Infection-Induced Kinin B₁ Receptors in Human Pulmonary Fibroblasts: Role of Intact Pathogens and p38 Mitogen-Activated Protein Kinase-Dependent Signaling

Stephen B. Phagoo, Krisanavane Reddi, Bertrand J. Silvallana, L. M. Fredrik Leeb-Lundberg, and David Warburton

Developmental Biology Program, Saban Research Institute, Children’s Hospital Los Angeles, Department of Surgery, Keck School of Medicine, University of Southern California, Los Angeles, California (S.B.P., K.R., B.J.S., D.W.); and Department of Physiological Sciences, Lund University, Lund, Sweden (L.M.F.L.-L.)

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ABSTRACT

Kinin B₁ receptors (B₁R) are involved in many pathophysiological processes, and its expression is up-regulated in inflammatory pulmonary disease. Although bacteria can generate kinin peptides, the molecular signaling mechanisms regulating B₁R during infection by intact pathogens is unknown. The serious opportunist clinical isolate Burkholderia cenocepacia (B. cen.) belongs to the important B. cepacia complex (Bcc) of gram-negative pathogens that rapidly causes fatal pulmonary disease in hospitalized and immunocompromised patients and those with cystic fibrosis. We demonstrate here that B. cen. infection induced a rapid increase in B₁R mRNA (1 h) proceeded by an increase in B₁R protein expression (2 h), without affecting B₂ receptor expression in human pulmonary fibroblasts. The B₁R response was dose-dependent and maximal by 6 to 8 h (3- to 4-fold increase), however, brief B. cen. infection could sustain B₁R up-regulation. In contrast, nonclinical Bcc phytopathogens were much less B₁R inducive. The protein synthesis inhibitor cycloheximide and transcriptional inhibitor actinomycin D abrogated the B₁ response to B. cen. indicating de novo B₁R synthesis. B. cen. activated p38 mitogen-activated protein kinase (MAPK), and blocking p38 MAPK with the specific inhibitor 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580) dramatically reduced B. cen.-induced B₁R. Furthermore, B. cen. regulation of B₁R was diminished by the anti-inflammatory glucocorticoid dexamethasone. In conclusion, this study is the first demonstration that infection with intact pulmonary pathogens like B. cen. positively modulates the selective expression of B₁R. Thus, providing evidence that B₁R regulation may be an important and novel mechanism in the inflammatory cascade in response to chronic pulmonary infection and disease.

Inflammatory processes and infection can cause the activation of the kallikrein-kinogen cascade generating the proinflammatory kinin peptide, bradykinin (BK). There have been many lines of evidence that the kinin cascade is important in the pathophysiology of acute lung injury and is associated with conditions such as sepsis and primary pneumonia. It has been reported that the concentration of BK in the bronchoalveolar lavage fluid of patients with pulmonary inflammation and pneumonia increases by about 5- to 10-fold (Baumgarten et al., 1992). Pathogens that are involved in serious lung diseases such as cystic fibrosis (CF) can promote the generation of BK (Khan et al., 1993; Mattsson et al., 2001), and furthermore kallikrein levels have been found to be increased in the saliva of these patients (Lieberman and Littenberg, 1969). BK is involved in the bacterial dissemination of pathogens in vivo (Sakata et al., 1996), and the use of kinin receptor antagonists may be beneficial in controlling infections (Ridings et al., 1995; Heitsch, 2000). Overall, these findings suggest an important role for kinins in the initiation and maintenance of inflammation in infection.

Kinins exert a wide range of biological actions including...
modulation of neuropeptide and cytokine release, increased epithelial transport, vasodilatation, smooth muscle contraction/relaxation, plasma exudation, pain, and cell proliferation. These actions are mediated through two kinin receptor subtypes, BK B2 and BK B1 (Leeb-Lundberg et al., 2005). The B2 receptor subtype mediates the action of BK and kallidin (KD), whereas the B1 receptor subtype mediates the action of metabolites of the B2 receptor ligands, desArg9BK and desArg10KD, respectively. Both receptor subtypes are members of the super family of seven transmembrane domain, G-protein-coupled receptors (Leeb-Lundberg et al., 2005). Under nonpathological conditions, B2 receptors are expressed widely. With only a few exceptions, B1 receptors are not expressed in significant levels in normal tissues. Instead, kinin B1 receptors are de novo expressed during inflammatory insult and tissue injury (Calixto et al., 2004) and have been demonstrated to be involved in regulating the accumulation of leukocytes at sites of airway inflammation (Perron et al., 1999; Gama Landgraf et al., 2003). Pathophysiological conditions including sepsis (Matsuda et al., 2004) and allergen challenge (Huang et al., 1999) have demonstrated B1 up-regulation in pulmonary tissues. Furthermore, up-regulated B1 receptor protein expression was observed in biopsies from patients with fibrotic lung tissue formation (Nadar et al., 1996). Thus, B1 receptors may be candidate therapeutic targets upstream in the cascade in airway inflammation processes (Calixto et al., 2004). In the current study, we hypothesized that lung infection regulates the expression and activity of kinin receptors during chronic inflammation, which is implicated in the pathobiology of infectious tissue diseases.

Here, we have investigated the effect of infection with the serious opportunistic pathogens from the Burkholderia cepacia (B. cepacia) complex (Bcc) on the regulation of kinin receptors. B. cepacia was originally described as a widespread phytopathogen and was formerly a member of the genus Pseudomonas. However, it is now clear that B. cepacia constitutes a complex of bacteria collectively known as the Bcc and consists of more than nine distinct species or genovars that are phenotypically similar but genotypically distinct. Bcc organisms are increasingly being isolated from CF patients (Govan et al., 1996). Strikingly, these pathogens are associated with more rapid progression of lung disease and severe infection than other CF organisms and causing increased rates of morbidity and mortality (Govan et al., 1996; Hutchinson and Govan, 1999; Jones et al., 2004). B. cepacia-infected CF patients can also develop cepacia syndrome causing fulminating pneumonia and resulting in fatal clinical deterioration (Isles et al., 1984). In addition to its devastating role as an important CF pathogen, B. cepacia has also been found to be significantly involved in other hospitalized and compromised patients (Mohr et al., 2001). Of the Bcc, the species B. cepacia (genovar III) has been associated with the great majority of serious infections and deaths in CF patients (Govan et al., 1996; Jones et al., 2004).

In the current study, we provide the novel demonstration that infection with intact serious human opportunistic pathogens like B. cepacia rapidly up-regulates B1 receptor expression, occurs through de novo protein synthesis, and signals through activation of the p38 mitogen-activated protein kinase (MAPK) pathway. This provides evidence that the induction of B1 receptors may have an important role in inflammatory pathologies that are driven by chronic bacterial infection.

**Materials and Methods**

**Culture of Human Lung Fibroblasts.** IMR-90, HEL 299, and WI-38 human pulmonary fibroblasts were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Fibroblasts were cultured in complete growth media comprised of Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 25 mM HEPES, 4 mM L-glutamine, and 1% nonessential amino acids (Invitrogen). The cells were maintained in a humidified atmosphere in 5% CO2 at 37°C and were subcultured by incubating with 0.05% trypsin-0.5 mM ethylenediaminetetraacetate (Invitrogen) at a weekly ratio of 1:2 to 1:3. For all experiments, cells were plated in six-well plates and grown to confluency. Prior to experimentation, the cells were washed once with growth medium containing fetal bovine serum before being incubated in the absence and presence of pathogens or interleukin-1β (IL-1β) (R&D Systems, Minneapolis, MN).

**Bacterial Strains and Culture Conditions.** All Bcc isolates were obtained from the Belgian Co-ordinated Collections of Microorganisms, Bruxelles, Belgium. *B. cenocepacia* is the predominant Bcc respiratory pathogen in CF patients, and strain J2315 (hence referred to as “B. cenocepacia”, strain LMG 16656, genovar III) is the reference strain of *B. cenocepacia* of the Bcc. This clinical isolate was obtained from a patient with CF and is a strain from the major transmissible lineage known as ET12 (Govan et al., 1993). To compare the kinin response to infection with *B. cenocepacia*, we used the CF clinical isolate *B. multivorans* (hence referred to as “B. multivorans”, LMG 13010, genovar II) and the phytopathogen *B. cepacia* (hence referred to as “B. cepacia”, strain LMG 1222, genovar I). All pathogens were routinely cultured on nutrient agar plates (Sigma-Aldrich) without any supplements and incubated aerobically at 37°C. One day prior to experiments, the organisms were cultured overnight in nutrient broth at 37°C and the number of organisms per milliliter assessed by measuring optical density at 600 nm. In experiments to kill extracellular bacteria, ceftazidime (1 mg/ml; GlaxoSmithKline, Uxbridge, Middlesex, UK) and gentamicin (0.5 mg/ml; Sigma-Aldrich) were added to the culture media. To verify that the organisms were nonviable following the antibiotic treatment, samples of the culture media were incubated for 48 h on blood agar plates and visually inspected.

**Measurement of Kinin B1 and B2 Receptor Expression.** Radioligand binding assays were performed at 4°C in six-well dishes in a final volume of 1.25 ml. Fibroblasts were incubated for 75 min in the presence of 1.25 nM [3H]desArg9KD (77–105 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) or [3H]HBBK (90 Ci/mmol; PerkinElmer Life and Analytical Sciences) in binding buffer (20 mM HEPES, pH 7.4, 125 mM N-methyl D-glucamine, 5 mM KCl, 0.14 g/l bacitracin, 1 mM 1,10-phenanthrolone, 1 mM teproe, and 1 g/l bovine serum albumin (Sigma-Aldrich)). For [3H]HBBK saturation studies, various concentrations of radioligand were used (0.1–2.0 nM). Nonspecific binding was defined as the amount of radiolabeled ligand bound in the presence of 5 μM nonradioactive ligand. After incubation, the assay buffer was removed, and the cells were washed with 2 × 4 ml of ice-cold phosphate-buffered saline. The cells were then lysed with 0.05% sodium dodecyl sulfate. Specific binding was expressed in femtomoles per milligram of protein, and protein concentrations were determined using a Bio-Rad kit (Bio-Rad, Hercules, CA). All assay plates were carried out in duplicate, and the variation between wells was ≤5%. **mRNA Analyses.** Fibroblasts were grown to confluence in six-well dishes and incubated in DMEM in the presence of *B. cenocepacia* at a multiplicity of infection (MOI, bacteria-to-cell ratio) of 50:1 for up to 6 h. Total RNA was extracted from cells using TRIzol reagent as described by the manufacturer (Invitrogen). Single-
stranded cDNA was generated using Superscript II reverse transcriptase (100 U; Invitrogen) in a 20-μl reaction mixture containing reaction buffer (50 mM Tris-Cl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, and 10 mM dithiothreitol), 0.5 mM dNTP, 0.5 μg of oligo(dT)_{12-18} (Invitrogen), 10 U rRNasin (Promega, Madison, WI), and 2 μg of total RNA. The reaction was carried out for 1 h at 42°C. Amplification of cDNA by PCR was performed using specific primers for the human B₁ receptor and β-actin, and the human B₁ receptor PCR product was 429 bp and β-actin was 661 bp (Phagoo et al., 2001). The reactions were carried out using a RoboCycler (Stratagene, La Jolla, CA) in a 50-μl reaction mixture containing reaction buffer (20 mM Tris-Cl, pH 8.4, 50 mM KCl, and 2.5 mM MgCl₂), 0.2 mM dNTP, 2.5 U Taq polymerase (Invitrogen), and 1 μl of cDNA. Each primer was added at a final concentration of 0.2 μM. PCR was for 30 to 35 cycles, each cycle consisting of a 1-min denaturation at 94°C, annealing at 55°C for 50 s, and extension at 72°C for 45 s. PCR reaction products were separated on 1% agarose gels containing 50 μg/ml ethidium bromide and visualized under UV light.

**IL-8 Release by Enzyme-Linked Immunosorbent Assay.** The confluent cell monolayer was infected with *B. cenocepacia* or *B. cenocepacia* at a MOI of 250:1 for 6 h. Controls consisted of nutrient broth as a negative control and IL-1β (500 pg/ml) as a positive control. The cell media were then removed, centrifuged at 13,000g for 15 min to remove bacteria and cell debris, and then stored at −80°C until assayed for IL-8 by enzyme-linked immunosorbent assay as described previously (Reddi et al., 2003). Samples were removed from triplicate wells and assayed in quadruplicate.

**Western Analysis of p38 MAPK Signaling Proteins.** Western analysis was used to assess whether there was an increase in the protein for phosphorylated p38 MAPK postexposure to *B. cenocepacia* in IMR-90 cells. Confluent cells were cultured in six-well plates and exposed to *B. cenocepacia* (MOI 50:1) in the absence and presence of 10 μM of the specific p38 MAPK inhibitor 4-(4-fluorophenyl)-2(4-methylsulfonylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580; Calbiochem, La Jolla, CA) for 10 min. The cells were then lysed with radioimmunoprecipitation assay lysis buffer (60 μM phenylmethylsulfonyl fluoride, 30 U/ml aprotinin, and 1 mM sodium orthovanadate; Sigma-Aldrich). Before loading onto 10% SDS polyacrylamide gels (Invitrogen), the samples were mixed with sample buffer and then denatured by boiling. Proteins were transferred onto Hybond-ECL (enhanced chemiluminescence) nitrocellulose paper (Millipore, Bedford, MA) in blotting buffer (20 mM Tris base, 192 mM glycine, 20% methanol). Membranes were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline (10 mM Tris base, 150 mM NaCl). For probing for phosphorylated p38 MAPK, the membranes were then incubated at 4°C with a polyclonal phosphorylated p38 antibody (1:1000 dilution as per manufacturer’s instructions; Cell Signaling Technology Inc., Beverly, MA). Bound antibodies were detected with a rabbit secondary horseradish peroxidase antibody (1:2000 dilution; Cell Signaling Technology Inc.). Antibody-labeled proteins were detected by enhanced chemiluminescence as described by the manufacturer (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK). To check protein loading, blots were subsequently stripped (as described by Amersham Biosciences UK, Ltd.) and probed for p38 MAPK using a polyclonal p38 MAPK antibody (1:1000 dilution as per manufacturer’s instructions; Cell Signaling Technology Inc.).

**Data Analysis.** Specific binding was processed using Origin (OriginLab Corp., Northampton, MA). Data are reported as the mean ± S.E. and were compared using Student’s *t* test. *p* values <0.05 were considered to be significant.

**Results**

**Modulation of B₁ Receptor Expression by Infection.** Human pulmonary fibroblasts have been demonstrated to express high levels of constitutive B₂ receptors and relatively low or undetectable levels of B₁ receptors under basal conditions (Schanstra et al., 1998; Haddad et al., 2000; Phagoo et al., 2000). To investigate the modulation of kinin B₁ and B₂ receptors, the experiments in this study were performed on human pulmonary fibroblasts incubated with pathogens or IL-1β in the absence of serum. Exposure of IMR-90 human lung fibroblasts to the clinical isolate *B. cenocepacia* (MOI 250:1) for 6 h significantly increased by 3- to 4-fold the gene expression of B₁ receptors at the protein level measured using receptor binding with the specific B₁ radioligand [³H]desArg⁵⁰KD (Fig. 1A). Incubation with an equivalent volume of nutrient broth vehicle had no significant effect on B₁ receptor expression. The proinflammatory cytokine IL-1β has previously been shown to be highly inducive for kinin B₁ receptors (Leeb-Lundberg et al., 2005). Interestingly, in contrast to the B₁ response to infection, incubation of IMR-90

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**Fig. 1.** The effect of *B. cenocepacia* infection and IL-1β on the expression of kinin B₁ and B₂ receptors. IMR-90 cells were treated with DMEM (Ctrl), 250:1 MOI of *B. cenocepacia* (B. cen.), equivalent volume of nutrient broth (NB), or 500 pg/ml IL-1β for 6 h at 37°C before being assayed. A: cells were assayed for specific B₁ binding measured using [³H]desArg⁵⁰KD at 4°C as described under Materials and Methods. The data shown are from at least five experiments. The results are presented as percentage of control where 100% refers to the response to DMEM (Ctrl) treatment alone. Comparison to Ctrl: *p* < 0.001. B, specific B₂ binding was measured using [³H]B₂K. The data shown are from at least five experiments. The results are presented as percentage of control where 100% refers to the response to DMEM (Ctrl) treatment alone. C, saturation binding analysis of [³H]desArg⁵⁰KD to IMR-90 cells treated with DMEM (Ctrl) or infected with *B. cenocepacia* at 250:1 MOI for 6 h at 37°C before being assayed. The data shown represent the means from four independent experiments.
cells with a peak concentration of IL-1β (500 pg/ml) for 6 h produced a 5-fold increase in B1 receptor expression. Parallel experiments showed that specific B2 receptor protein measured using the B2 receptor agonist [3H]BK indicated no significant modulation at the cell surface in IMR-90 human fibroblasts after exposure to either B. cenocepacia or IL-1β for up to 6 h (Fig. 1B).

Figure 1C shows the saturation-binding isotherm of [3H]desArg^{10}KD to cell surface B1 receptors in IMR-90 cells after incubation in DMEM for 6 h. Under these conditions, [3H]desArg^{10}KD identified a relatively small number of B1 receptors (B_{max} = 23 ± 2 fmol/mg protein) with high affinity (K_D = 0.24 ± 0.03 nM; Table 1). Exposure of the cells to B. cenocepacia at 250:1 MOI for 6 h resulted in a 3- to 4-fold increase in the number of B1 receptors available for [3H]desArg^{10}KD binding (B_{max} = 87 ± 15 fmol/mg protein) without any significant effect on the affinity of [3H]desArg^{10}KD (K_D = 0.37 ± 0.04 nM; Fig. 1C; Table 1). These results suggest that infection of IMR-90 cells with B. cenocepacia results in an increase in specific B1 receptor binding sites without changing the receptor binding affinity.

B1 Receptor Induction by Clinical Isolates versus Nonclinical Phytopathogens of the B. cepacia Complex. B. cenocepacia, a clinically isolated type strain of genomovar III of the Bcc, has been recognized as a highly invasive and transmissible pathogen compared with other strains of the Bcc and can cause terminal lung destruction in patients with CF, chronic granulomatous disease, and underlying lung disorders. Consistent with the effect in IMR-90 fibroblasts, similar levels of B1 receptor up-regulation were obtained in other human pulmonary fibroblast cells including HEL 299 and WI-38 following a 6-h infection with B. cenocepacia (Fig. 2). This effect was MOI-dependent, with a maximal response for HEL 299 fibroblasts at 250:1 MOI and WI-38 cells at 50:1 MOI.

A lower, although significant, B1 response was obtained in IMR-90 cells after infection with the clinical isolate B. multivorans, obtained from a patient with CF (Fig. 3A). This was only apparent at a high infection ratio. Interestingly, compared with the clinical isolates, B1 receptor expression was not changed significantly by infection with the Bcc phytopathogen B. cepacia at densities of up to 250:1 MOI (Fig. 3A).

To demonstrate the difference in the ability of the clinical isolate B. cenocepacia and phytopathogen to generate an inflammatory cellular response, we compared the IL-8 production induced by the bacteria in IMR-90 cells infected for 6 h. In contrast to the phytopathogen B. cepacia, infection with B. cenocepacia produced up to 5-fold higher levels of IL-8 demonstrating the difference in the ability of the clinical and environmental organisms to illicit a proinflammatory response (Fig. 3B). A similar difference between these organisms was observed in their interleukin-6 stimulatory potential (data not shown).

**Materials and Methods**

**Fig. 2.** Comparison of the B1 up-regulation response to B. cenocepacia in human pulmonary fibroblasts. Confuent HEL 299 or WI-38 human pulmonary fibroblasts were treated with DMEM (Ctrl) or B. cenocepacia (B. cen.) at 50:1 and 250:1 MOI for 6 h at 37°C before being assayed for specific B1 binding measured using [3H]desArg^{10}KD as described under Materials and Methods. The data shown are from at least three experiments. The results are presented as percentage of control where 100% refers to the response to DMEM (Ctrl) treatment alone. Comparison of B. cenocepacia treatment with Ctrl for each cell type: ***, p < 0.01, **, p < 0.05, *, p < 0.01.

**Fig. 3.** Clinical isolates of the B. cepacia complex promote B1 receptor expression more potently than the environmental phytopathogen B. cepacia. IMR-90 cells were treated with DMEM (Ctrl), the clinical isolate B. cenocepacia (B. cen.) or B. multivorans (B. mul.) and the nonclinical phytopathogen B. cepacia (B. cep.) at the bacterial densities shown, or 500 pg/ml IL-1β for 6 h at 37°C. A, cells were assayed for specific B1 binding measured using [3H]desArg^{10}KD at 4°C as described under Materials and Methods. The data shown are from at least four experiments. The results are presented as percentage of control where 100% refers to the response to DMEM (Ctrl) treatment alone. CF, clinical isolate from patient with CF; ENV, environmental phytopathogen. Comparison to Ctrl: +, p < 0.05, ***, p < 0.001. B, supernatants from cells treated with B. cenocepacia (B. cen.) and B. cepacia (B. cep.) at a MOI of 250:1 or IL-1β for 6 h were tested for the production of IL-8 as described under Materials and Methods. The data shown are from at least four experiments. Comparison to Ctrl: +, p < 0.05, ***, p < 0.001.

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>K_D (nM)</th>
<th>B_{max} (fmol/mg protein)</th>
<th>ΔB_{max} (% of basal)</th>
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<tbody>
<tr>
<td>Basal</td>
<td>0.24 ± 0.03</td>
<td>23 ± 2</td>
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<tr>
<td>+ B. cenocepacia</td>
<td>0.37 ± 0.04</td>
<td>87 ± 15</td>
<td>381 ± 72</td>
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**Notes:**

- **Values for binding constants were calculated from saturation curves of radioligated binding as shown in Fig. 1C. Values are averages of four independent experiments with each assay performed in duplicate, and values differed by <10%.

**Fig. 1.** A, cells were assayed for specific B1 binding measured using [3H]desArg^{10}KD at 4°C as described under Materials and Methods.
Infection involved an increase in B1 receptor mRNA, we assessed changes in mRNA using reverse transcriptase-PCR. IMR-90 cells were exposed to 50:1 MOI of B. cenocepacia for 1 h and then washed several times to remove any residual bacteria before being replaced with fresh media containing the antibiotics ceftazidime and gentamicin for a total time of 6 h. Following treatment with antibiotics, inoculation of the cell supernatants onto blood agar plates indicated no bacterial growth. Despite the removal of extracellular bacteria, the B1 receptor response obtained after an initial exposure period of 0.5 h was sufficient to produce approximately 60% of the response compared with the IMR-90 cells that had been continuously exposed to B. cenocepacia for the full 6 h (Fig. 5B). Overall, these results indicate that the clinical isolate B. cenocepacia can rapidly up-regulate the expression of B1 receptors and requires only very low densities of bacteria, and short infection times produce a sustained B1 receptor up-regulation response.

**Inhibition of p38 MAPK Activation Protects against Infection-Induced B1 Receptor Expression.** Various cellular stresses can activate several MAPK pathways, which act as effectors for inflammatory cellular responses. Three MAPK families have been identified: extracellular-regulating kinase (ERK) MAPK, c-Jun N-terminal kinase (JNK) MAPK, and p38 MAPK. Inhibitors of these cascades are effective in preventing the induction of proinflammatory genes. To investigate whether MAPK activation plays a contributory role in B. cenocepacia-stimulated B1 receptor up-regulation, selective inhibitors were used. Figure 6A shows the effect of inhibiting ERK, JNK, and p38 MAPK on B1 receptor expression induced by exposure of IMR-90 cells to B. cenocepacia for 6 h. The presence of the specific ERK MAPK inhibitor 2'-amino-3'-methoxyflavone (PD 98059, 30 μM; Calbiochem) or specific JNK inhibitor antra[1,9-cd]pyrazole-6 (2H)-one (SP600125, 10 μM; BIOMOL Research Laboratories, Plymouth Meeting, PA) produced a small suppressive effect on the B1 response to B. cenocepacia, although it was not significantly altered (Fig. 6A). However, pretreatment of IMR-90 cells with the specific p38 MAPK inhibitor SB 203580 (20 μM) dramatically attenuated the response to infection. The effect of SB 203580 was dose-responsive with inhibition occurring at a SB 203580 concentration of 2 μM (Fig. 6B).

Western blot analysis showed that there was a significant increase in phosphorylated p38 MAPK protein after exposing IMR-90 cells to B. cenocepacia for 10 min (Fig. 6C). This phosphorylation for p38 protein was inhibited in the presence of the p38 MAPK inhibitor SB 203580, confirming that p38
MAPK is an integral pathway during B. cenocepacia-induced B1 receptor up-regulation. There was little or no difference in the basal levels of phosphorylated p38 protein in cells exposed to media and SB 203580 alone (data not shown).

**Effect of the Anti-Inflammatory Glucocorticoid Dexamethasone on Infection-Induced B1 Receptor Up-Regulation.** Glucocorticoids are potent suppressors of inflammation and are widely used in the treatment of chronic lung diseases (Payne and Adcock, 2001). We used dexamethasone to investigate whether this anti-inflammatory drug could interfere with the induction of B1 receptors by B. cenocepacia infection. Pulmonary fibroblasts were exposed to various concentrations of dexamethasone (1–1000 nM; Sigma-Aldrich) for 1 h prior to infection with B. cenocepacia. Dexamethasone produced a dose-dependent decrease in infection-induced B1 receptor levels in IMR-90 cells, and a maximal inhibition of the B. cenocepacia-induced B1 response occurred at a concentration of 100 nM (Fig. 7).

**Discussion**

In this study, we have provided the first demonstration that infection by intact, serious opportunistic gram-negative pathogens potently modulates the expression of B1 receptor protein and B1 receptor mRNA. We have shown that the B. cenocepacia-induced B1 receptors required small numbers of bacteria at MOIs as low as 3:1, was rapid, and occurred through de novo protein synthesis. Furthermore, we have also demonstrated that the p38 MAPK signaling pathway is crucial in the B1 receptor up-regulation process by infection and that the induction of B1 receptor protein is sensitive to treatment with the anti-inflammatory glucocorticoid dexamethasone. Overall, the data from this study supports the notion that the induction of kinin B1 receptors is involved in the chronic inflammatory response to injury and thus may be an important and novel mechanism in the inflammatory response to bacterial infection and disease.

There is substantial evidence that the kininogen-kallikrein-kinin system is important in manifestations of inflammation and infection. Several factors, including tissue damage and infection, activates the generation of BK in tissues and plasma, and this occurs in infections with both gram-negative and gram-positive pathogens (Khan et al., 1993; Sakata et al., 1996; Mattsson et al., 2001). The formation of B1 agonist through degradation of B2 agonists is likely to occur and has been demonstrated in models of inflammation (Raymond et al., 1995) and following long-term incubation of BK on pulmonary fibroblasts (Koyama et al., 2000). Furthermore, as infection-induced BK production can cause increased vascular permeability, fluid accumulation in inflamed tissues may act to enhance dissemination and transport pathogens deeper into tissues, a mechanism for severe infection (Travis et al., 1995). As such, kinin receptors have been demonstrated to be involved in controlling the response to infection (Ridings et al., 1995; Fein et al., 1997; Heitsch, 2000) and therefore are important cellular targets for inflammation and infection therapies.

To study the effect of infection, we used human pulmonary fibroblasts as kinin receptors have been proposed to play an important role in inflammation of the airways (Calixto et al., 2004). Activation of B1 receptors in pulmonary fibroblasts has been demonstrated to stimulate cell proliferation (Goldstein and Wall, 1984) and the production of collagen (Ricupero et al., 2000). In addition to pulmonary fibroblasts being widely used to model human kinin receptors in inflammatory conditions (e.g., Schanstra et al., 1998; Haddad et al., 2000), these cells can produce cytokines, monocyte and neutrophil chemoattractant molecules in response to long-term exposure to BK. Because this effect was partially inhibited by B1 receptor antagonists, the B1 receptors may participate in the recruitment of cells in inflammation (Koyama et al., 2000).

Thus, during infection, resident pulmonary cells may poten-
tiate the response to inflammation through kinin B₁ receptor activation.

In the current study, we investigated the effect of infection with \textit{B. cepacia} on the modulation of kinin receptors. These pathogens are responsible for causing major life-threatening problems in immunocompetent and nosocomial patients (Govan et al., 1996; Mohr et al., 2001), and eradication of these organisms in patients is extremely problematic (Hutchison and Govan, 1999). The organisms have a particular predilection for causing serious lung inflammation in CF patients and being opportunistic, is easily transmitted in hospitalized persons where it has a major impact on morbidity and mortality (Govan et al., 1996; Hutchison and Govan, 1999; Jones et al., 2004). For example, \textit{B. cepacia} causes pneumonia among chronic granulatous disease patients and is a leading cause of death in these patients (Mohr et al., 2001). Furthermore, \textit{B. cepacia} has also been implicated in causing pneumonia among cancer patients and in human immunodeficiency virus patients with acute bronchiectasis (Mohr et al., 2001). Those patients who have underlying diseases appear to be particularly vulnerable to these pathogens (Huang et al., 2001).

Of the species comprising the Bcc, the \textit{B. cenocepacia} group of pathogens accounts for the majority of serious infections in CF patients. However, \textit{B. multivorans} has also been found to play a significant role in the inflammatory CF lung response (Jones et al., 2004). We found that B₁ receptor up-regulation was greater after exposure of IMR-90 fibroblasts to the clinical isolate \textit{B. cenocepacia} than to \textit{B. multivorans}, however, the phytopathogen \textit{B. cepacia} did not produce a significant B₁ induction. As suggested by the poor B₁ response, \textit{B. cepacia} was weakly active in producing IL-8 synthesis compared with the clinical isolate \textit{B. cenocepacia}. Currently it is unclear why \textit{B. cenocepacia} are isolated more frequently among CF patients. This may be due to these novospecies being more virulent than others. However, it is known that \textit{B. cenocepacia} causes lethal infection in CF patients, is highly transmissible, and is responsible for the fatal cepacia syndrome observed in some CF patients.

Interaction of the pathogen with the host cell may be an important requirement for the B₁ up-regulation response and may be initiated by the pathogen adhering to the host cell and therefore dependent on the infection time. Although continuous exposure with \textit{B. cenocepacia} for 6 h was found necessary to obtain maximal B₁ up-regulation to infection, our data indicates that an inoculation time of 0.5 h was sufficient to produce a sustained B₁ response. Because the accumulation of B₁ receptor mRNA was detected after only 1 h of infection and preceded the increase in B₁ receptor protein suggests that the stimulatory effect of \textit{B. cenocepacia} is likely to have occurred through a direct bacterial-host response mechanism. Furthermore, the B₁ response was completely abrogated by the protein translation inhibitor cycloheximide and transcriptional inhibitor actinomycin D. Taken together, these results indicate that a short period of infection with \textit{B. cenocepacia} can produce a prolonged B₁ response, and \textit{B. cenocepacia}-induced B₁ expression involves de novo B₁ receptor protein synthesis and occurs at the level of B₁ receptor gene expression.

Because the cellular mechanisms causing the B₁ up-regulation by infection has not been investigated, we examined the role of the MAPK pathways. Our data indicate that the p38 MAPK pathway is crucial in the signaling of \textit{B. cenocepacia}-induced B₁ receptors. However, as the ERK and JNK inhibitor treatments caused a small although insignificant suppression of the \textit{B. cenocepacia}-induced response, we cannot exclude the possibility that the JNK or ERK MAPK pathways may play a minor role. Overall, our observation agrees with the finding of other studies that show a pivotal role for the p38 MAPK pathway in B₁ receptor up-regulation in isolated rabbit tissues and in inflammatory hyperalgesia in rats (Larrivee et al., 1998; Ganju et al., 2001). The present data are also consistent with our recent report that the p38 MAPK pathway is activated primarily by \textit{B. cenocepacia} in the production of the chemoattractant IL-8 in lung epithelial cells (Reddi et al., 2003).

Glucocorticoids are inhibitors of transcription factors and have been used as anti-inflammatory drugs in CF lung disease (Kazachkov et al., 2001). Thus, we used dexamethasone to investigate whether this synthetic glucocorticosteroid could interfere with the induction of B₁ receptors by \textit{B. cenocepacia}. Our results demonstrated that dexamethasone treatment provided significant protection against infection-induced B₁ receptors. The inhibitory effect of dexamethasone may occur through several routes including post-transcriptional mechanisms (Haddad et al., 2000). Also, it is widely known that dexamethasone may affect the activation of nuclear factor-κB which is one of the transcriptional control elements for the B₁ receptor gene (Ni et al., 1998; Schanstra et al., 1998). However, it should be noted that the B₁ receptor gene also contains other potential regulatory elements such as activator protein-1 which may be required for full B₁ promoter activity (Yang et al., 2001). Further experiments are in progress to determine the transcriptional pathways used by the B₁ receptor for its up-regulation during bacterial infection.

Understanding how kinin receptors are influenced in response to infection will require considerable investigation. The \textit{B. cepacia} group of organisms have been reported to produce a host of either cleaved products, proteases, lipase, secreted products, and surface molecules that can act as virulence factors (Mohr et al., 2001). Furthermore, proteinaceous components of the organism including flagella and pili have been found to contribute to the inflammatory mechanism of these organisms (Urban et al., 2004). Thus, we are currently identifying the components on \textit{B. cepacia} that are responsible for this induction of B₁ receptors.

We conclude from this study that serious human opportunistic lung pathogens such as \textit{B. cenocepacia} can rapidly promote B₁ receptor up-regulation in human pulmonary fibroblasts. In contrast, B₂ receptor expression remains unaffected. The up-regulation of B₁ receptors during infection would further enhance the responsiveness of surrounding tissues to kinins promoting an increase in the production of inflammatory mediators or sensitizing other cells. This could act through constitutive B₁ receptor activity and enhancement of intracellular signaling mechanisms (Leeb-Lundberg et al., 2001). Furthermore, the B₁ receptor does not desensitize, internalize, or desensitize compared with the B₂ receptor (Leeb-Lundberg et al., 2005). Because the induction of B₁ receptors has been strongly linked to inflammatory pathologies and has been suggested to be important in the chronic state of the disease, kinin receptors may provide new strat-
egies for controlling infection and its associated inflammation.

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References


Address correspondence to: Dr. Stephen B. Phagoo, Developmental Biology Program, Saban Research Institute, Childrens Hospital Los Angeles, Department of Surgery, Keck School of Medicine, University of Southern California, 4650 Sunset Boulevard, MS #35, SABAN RM 507, Los Angeles, CA 90027. E-mail: spagoo@chla.usc.edu