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Title page

INFECTION-INDUCED KININ B_1 RECEPTORS IN HUMAN PULMONARY FIBROBLASTS: ROLE OF INTACT PATHOGENS AND P38 MITOGEN-ACTIVATED PROTEIN KINASE-DEPENDENT SIGNALING

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Running title page

Running title: Induction of kinin receptors by intact pathogens

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Abbreviations: Bcc, *Burkholderia cepacia* complex; BK, bradykinin; B_{max}, maximum number of binding sites; CGD, chronic granulatomous disease; CF, cystic fibrosis; DMEM, Dulbecco's modified Eagle's medium; EC_{50}, concentration required for half-maximal response; ERK, extracellular signal-regulated kinase; GPCR, G-protein coupled receptor; JNK, c-Jun N-terminal kinase; K_D, equilibrium dissociation constant; KD, kallidin; IL-1β, interleukin-1β; IL-8, interleukin-8; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; NF-κB, nuclear factor kappa-B; PD 98059, 2'-amino-3'methoxyflavone; RT-PCR, reverse transcriptase polymerase chain reaction; SB 203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; SP600125, anthra[1,9-*cd*]pyrazole-6 (2*H*)-one.

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ABSTRACT

Kinin B_1 receptors (B_1R) are involved in many pathophysiological processes, and its expression is upregulated in inflammatory pulmonary disease. Although bacteria can generate kinin peptides, the molecular signaling mechanisms regulating B_1R during infection by intact pathogens is unknown. The serious opportunistic 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 immuno-compromised patients and those with cystic fibrosis. We demonstrate here that *B. cen.* infection induced a rapid increase in B_1R mRNA (1 h) proceeded by an increase in B_1R protein expression (2 h), without affecting B_2 receptor expression in human pulmonary fibroblasts. The B_1R response was dose-dependent and maximal by 6-8 h (3- to 4-fold increase), however brief *B. cen.* infection could sustain B_1R upregulation. In contrast, non-clinical Bcc phytopathogens were much less B_1R inducive. The protein synthesis inhibitor cycloheximide and transcriptional inhibitor actinomycin D abrogated the B_1 response to *B. cen.* indicating *de novo* B_1R 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)1*H*-imidazole (SB 203580) dramatically reduced *B. cen.*-induced B_1R. Furthermore, *B. cen.* regulation of B_1R 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 B_1R. Thus, providing evidence that B_1R regulation may be an important and novel mechanism in the inflammatory cascade in response to chronic pulmonary infection and disease.

INTRODUCTION

Inflammatory processes and infection can cause the activation of the kininogen-kallikrein cascade generating the pro-inflammatory 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 et al., 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 B_2 and BK B_1 (Leeb-Lundberg et al., 2005). The B_2 receptor subtype mediates the action of BK and kallidin (KD), whereas the B_1 receptor subtype mediates the action of metabolites of the B_2 receptor ligands, desArg^{9}BK and desArg^{10}KD, respectively. Both receptor subtypes are members of the

super family of seven transmembrane domain, G-protein coupled receptors (GPCR; Leeb-Lundberg et al., 2005). Under non-pathological conditions, B_2 receptors are expressed widely. With only a few exceptions, B_1 receptors are not expressed in significant levels in normal tissues. Instead, kinin B_1 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 B_1 upregulation in pulmonary tissues. Furthermore, upregulated B_1 receptor protein expression was observed in biopsies from patients with fibrotic lung tissue formation (Nadar et al, 1996). Thus, B_1 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 genomovars 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; Hutchison 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. cenocepacia* (genomovar 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. cenocepacia* rapidly upregulates B_1 receptor expression; occurs through *de novo* protein synthesis and signals through activation of the p38 MAPK pathway. This provides evidence that the induction of B_1 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; Gibco, NY) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), 25mM HEPES, 4 mM L-glutamine, and 1% non-essential amino acids (Gibco). The cells were maintained in a humidified atmosphere in 5% CO_2 at 37°C and were subcultured by incubating with 0.05% trypsin-0.5 mM ethylenediaminetetraacetate (Gibco) at a ratio of 1:2-1:3, weekly. For all experiments, cells were plated in 6-well plates and grown to confluency. Prior to experimentation, the cells were washed once with growth medium excluding fetal bovine serum before being incubated in the absence and presence of pathogens or IL-1β (R&D Systems, MN).

Bacterial strains and culture conditions. All Bcc isolates were obtained from the Belgian Co-ordinated Collections of Micro-organisms, Belgium. *B. cenocepacia* is the predominant Bcc respiratory pathogen in CF patients, and strain J2315 (hence referred to as "*B. cenocepacia*", strain LMG 16656, genomovar 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 *B. cenocepacia*, we used the CF clinical isolate *B. multivorans* (hence referred to as "*B. multivorans*", LMG 13010, genomovar II), and the phytopathogen *B. cepacia* (hence referred to as "*B. cepacia*", strain LMG 1222,

genomovar 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/ml assessed by measuring optical density at 600 nm. In experiments to kill extracellular bacteria, ceftazidime (1 mg/ml; GlaxoWellcome) and gentamicin (0.5 mg/ml; Sigma-Aldrich) were added to the culture media. To verify that the organisms were non-viable following the antibiotic treatment, samples of the culture media were incubated for 48 h on blood agar plates and visually inspected.

Measurement of kinin B_1 and B_2 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 [^{3}H]desArg^{10}KD (77-105 Ci/mmol, NEN DuPont, MA) or [^{3}H]BK (90 Ci/mmol, NEN DuPont, MA) 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-phenanthroline, 1 μM teprotide and 1 g/l bovine serum albumin (Sigma-Aldrich)). For [^{3}H]desArg^{10}KD saturation studies, various concentrations of radioligand were used (0.1-2.0 nM). Non-specific binding was defined as the amount of radiolabelled ligand bound in the presence of 5 μM non-radioactive ligand. After incubation, the assay buffer was removed and the cells were washed with 2 x 4 ml of ice-cold PBS. The cells were then lysed with 0.05% sodium dodecyl sulfate. Specific binding was expressed in fmol per mg of protein, and protein concentrations were determined using a BioRad kit (BioRad Laboratories, CA). All assay plates were carried out in duplicate and the variation between wells was ≤8%.

mRNA Analyses - Fibroblasts were grown to confluence in 6-well dishes and incubated in DMEM in the presence of *B. cenocepacia* at a multiplicity of infection (bacteria to cell ratio, MOI) of 50:1 for up to 6 h. Total RNA was extracted from cells using TRIZOL reagent as described by the manufacturer (Gibco). Single-stranded cDNA was generated using Superscript II reverse transcriptase (100 U; Gibco) in a 20 μl reaction mixture containing reaction buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol), 0.5 mM dNTP, 0.5 μg $oligo(dT)_{12-18}$ (Gibco), 10 U rRNasin (Promega Madison, WI), and 2 μg 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 B_1 receptor and β-Actin and the human B_1 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/HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$), 0.2 mM dNTP, 2.5 U Taq polymerase (Gibco) and 1 μl cDNA. Each primer was added at a final concentration of 0.2 μM. PCR was for 30-35 cycles, each cycle consisting of 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 ELISA - The confluent cell monolayer was infected with *B. cenocepacia* or *B. cepacia* at an MOI of 250:1 for 6 h. Controls consisted of nutrient broth as a negative control and interleukin-1β (IL-1β, 500 pg/ml) as a positive control. The cell media was then removed, centrifuged at 13,000 g for 15 min to remove bacteria

and cell debris and then stored at -80°C until assayed for IL-8 by ELISA as described previously (Reddi et al, 2003). Samples were removed from triplicate wells, and assayed in quadruplicates.

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 post-exposure to *B. cenocepacia* in IMR-90 cells. Confluent cells were cultured in 6-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-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB 203580; Calbiochem, La Jolla, CA) for 10 min. The cells were then lysed with RIPA lysis buffer (60 μM PMSF, 30 U/mL aprotinin, 1 mM sodium orthovanadate; Sigma-Aldrich). Before loading onto 10% SDS polyacrylamide gels (Invitrogen, Carlsbad, CA), the samples were mixed with sample buffer and then denatured by boiling. Proteins were transferred onto Hybond-ECL nitrocellulose paper (Millipore, Bedford, MA) in blotting buffer (20 mM Tris-base, 192 mM glycine, 20% methanol). Membranes were blocked with 5% (wt/vol) non-fat 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^{o}C with a polyclonal phosphorylated p38 antibody (1:1000 dilution as per manufacturers instructions, Cell Signaling, Beverly, MA). Bound antibodies were detected with a rabbit secondary horseradish peroxidase antibody (1:2000 dilution; Cell Signaling). Antibody-labeled proteins were detected by Enhanced Chemiluminescence as described by the manufacturer (Amersham Pharmacia Biotech, Buckinghamshire, UK). To check protein loading, blots were subsequently stripped (as

described by Amersham) and probed for p38 MAPK using a polyclonal p38 MAPK antibody (1:1000 dilution as per manufacturers instructions; Cell Signaling).

Data Analysis - Specific binding was processed using ORIGIN (Microcal Software Inc., USA). 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.

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RESULTS

Modulation of B_1 Receptor Expression by Infection:

Human pulmonary fibroblasts have been demonstrated to express high levels of constitutive B_2 receptors and relatively low or undetectable levels of B_1 receptors under basal conditions (Schanstra et al, 1998; Haddad et al, 2000; Phagoo et al., 2000). To investigate the modulation of kinin B_1 and B_2 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 B_1 receptors at the protein level measured using receptor binding with the specific B_1 radioligand [^{3}H]desArg^{10}KD (Fig. 1A). Incubation with an equivalent volume of nutrient broth vehicle had no significant effect on B_1 receptor expression. The pro-inflammatory cytokine IL-1β has previously been shown to be highly inducive for kinin B_1 receptors (Leeb-Lundberg et al, 2005). Interestingly, in contrast to the B_1 response to infection, incubation of IMR-90 cells with a peak concentration of IL-1β (500 pg/ml) for 6 h produced a 5-fold increase in B_1 receptor expression.

Parallel experiments showed that specific B_2 receptor protein measured using the B_2 receptor agonist [^{3}H]BK indicated no significant modulation at the cell surface in IMR-90 human fibroblasts after exposure to either *B. cenocepacia* nor IL-1β for up to 6 h (Fig. 1B).

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Figure 1C shows the saturation-binding isotherm of [^{3}H]desArg^{10}KD to cell surface B_1 receptors in IMR-90 cells after incubation in DMEM for 6 h. Under these conditions, [^{3}H]desArg^{10}KD identified a relatively small number of B_1 receptors (B_{max} = 23$\pm$2 fmol/mg protein) with high affinity (K_D = 0.24$\pm$0.03 nM; Table 1). Exposure of the cells to *B. cenocepacia* at 250:1 MOI for 6 h resulted in a 3- to 4-fold increase in the number of B_1 receptors available for [^{3}H]desArg^{10}KD binding (B_{max}= 87$\pm$15 fmol/mg protein) without any significant effect on the affinity of [^{3}H]desArg^{10}KD (K_D = 0.37$\pm$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 B_1 receptor binding sites without changing the receptor binding affinity.

B_1 Receptor Induction by Clinical Isolates Versus 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 to other strains of the Bcc and can cause terminal lung destruction in patients with CF, chronic granulatomous disease (CGD) and underlying lung disorders. Consistent with the effect in IMR-90 fibroblasts, similar levels of B_1 receptor upregulation 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 B_1 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, in comparison to the clinical isolates, B_1 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 interleukin-8 (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 pro-inflammatory response (Fig. 3B). A similar difference between these organisms was observed in their interleukin-6 stimulatory potential (data not shown).

Brief Exposure to Lung Pathogens Produces a Sustained B_1 Receptor Upregulation Response and Increase in B_1 Receptor mRNA:

Infection with *B. cenocepacia* caused a time-dependent increase in B_1 receptor expression in IMR-90 cells. The increase in B_1 receptors was apparent at 2 h with a maximal effect at 6 to 8 h (Fig 4A). The B_1 response to infection was dependent upon bacterial density, with a significant increase obtained at 3:1 MOI and an EC_{50} value for *B. cenocepacia* of 15:1 MOI (Fig. 4B). Bacterial concentrations of 50:1-100:1 MOI produced a maximal effect on B_1 expression over 6 h. Hence, in future experiments, we used an MOI of 50:1 that was at an optimal dose of *B. cenocepacia* over a 6 h period.

To determine whether the upregulation of B_1 receptors by infection involved an increase in B_1 receptor mRNA, we assessed changes in mRNA using RT-PCR. IMR-90

cells were exposed to 50:1 MOI of *B. cenocepacia* for time points up to 8 h before analysis of expression for B_1 receptor mRNA. Under basal conditions, IMR-90 cells expressed little B_1 mRNA as shown in Fig. 4C. The PCR product encoding for the human B_1 receptor mRNA increased 1 h after exposure to *B. cenocepacia* and continued to increase up to 6 h.

To investigate whether the infection-promoted cell surface B_1 receptor upregulation occurs at the level of gene expression, we examined whether protein synthesis was required for the upregulation. IMR-90 cells were therefore pretreated for 1 h with cycloheximide (10 μg/ml), a protein synthesis inhibitor, before infection. Cycloheximide treatment completely prevented the increase in [^{3}H]desArg^{10}KD binding sites induced by *B. cenocepacia* infection (Fig. 5A). Pre-incubating the cells with the transcription inhibitor actinomycin D (5 μg/ml) diminished the B_1 receptor response down to basal levels (Fig. 5A). These results show that infection-promoted upregulation of B_1 receptor expression involves *de novo* receptor synthesis, occurs at the level of gene transcription, and is preceded by an increase in B_1 receptor mRNA.

To determine whether the B_1 response was dependent upon the period of initial infection or bacterial contact, IMR-90 cells were exposed to *B. cenocepacia* for 0.5 h or 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 B_1 response obtained after an initial exposure period of 0.5 h was sufficient to produce approximately 60% of the response compared to 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 upregulate the expression of B_1 receptors and requires only very low densities of bacteria, and short infection times produce a sustained B_1 receptor upregulation response.

Inhibition of p38 MAPK Activation Protects Against Infection-Induced B_1 Receptor expression:

Various cellular stresses can activate several mitogen-activated protein kinase (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 pro-inflammatory genes. To investigate whether MAPK activation plays a contributory role in *B. cenocepacia*-stimulated B_1 receptor upregulation, selective inhibitors were used. Figure 6A shows the effect of inhibiting ERK, JNK and p38 MAPK on B_1 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, La Jolla, CA) or specific JNK inhibitor anthra[1,9-*cd*]pyrazole-6 (2*H*)-one (SP600125, 10 μM; Biomol, Plymouth Meeting, PA) produced a small suppressive effect on the B_1 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 the p38 MAPK pathway is an integral pathway during *B. cenocepacia*-induced B_1 receptor upregulation. 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 B_1 Receptor Upregulation:

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 B_1 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 B_1 receptor levels in IMR-90 cells and a concentration as low as 10 nM was able to significantly inhibit the B_1 response in cells exposed to *B. cenocepacia* (Fig. 7). Maximal inhibition of the *B. cenocepacia* induced B_1 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 B_1 receptor protein and B_1 receptor mRNA. We have shown that the *B. cenocepacia*-induced B_1 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 B_1 receptor upregulation process by infection, and that the induction of B_1 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 B_1 receptors are 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 B_1 agonist through degradation of B_2 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 et al., 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 B_1 receptors in pulmonary fibroblasts has been demonstrated to stimulate cell proliferation (Goldstein & Wall, 1994) 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 (eg. 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. As this effect was partially inhibited by B_1 receptor antagonists, suggests that B_1 receptors may participate in the recruitment of cells in inflammation (Koyama et al., 2000). Thus, during infection, resident pulmonary cells may potentiate the response to inflammation through kinin B_1 receptor activation.

In the current study, we investigated the effect of infection with *B. cepacia* on the modulation of kinin receptors. These pathogens are responsible for causing major life-threatening problems in immuno-competent 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, *B. cepacia* causes

pneumonia among CGD patients and is a leading cause of death in these patients (Mohr et al., 2001). Furthermore, *B. cepacia* has also been implicated in causing pneumonia among cancer patients and in HIV 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 *B. cenocepacia* group of pathogens accounts for the majority of serious infections in CF patients. However, *B. multivorans* has also been found to play a significant role in the inflammatory CF lung response (Jones et al., 2004). We found that B_1 receptor upregulation was greater after exposure of IMR-90 fibroblasts to the clinical isolate *B. cenocepacia* than to *B. multivorans*, however the phytopathogen *B. cepacia* did not produce a significant B1 induction. As suggested by the poor B_1 response, *B. cepacia* was weakly active in producing IL-8 synthesis compared to the clinical isolate *B. cenocepacia*. Currently it is unclear why *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 *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 B_1 upregulation response and may be initiated by the pathogen adhering to the host cell and therefore dependent on the infection time. Although continuous exposure with *B. cenocepacia* for 6 h was found necessary to obtain maximal B_1 upregulation to infection, our data indicates that an inoculation time of 0.5 h was sufficient to produce a sustained B_1 response. As the accumulation of B_1 receptor mRNA was detected after only

1 h of infection and preceded the increase in B_1 receptor protein suggests that the stimulatory effect of *B. cenocepacia* is likely to have occurred through a direct bacterial-host response mechanism. Furthermore, the B_1 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 *B. cenocepacia* can produce a prolonged B_1 response and *B. cenocepacia*-induced B_1 expression involves *de novo* B_1 receptor protein synthesis and occurs at the level of B_1 receptor gene expression.

As the cellular mechanisms causing the B_1 upregulation 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 *B. cenocepacia*-induced B_1 receptors. However, as the ERK and JNK inhibitor treatments caused a small although insignificant suppression of the *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 B_1 receptor upregulation in isolated rabbit tissues and in inflammatory hyperalgesia in rats (Larrivee et al., 1998; Ganju et al., 2001). The present data is also consistent with our recent report that the p38 MAPK pathway is activated primarily by *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 B_1 receptors by *B. cenocepacia*. Our results demonstrated that

dexamethasone treatment provided significant protection against infection-induced B_1 receptors. The inhibitory effect of dexamethasone may occur through several routes including through post-transcriptional mechanisms (Haddad et al., 2000). Also, it is widely known that dexamethasone may affect the activation of NF-κB which is one of the transcriptional control elements for the B_1 receptor gene (Ni et al., 1998; Schanstra et al., 1998). However, it should be noted that the B_1 receptor gene also contains other potential regulatory elements such as activator protein-1 which may be required for full B_1 promoter activity (Yang et al., 2001). Further experiments are in progress to determine the transcriptional pathways used by the B_1 receptor for its upregulation during bacterial infection.

Understanding how kinin receptors are influenced in response to infection will require considerable investigation. The *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 *B. cepacia* that are responsible for this induction of B_1 receptors.

We conclude from this study that serious human opportunistic lung pathogens such as *B. cenocepacia* can rapidly promote B_1 receptor upregulation in human pulmonary fibroblasts. In contrast, B_2 receptor expression remains unaffected. The upregulation of B_1 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 B_1 receptor activity and enhancement of intracellular signaling mechanisms (Leeb-Lundberg et al., 2001). Furthermore, the B_1 receptor does not desensitize, internalize or resensitize compared to the B_2 receptor (Leeb-Lundberg et al., 2005). As the induction of B_1 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 strategies for controlling infection and its associated inflammation.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1 - The effect of *B. cenocepacia* infection and IL-1β on the expression of kinin B_1 and B_2 receptors. IMR-90 cells were treated with DMEM (Ctrl), 250:1 Multiplicity of Infection (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 B_1 binding measured using [^{3}H]desArg^{10}KD 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 B_2 binding was measured using [^{3}H]BK. 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 [^{3}H]desArg^{10}KD to IMR-90 cells treated with DMEM (Ctrl) or infected with *B. cenocepacia* (*B. cen.*) at 250:1 MOI for 6 h at 37°C before being assayed. The data shown represent the means from four independent experiments.

Figure 2 – Comparison of the B_1 upregulation response to *B. cenocepacia* in human pulmonary fibroblasts. Confluent 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 B_1 binding measured using [^{3}H]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*<0.001.

Figure 3 – Clinical isolates of the *B. cepacia* complex promote B_1 receptor expression more potently than the 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 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 B_1 binding measured using [^{3}H]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 an MOI of 250:1, or IL-1β for 6 h were tested for the production of IL-8 as described in the *Materials and Methods*. The data shown are from at least four experiments. Comparison to Ctrl: **p*<0.05, ****p*<0.001.

Figure 4 – The effect of *B. cenocepacia* infection density and exposure time on the expression of B_1 receptors and B_1 mRNA. **A**, IMR-90 cells were treated with DMEM (Ctrl), or *B. cenocepacia* (50:1 MOI) for various times at 37°C before being assayed for specific B_1 binding measured using [^{3}H]desArg^{10}KD 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*<0.05, ***p*<0.01, ****p*<0.001. **B**, cells were

treated with various MOI of *B. cenocepacia* for 6 h at 37°C before being assayed for binding. The data shown are from at least three experiments. The results are presented as percentage of maximum. Comparison to Ctrl: **p*<0.05, ***p*<0.01, ****p*<0.001. **C**, Cells were treated as described in A and then analyzed for B_1 receptor mRNA as described in *Materials and Methods.* β-Actin mRNA was used as a loading control. The results are a representative of two experiments.

Figure 5 – Effect of cycloheximide, actinomycin D and a short infection period on B_1 receptor upregulation in *B. cenocepacia* exposed fibroblasts. **A**, IMR-90 cells were pre-treated for 1 h with cycloheximide (70 μM) or actinomycin D (5 μg/ml) in the presence or absence of *B. cenocepacia* (*B. cen.*; 50:1 MOI) for 6 h before assaying for specific [^{3}H]desArg^{10}KD binding. The data shown are from three independent experiments. The results are presented as percentage of control. Comparison to *B. cenocepacia*: ****P*<0.001. **B**, IMR-90 cells were treated with 50:1 MOI *B. cenocepacia* (*B. cen.*) for the times indicated at 37°C before being assayed for specific B_1 binding. For the cells that had been infected for 0.5 or 1 h, they were vigorously washed, antibiotics added as described in the *Materials and Methods* to kill extracellular bacteria and incubation at 37°C continued for a total time of 6 h. The data shown are from at least three experiments. The results are presented as percentage of maximum response where 100% refers to the response to cells treated continuously with *B. cenocepacia* for 6 h.

Figure 6 - Effect of MAPK (ERK, JNK and p38) inhibitors on the expression of B_1 receptors induced by infection with *B. cenocepacia*. **A**, IMR-90 cells were infected for 6 h at 37°C with *B. cenocepacia* (*B. cen.*; 50:1 MOI) as indicated, or pretreated for 1 h

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with the specific ERK MAPK inhibitor PD 98059 (30 μM), the specific JNK MAPK inhibitor SP600125 (10 μM) or the specific p38 MAPK inhibitor SB 203580 (20 μM) prior to infection. Cells were then assayed for specific [^{3}H]desArg^{10}KD binding as described under *Materials and Methods*. The results are from at least four experiments and presented as percentage of control. Comparison to *B. cenocepacia* in the absence of inhibitor: ***$p < 0.001$. **B**, cells were pretreated for 1 h with SB 203580 at the concentrations indicated, stimulated with *B. cenocepacia* (*B. cen.*) for 6 h, and then assayed for B_1 binding. The result is the average of three experiments. Comparison to *B. cenocepacia* in the absence of inhibitor: ***$p < 0.001$. **C**, cells were exposed to *B. cenocepacia* (*B. cen.*) for 10 min, in the absence or presence of a 1 h pretreatment with SB 203580 (10 μM) before processing cells for Western blot analysis and probing with a specific polyclonal antibody for p-p38 as described in the *Materials and Methods*. Following stripping, the membrane was re-probed with a p38 antibody to verify protein loading.

Figure 7 - Effect of the anti-inflammatory glucocorticoid dexamethasone on B_1 receptor expression induced by infection with *B. cenocepacia*. IMR-90 cells were treated with DMEM (Ctrl), or *B. cenocepacia* (*B. cen.*; 50:1 MOI), or pretreated for 1 h with various concentrations of dexamethasone as indicated at 37^{o}C before being assayed for specific B_1 binding 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 *B. cenocepacia* in the absence of inhibitor: *$p<0.05$, **$p<0.01$.

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TABLE 1:

Binding constants of [^{3}H]desArg^{10}KD to IMR-90 cells before and after infection with *B. cenocepacia*. Values for binding constants were calculated from saturation curves of radioligand binding as shown in Fig. 1C. Values are averages of four independent experiments with each assay performed in duplicate, and values differed by <10%.

Treatment	K_D (nM)	B_{max} (fmol / mg protein)	ΔB_{max} (% of basal)
Basal	0.24±0.03	23±2	-
+ *B. cenocepacia*	0.37±0.04	87±15	381±72

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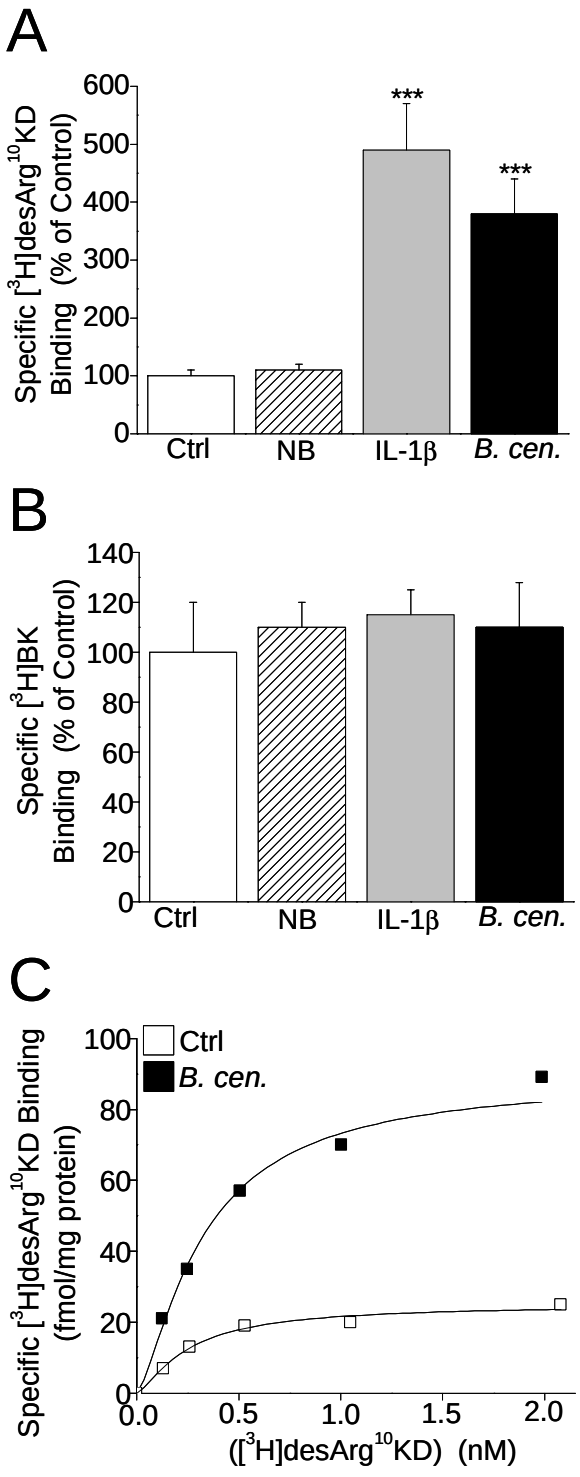

Figure 1

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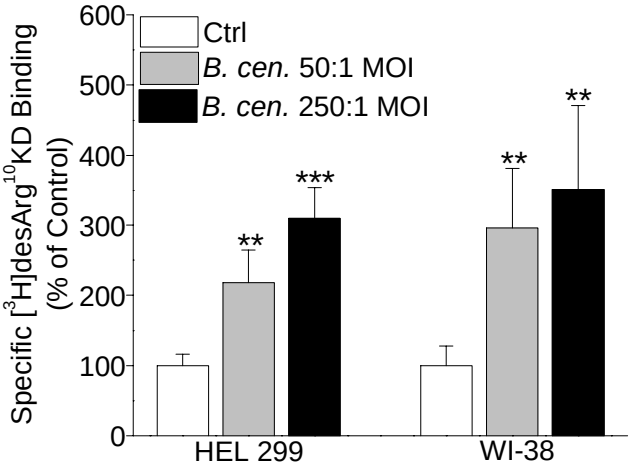

Figure 2

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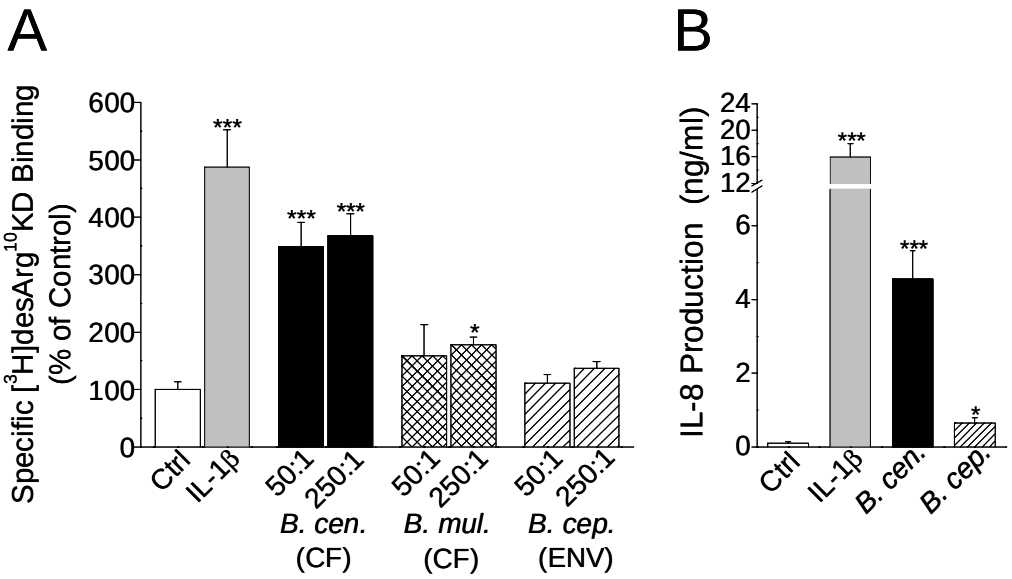

Figure 3

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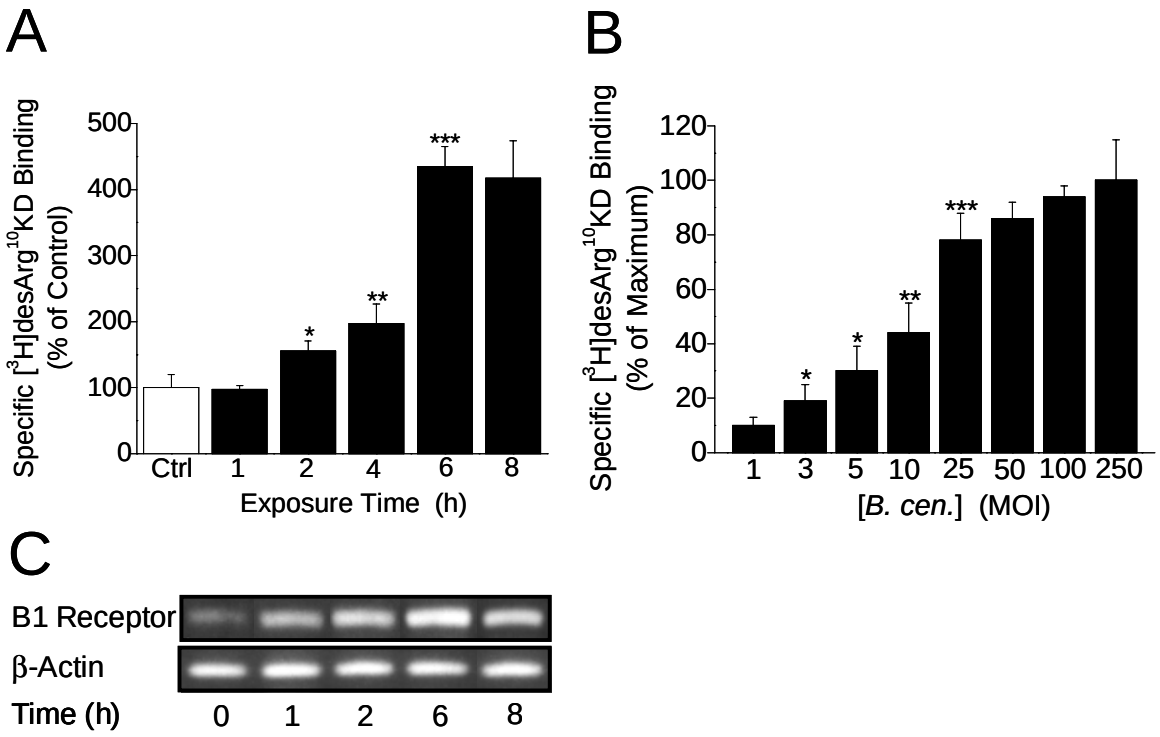

Figure 4

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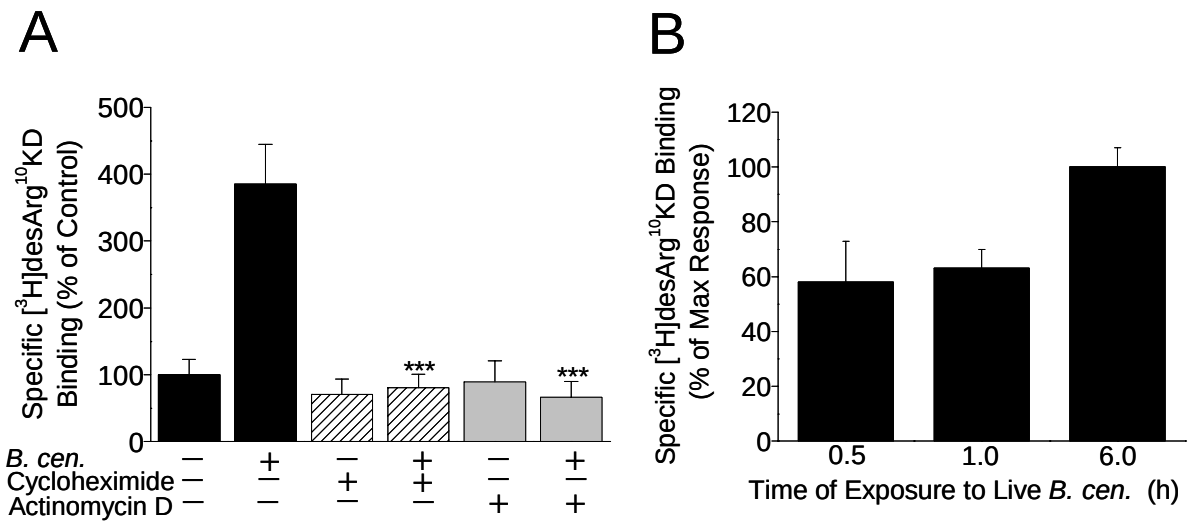

Figure 5

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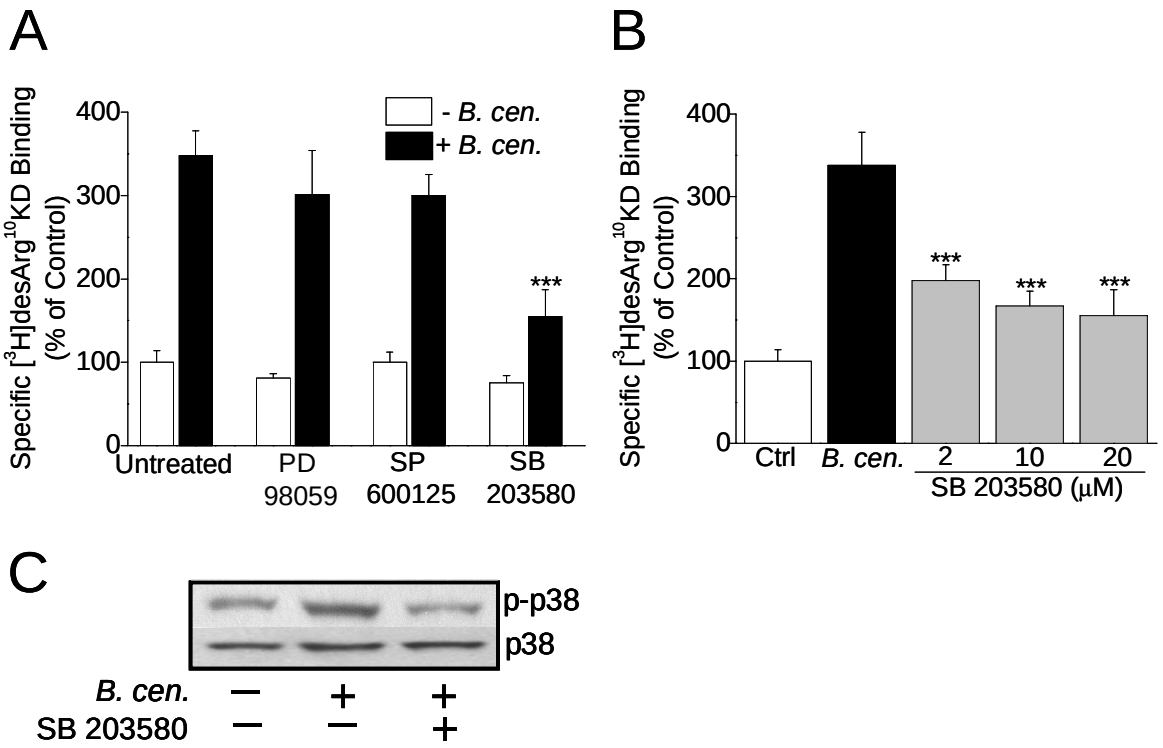

Figure 6

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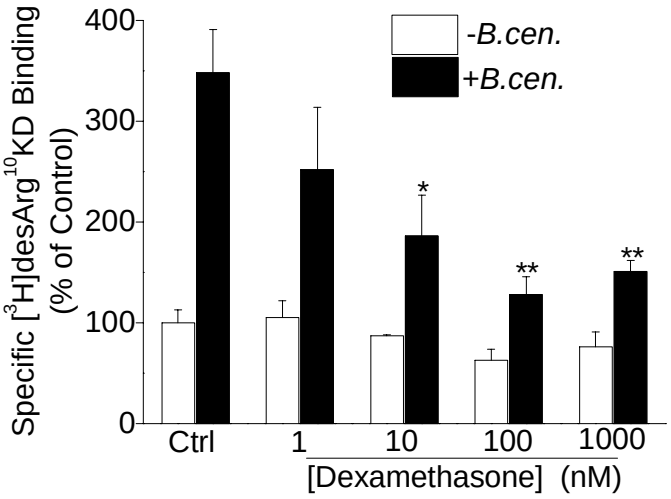

Figure 7