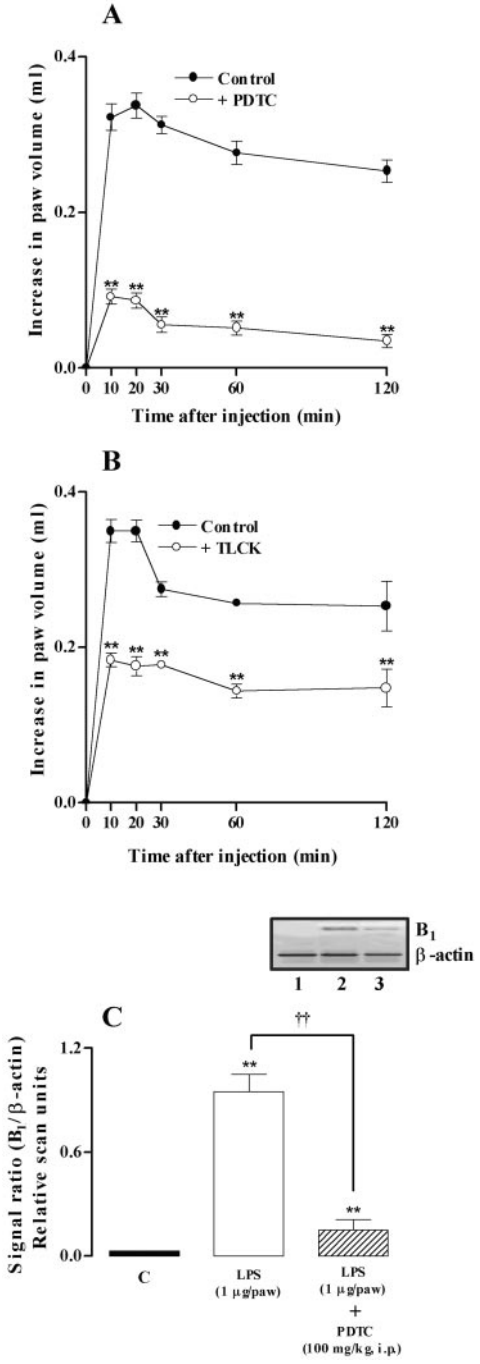

FIGURE 4. Effect of NF-κB inhibitors on des-Arg^9-BK induced paw edema in rats pretreated with LPS. PDTC (100 mg/kg i.p.; *A*) or TLCK (2 mg/kg i.p.; *B*) was administrated systemically 30 min before LPS (1 μg/paw). After 12 h, animals received des-Arg^9-BK (100 nmol/paw), and edematogenic responses were evaluated. Values represent the differences between volume (in milliliters) of vehicle-injected (0.1 ml of PBS solution) and drug-injected paws. Each point represents the mean ± SEM of four or five animals. *C*, Effect of PDTC treatment (100 mg/kg i.p., 30 min before LPS) on kinin mRNA B_1 receptor expression in rats pretreated with LPS (1 μg/paw, 6 h). *Bottom*, Graphic representation of B_1/β-actin signals ratio. *Lane 1*, PBS; *lane 2*, LPS, 6 h; *lane 3*, LPS, 6 h, plus PDTC. Number of replicates = 4. Significantly different from PBS-injected (**) or LPS-injected (††) values, $p < 0.01$.

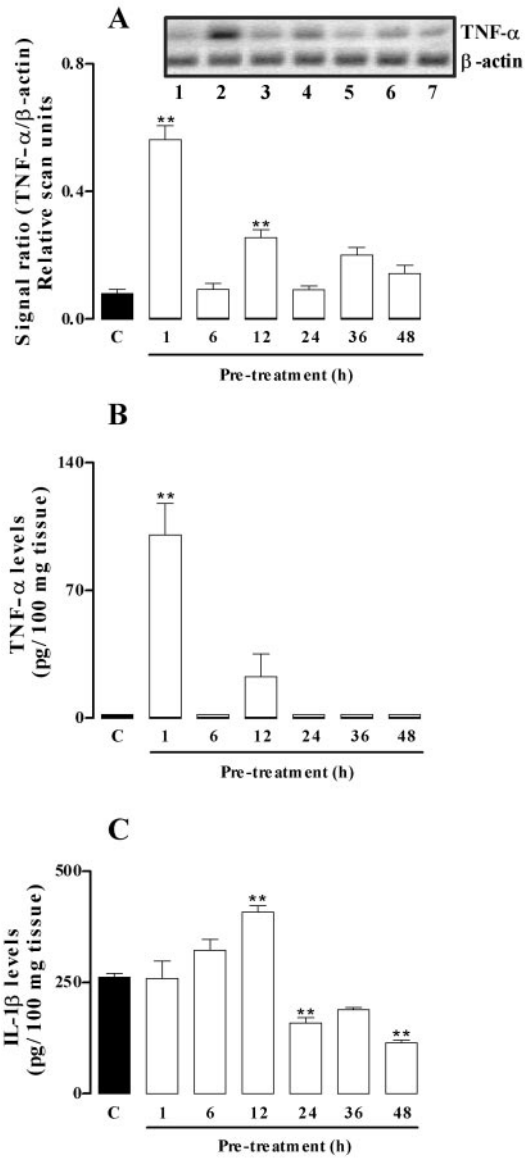

FIGURE 5. Time-dependent effect of LPS treatment on TNF-α mRNA, TNF-α, and IL-1β levels in the rat paw. *A*, Effect of LPS injection (1 μg/paw, 1–48 h) on TNF-α mRNA levels in the rat paw. *Bottom*, Graphic representation of TNF-α/β-actin signals ratio. *Lane 1*, PBS; *lane 2*, LPS, 1 h; *lane 3*, LPS, 6 h; *lane 4*, LPS, 12 h; *lane 5*, LPS, 24 h; *lane 6*, LPS, 36 h; *lane 7*, LPS, 48 h. TNF-α (*B*) or IL-1β (*C*) levels in the rat paw after LPS treatment (1 μg/paw, 1–48 h) are shown. Number of replicates = 4. ■, Response of PBS-treated animals. **, $p < 0.01$.

Assessing the role of proinflammatory cytokines

The injection of LPS induced an increase in TNF-α mRNA expression that was prominent at 1 h after treatment (Fig. 5*A*). At later time points, TNF-α expression levels were similar to or just slightly greater than background. Of note, mRNA expression was markedly consistent with the detection of TNF-α protein by ELISA (Fig. 5*B*). Not only was TNF-α expressed, but, more importantly, its inhibition with anti-TNF-α Ab was associated with a significant suppression (36 ± 5% inhibition) of the ability of LPS to induce functional up-regulation of B_1 receptors (Fig. 6*B*). There was also a marked increase in the expression of IL-1β immunoreactivity after LPS administration in the paw (Fig. 5*C*). Likewise, coinjection of anti-IL-1β or recombinant IRA (100 μg/paw) significantly prevented (62 ± 8 and 55 ± 6% inhibition, respectively) the ability of LPS to induce functional B_1 receptors (Fig. 6, *A* and *C*). Finally, injection of IL-10, a cytokine known to counteract the actions of TNF-α and IL-1β (29), also suppressed (37 ± 4% inhibition) LPS-induced B_1 receptor up-regulation (Fig. 6*D*).

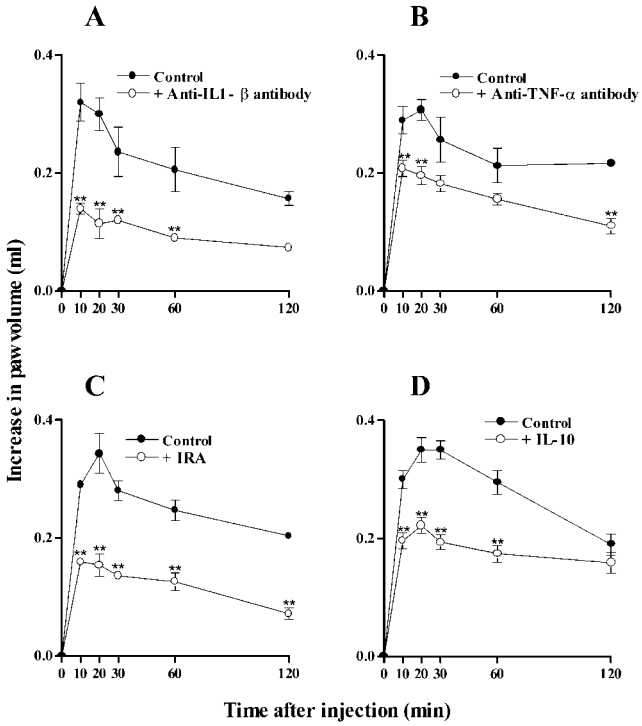

FIGURE 6. Effect of Abs anti-IL-1β, anti-TNF-α, or anti-inflammatory cytokines on des-Arg^9-BK-induced paw edema in rats pretreated with LPS. The anti-murine Abs anti-IL-1β (50 ng/paw; *A*), anti-TNF-α (50 ng/paw; *B*), the recombinant human IRA (100 μg/paw; *C*), or IL-10 (10 ng/paw; *D*) was coadministrated with LPS (1 μg/paw) 12 h before injection of des-Arg^9-BK (100 nmol/paw). Values represent the differences between volume (in milliliter) of vehicle-injected (0.1 ml of PBS solution) and drug-injected paws. Each point represents the mean ± SEM of four or five animals. **, $p < 0.01$.

Involvement of neutrophil influx

The migration of neutrophil to the rat paws in response to LPS injection was assessed indirectly by means of MPO assay. As shown in Fig. 7, injection of LPS (6–36 h) induced a marked (~7-fold) and time-related increase in MPO levels, which peaked at 12 h and remained significantly increased up to 36 h. Of note, MPO levels were significantly greater than background only 6 h after LPS injection, hence, after the LPS-induced increases in NF-κB activation and TNF-α production (compare Figs. 3, 5, and 7).

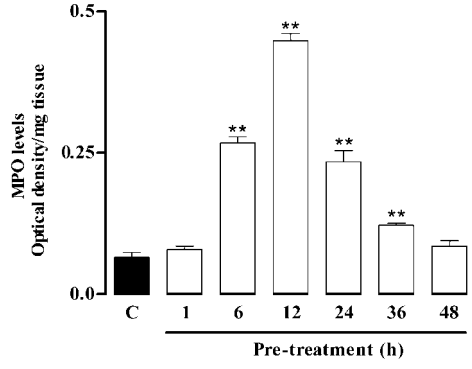

FIGURE 7. Time-related effect of LPS pretreatment on MPO levels. LPS (1 μg/paw) was administrated 1–48 h before experiments. ■, Response of PBS-treated animals. Each point represents the mean ± SEM of four or five animals. ■, Response of PBS-treated animals. **, $p < 0.01$.

To assess the possible contribution of neutrophil influx to functional up-regulation of B_1 receptors, several strategies known to modulate cell migration were tested. Coinjection of the PAF receptor antagonist, WEB2086 (15 μg/paw), with LPS suppressed functional up-regulation of B_1 receptors by 54 ± 6% (Fig. 8*A*). Similarly, prevention of neutrophil influx by the treatment with the nonspecific selectin inhibitor, fucoidan (10 mg/kg i.v., 15 min before LPS), or a monoclonal anti-CD18 (integrin β_2-chain) Ab (WT3, 1 mg/kg i.v., 15 min before LPS treatment) significantly suppressed (60 ± 5 and 41 ± 4% inhibition, respectively) the functional up-regulation of B_1 receptors, as assessed by edema formation in the rat paw in response to des-Arg^9-BK injection (Fig. 8, *B* and *C*). Together, the latter results firmly support the idea that neutrophil influx is necessary for the ability of LPS to induce functional B_1 receptors.

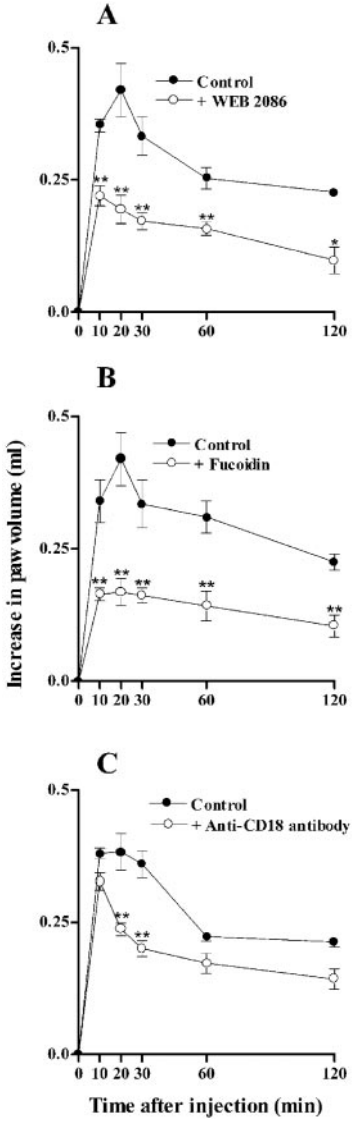

FIGURE 8. Effects of migration inhibitors on des-Arg^9-BK induced paw edema in rats pretreated with LPS. Animals were treated with WEB 2086 (15 μg/paw; *A*), fucoidan (10 mg/kg i.v., 15 min; *B*), or monoclonal (integrin β_2-chain) anti-CD18 Ab (WT3, 1 mg/kg i.v., 15 min; *C*) and received des-Arg^9-BK (100 nmol/paw) 12 h after LPS injection (1 μg/paw). Values represent the differences between volume (in milliliters) of vehicle-injected (0.1 ml of PBS solution) and drug-injected paws. Each point represents the mean ± SEM of four or five animals. *, $p < 0.05$; **, $p < 0.01$.

Discussion

LPS is a component of the outer membrane of Gram-negative bacteria widely recognized as a potent immune system activator, capable of inducing a plethora of inflammatory genes (2). Most of their effects seem to be mediated by a cascade of cellular events governed by interaction with a member of the Toll-like receptor family (TLR-4), resulting in the stimulation of transcriptional factors, such as NF-κB, and the release of proinflammatory cytokines, such as IL-1β and TNF-α (44, 45). There is compelling evidence showing that there is up-regulation of kinin B_1 receptors after LPS injection or tissue trauma, and that proinflammatory cytokines and activation of transcriptional factors are involved in this process (46–48). Several studies performed with different cell lines, such as HEK-293 and IMR-90, or primary culture of vascular umbilical smooth muscle cells demonstrated that the human B_1 receptor gene contains three exons separated by two introns. The first and second exons are noncoding, whereas the coding region and the 3′-flanking region are located entirely at the third exon. Sequence analysis of the 5′-flanking region has revealed the presence of a consensus TATA box and several transcription factor binding sequences, a putative enhancer, and silencer elements (49, 50). In the present study we have analyzed, by means of molecular and functional approaches, the sequence of events leading to B_1 receptor up-regulation after in vivo administration of LPS.

Treatment with LPS resulted in a rapid and prolonged functional up-regulation of B_1 receptors in the rat paw, as assessed by the increase in paw edema in response to des-Arg^9-BK. This functional response appeared to be critically dependent on protein synthesis, as shown by the rapid and transient (1–6 h after LPS) increase in mRNA levels for B_1 receptors and the effects of dexamethasone or cycloheximide. The latter agents are known to interfere with de novo protein synthesis and were capable of consistently preventing the increase in B_1 receptor-mediated edematogenic response. The ability of des-Arg^9-BK to detect functional B_1 receptors was reinforced by the data obtained with two selective B_1 receptor antagonists, des-Arg^9[Leu^8]-BK and R-715. Both of these antagonists markedly prevented the edematous response to des-Arg^9-BK injection. Thus, our study clearly demonstrates the ability of LPS to induce the novel synthesis and functional expression of B_1 receptors when injected into the rat paw.

Several studies have indicated that LPS and other microbial agents are capable of activating NF-κB and that activation of this transcription factor is important for their action (2, 51–53). Similar to the functional up-regulation of B_1 receptors, the injection of LPS induced a rapid activation of the transcriptional factor NF-κB. Not only was NF-κB activated, but results with PDTC and TLCK demonstrate that NF-κB activation was functionally important in our system. Indeed, there was a close temporal association between NF-κB activation and functional B_1 receptor expression (compare Figs. 1 and 3). Thus, akin to in vitro studies evaluating the expression of B_1 receptors (24, 54, 55), our results clearly demonstrate that LPS-induced NF-κB activation is necessary for the increase in mRNA and functional up-regulation of B_1 receptors. However, as will be discussed below and shown schematically in Fig. 9, additional interactions are apparently necessary for a full activation of NF-κB and B_1 receptor functional up-regulation.

Several studies have now shown that proinflammatory cytokines are capable of inducing B_1 receptor up-regulation in several tissues and under various conditions both in vitro and in vivo (16, 36, 37, 46, 55–58). For example, we have previously demonstrated the ability of IL-1β to act in cooperation with TNF-α to induce the functional up-regulation of B_1 receptors in the rat paw (36, 37).

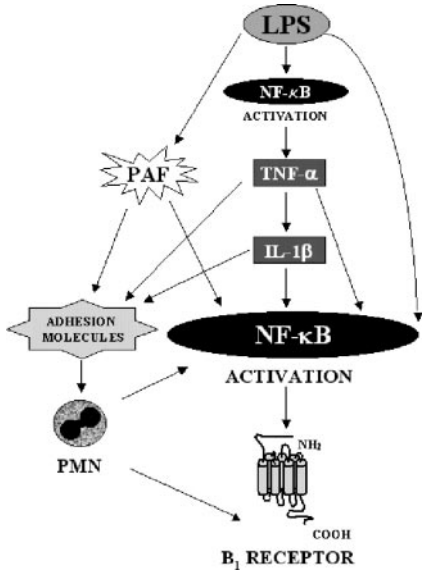

FIGURE 9. Possible sequence of events leading to B_1 receptor up-regulation after local administration of LPS in vivo. PMN, polymorphonuclear cells.

The present data provide good evidence to indicate that the generation of proinflammatory cytokines is necessary for LPS to induce B_1 receptor-mediated inflammatory responses in the rat paw: 1) injection of LPS resulted in a significant increase in both TNF-α and IL-1β release in the rat paw; and 2) blockade of TNF-α action with Abs or blockade of IL-1β action with Abs or IRA prevented B_1 receptor up-regulation. Interestingly, administration of the anti-inflammatory cytokine, IL-10, also suppressed the ability of LPS to facilitate B_1 receptor-mediated edema. The cooperative actions of TNF-α and IL-1β to induce B_1 receptor up-regulation are probably due to their ability to further enhance NF-κB activation (Fig. 9) and to facilitate the influx of leukocytes (see below). Indeed, TNF-α and IL-1β are well-known activators of NF-κB in many in vivo situations, including local and systemic administration of LPS (59, 60). Furthermore, both these cytokines are good inducers of the expression of cell adhesion molecules and chemoattractant mediators, such as PAF, which are capable of supporting neutrophil recruitment in vivo (59, 61–64). Of note, our previous studies have clearly demonstrated that proinflammatory cytokines are able to activate NF-κB and induce neutrophil accumulation in the rat paw (37).

The administration of LPS in the rat paw induced a marked influx of neutrophils that was first noticed at 6 h and peaked 12 h after LPS administration. The neutrophil influx was temporally very similar to the activation of NF-κB and the functional up-regulation of B_1 receptors, with the exception of the first hour. Not only were these phenomena temporally related, but the blockade of neutrophil influx using different strategies markedly prevented the up-regulation of B_1 receptors. Thus, the administration of fucoidin, an anti-CD18, or a selective PAF receptor antagonist consistently prevented LPS from facilitating des-Arg^9-BK-induced edema. This is consistent with our previous studies demonstrating the involvement of neutrophils in the modulation of B_1 receptors in vivo (37, 65). Overall, these studies firmly establish a role for neutrophils in the sequence of events leading to the functional up-regulation of B_1 receptors after several inflammatory stimuli in vivo (Fig. 9).

PAF has been described as a pivotal molecule responsible for many of the pathophysiological effects of LPS and other bacterial

products in vivo (61–63). In addition, it has been recently demonstrated that PAF is a main mediator underlying NF-κB activation and cytokine production after LPS treatment in vivo (59). Thus, in addition to inducing neutrophil influx in response to LPS administration in the rat paw, PAF could be contributing directly to B_1 receptor up-regulation via receptor-mediated NF-κB activation in the target cell. In this regard, we have recently reported that local treatment with PAF results in the functional and molecular up-regulation of B_1 receptors in the rat paw (66).

Together the data presented above suggest the following sequence of events leading to B_1 receptor up-regulation after local LPS administration in vivo (shown schematically in Fig. 9); there is a rapid activation of the transcription factor NF-κB that appears to facilitate the local release of proinflammatory cytokines, including TNF-α and IL-1β. These cytokines promote the influx of neutrophils by inducing the local expression of cell adhesion molecules and secretion of chemoattractant molecules (such as PAF) and may also enhance NF-κB activation. The incoming neutrophils are necessary for the up-regulation of B_1 receptors, possibly by enhancing NF-κB activation or releasing yet unidentified molecules. It is unclear at present which cells express B_1 receptors, but based on the functional response assessed in this study, it is likely that endothelial cells are major targets for the inflammatory circuit suggested above. Thus, by acting in cooperation, TNF-α, IL-1β, neutrophils, and the transcription factor NF-κB are major and essential players in the ability of LPS to induce B_1 receptor expression in vivo.

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