Transcriptional Regulation of Interleukin (IL)-8 by Bradykinin in Human Airway Smooth Muscle Cells Involves Prostanoid-dependent Activation of AP-1 and Nuclear Factor (NF)-IL-6 and Prostanoid-independent Activation of NF-κB*

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Bradykinin (BK) is a potent neutrophil chemotactic, pro-inflammatory mediator, and angiogenic factor, which acts through G protein-coupled receptors (GPCRs). Here we studied the mechanisms involved in IL-8 generation by BK in human airway smooth muscle cells focusing on the transcription factors involved and role of endogenous prostanoids in transcription factor activation. Transfection experiments with wild-type IL-8 promoter constructs or constructs with NF-κB, AP-1, and NF-IL-6 binding site mutations suggested that all three transcription factors were necessary for optimal IL-8 expression. BK increased NF-κB, AP-1, and NF-IL-6 binding to the IL-8 promoter by electrophoretic mobility shift assay. NF-κB, the most important transcription factor in the current study, was translocated to the nucleus after BK stimulation. Indomethacin, a cyclooxygenase inhibitor, partially inhibited IL-8 release and the promoter binding of AP-1 and NF-IL-6, but not NF-κB. Furthermore, exogenous prostaglandin E2 stimulated AP-1 and NF-IL-6 binding to the IL-8 promoter. The anti-inflammatory glucocorticoid dexamethasone inhibited NF-κB translocation and the promoter binding of NF-κB, AP-1, and NF-IL-6. These results are the first to delineate the transcription factors involved in BK induced IL-8 release. Transcriptional activation of the IL-8 promoter by BK involves the prostanoid-independent activation of NF-κB, and prostanoid-dependent activation of AP-1 and NF-IL-6 plays a key role in augmenting the response.

Endogenous prostanoid generation in response to GPCR ligands such as BK may be an important mechanism whereby GPCRs signal to the nucleus to maximize the transcription of inflammatory response genes.

Bradykinin (BK) is an important pro-inflammatory mediator in a number of inflammatory diseases such as asthma, allergic rhinitis, and rheumatoid arthritis (1, 2). BK is also a potent vasodilator that plays an important role in regulating blood pressure in a several vascular beds (3). While some of the effects of BK occur directly, many of its effects are mediated by complex autocrine or paracrine loops involving intermediate factors such as prostaglandin or nitric oxide (4–7). Most of the biological effects of BK are mediated by the B2 receptor, a receptor of the seven-transmembrane G protein-coupled receptor (GPCR) family, which are capable of generating a broad spectrum of physiological responses. Increasing evidence suggests that G protein coupled receptors actively regulate transcription and gene expression events. This provides an effective means for local production of cytokines and growth factors because of the wide distribution of GPCR in various tissues and organs. Furthermore, GPCR may also synergize with cytokine mediated gene transcription. Although nuclear signaling by cytokines has been extensively studied there is a paucity of information on how GPCRs such as the BK receptor send signals to the nucleus. Recent evidence suggests that BK may actively regulate transcription and gene expression events through activation of the transcription factor nuclear factor κB (NF-κB), but the downstream targets for BK-mediated NF-κB activation have not been extensively studied (8, 9). Whether other transcription factors are also activated by BK and which genes they activate is also poorly understood.

IL-8, first isolated from monocytes as a neutrophil chemotactant, is an 8.5-kDa C-X-C chemokine that is also chemotactic for T lymphocytes, monocytes, and eosinophils. IL-8 plays a major role in the initiation and maintenance of inflammatory responses (10). IL-8 also functions as an angiogenic factor and may contribute to angiogenesis, a prominent feature of the histopathology of several inflammatory diseases such as asthma, rheumatoid disease, and inflammatory bowel disease (11, 12). Structural mesenchymal derived cells have increasingly been shown to be an important source of chemokines such as IL-8. Our studies and others in recent years have advanced the paradigm that human airway smooth muscle (HASM) has important synthetic functions in asthma and is a rich source of cytokines, chemokines, inflammatory mediators, and growth factors, which contribute to the inflammatory process and airway remodeling (13–17).

Recent studies from our laboratory and others (18, 19, 20–22) have shown that HASM cells release IL-8 in response to cytokines such as tumor necrosis factor-α, IL-1β, and transforming growth factor-β. However, of greater interest was the finding that BK, a non-cytokine mediator, could also cause IL-8 release as this was the first report that BK could do so in any biological system (23). Subsequent studies have shown that BK
can also cause IL-8 release by epithelial and fibroblast cells (24). There are various motifs within the 5′-flanking region of the human IL-8 promoter with the potential to bind a number of important transcription factors in a cell and stimulus-dependent manner. Although other stimuli can regulate both transcriptional and post-transcriptional levels, there is little information on the transcriptional and post-transcriptional mechanisms used by BK or other GPCR ligands. Here we study the transcriptional mechanisms involved in IL-8 production in response to BK in HASM cells. We report that the transcription factors NF-κB, AP-1, and NF-IL-6 are all involved in IL-8 induction by BK. Activation of AP-1 and NF-κB was prostanoid-dependent, as it was mimicked by exogenous PGE₂ and inhibited by indomethacin, a cyclooxygenase (COX) inhibitor. In contrast activation of NF-κB occurred mainly via prostanoid-independent pathways. The glucocorticoid dexamethasone suppressed BK-induced IL-8 production by inhibiting binding of NF-κB, AP-1, and NF-IL-6 to the IL-8 promoter.

MATERIALS AND METHODS

Cell Culture—HASM cells were purchased from BioWhittaker. Cells at passages 4 to 6 were used for all experiments. The cells were cultured to confluence in 10% fetal calf serum (Harlan Sera-lab, Loughbor-ough, Leicestershire, UK)- Dulbecco’s modified Eagle’s medium (Sigma, Poole, Dorset, UK) in humidified 5% CO₂, 95% air at 37 °C and growth arrested in fetal calf serum-free Dulbecco’s modified Eagle’s medium for 24 h before the experiments. Immediately before each experiment, fresh fetal calf serum-free Dulbecco’s modified Eagle’s medium containing BK was added. The culture media were harvested at the indicated times and stored at −20 °C before analysis by IL-8 ELISA. The cells were collected for extraction of protein or mRNA.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—After the cells in the 6-well plates were treated with BK at a final concentration of 1 μM, cells were collected at time 0, 0.5, 2, 4, and 8, respectively. Total RNA was isolated by using the RNeasy mini kit (Qiagen, West Sussex, UK) following the manufacturer’s protocol. 1 μg of total RNA was reverse-transcribed in a total volume of 20 μl, including 200 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI), 25 units of RNase inhibitor (Promega), 0.5 μg of oligo(dT)₁₇ primer, a 0.5 mM concentration of each dNTP, 1× first-strand buffer provided by Promega. The reaction was incubated at 42 °C for 90 min.

Aliquots of the RT products were subsequently used for PCR amplification. 10 μl of RT products was brought to a volume of 50 μl containing 1 μM MgCl₂, 0.12 mM of each dNTP, 1 unit of Taq polymerase (Sigma), a 0.5 mM concentration of both the upstream and downstream PCR primers, 1× PCR buffer supplied by Sigma. Two pairs of primers were used for this study. The primer sequences were as follows: IL-8 sense, 5′-ATG ACT CCT AAC GCT GGC GTT CCT-3′; IL-8 antisense, 5′-TCT CAC CCC TCT TCA AAA ACT TCT C-3′; GAPDH sense, 5′-CCA ATT GGA AAT TTC AGT GCA-3′; GAPDH anti-sense, 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′. Amplification was carried out in Techne PTC-3 thermal cycler (Cambridge, Cam-bridgehire, UK) after an initial denaturation at 94 °C for 3 min. This was followed by 35 cycles of PCR using the following temperature and time profile: denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, primer extension at 72 °C for 1 min, and a final extension of 72 °C for 10 min. The PCR products were visualized by electrophoresis on 1.5% agarose gels in 0.5× TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.3) after staining with 0.5 μg/ml ethidium bromide. The ultraviolet (UV)-illuminated gels were photographed, and the densitometry was analyzed by GeneGenius gel documentation and analysis system (Syngene, Cambridge, Cam-bridgehire, UK) after an initial denaturation at 94 °C for 3 min. This was followed by 35 cycles of PCR using the following temperature and time profile: denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, primer extension at 72 °C for 1 min, and a final extension of 72 °C for 10 min. The PCR products were visualized by electrophoresis on 1.5% agarose gels in 0.5× TBE buffer (89 mM Tris borate, 2 mM EDTA, pH 8.3) after staining with 0.5 μg/ml ethidium bromide. The ultraviolet (UV)-illuminated gels were photographed, and the densitometry was analyzed by GeneGenius gel documentation and analysis system (Syngene, Cambridge, Cam-bridgehire, UK).

IL-8 Assay—IL-8 concentrations in the culture medium were determined by ELISA (ELISA, CLR, Amsterdam, Netherlands) according to the manufacturer’s instructions, and we have reported the method in detail elsewhere (23).

Vectors and Transfections—The vectors encoding either the wild-type IL-8 promoter (162 bp/44 luciferase reporter, or site mutations of one of the binding sites (AP-1, NF-IL-6, or NF-κB) in the IL-8 promoter region) were provided by D. R. Braiser (Department of Medicine, Sealy Center for Molecular Science, Galveston, TX). The constructs were described in detail in the publications from Braiser’s laboratory (25, 26). HASMC were co-transfected using LipofectAMINE 2000 (In-vitrogen) with one of these vectors and control vector pRL-TK, Pro-moter) for 4 h, then cultured in fresh serum for a further 16 h before the BK treatment for 60 min. The efficiency of transfection by LipofectAMINE 2000 was 22%. The cells were then collected and lysed. Firefly and Renilla luciferase activities were measured by luminometer using the dual-luciferase reporter assay system (Promega). The normalized luciferase activities were calculated by dividing firefly activities by Renilla activities.

Western Blotting—Nuclear and cytoplasmic extracts from stimulated (by BK) or untreated control cells were prepared by Nu-Clear extraction kit (Sigma) following the manufacturer’s protocol before Western blot analysis to measure NF-κB expression. Protein was extracted by incubating the cells for 5 min with 300 μl/well protein extraction buffer (0.9% NaCl, 20 mM Tris-HCl, pH 7.6, 0.1% Triton X-100, 1 mM phenyl-methylsulfonyl fluoride, and 0.01% leupeptin; all from Sigma) and shaking gently. The protein extraction buffer was then transferred and stored at −20 °C for subsequent Western blot analysis. The protein concentration of cell extracts was determined with the Bio-Rad protein assay reagent (Bio-Rad, Hemel Hempstead, UK). Sufficient aliquots of sample (30 μg of protein/track) were mixed 5:1 with 5× sample buffer and boiled for 5 min before electrophoresis. Electrophoresis was performed on these samples on a SDS-polyacrylamide gel (100 V, 1 h). The separated proteins were then electroblotted (100 V, 1 h) to pure nitrocellulose membranes (Gelman Sciences, Northampton, MA) and then blocked for 2 h at 4 °C in blocking reagent (5% fat-free dried milk powder in PBS, pH 7.4, with 0.3% Tween 20 (PBS-T), incubated with primary polyclonal anti-human IκB-α antibody (1:2,000) (Santa Cruz) or polyclonal anti-human NF-κB antibody (1:2,000) (Santa Cruz) for 2 h at room temperature before being washed with PBS-T and incubated with goat anti-rabbit antibody coupled with horseradish peroxidase (1:2,000) (Amersham Pharmacia Biotech, Piscataway, NJ) for 1 h at room temperature. Enhanced chemiluminescence detection was performed by washing the blot with PBS-T, incubating it with the SuperSignal CL-HRP substrate system (Pierce) for 1 min, and finally exposing it to Hyperfilm ECL (Amersham Biosciences, Little Chalfont, Bucks, UK).

Immunofluorescence—HASM cells were seeded and cultured on coverslips. Cells were then transfected by the HASM cells treated by BK in 4% paraformaldehyde in PBS for 30 min at room temperature, washed three times in PBS (10 min each), permeabilized with 0.2% Triton X-100 for 10 min, and then washed again three times in PBS. Following fixation and permeabilization, each coverslip was incubated with 500 μl of blocking solution (1.5% goat serum in PBS) for 1 h at room temperature in a box to block nonspecific binding. 500 μl of diluted rabbit anti-human NF-κB (1:200 dilution, Santa Cruz) antibody with blocking solution was added to each coverslip. Coverslips were incubated for 1 h at room temperature in a box and then were washed three times with PBS. Fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody was diluted 1:160 in blocking solution, and 500 μl of diluted antibody was incubated with each coverslip for 1 h at room temperature. Coverslips were washed three times with PBS. Mounting medium with VECTASHIELD mounting medium (Vector Laboratories, Inc., Burlingame, CA). Fluorescence was observed and photographed on an Olympus BX40 microscope.

Electrophoretic Mobility Shift Assay (EMSA)—The nuclear fractions for EMSA were prepared using Nu-Clear extraction kit (Sigma) following the manufacturer’s protocol. Protein concentrations of nuclear fractions were determined using the Bio-Rad protein assay. Oligonucleotides containing the NF-κB site (5′-TGG TAA TTC CTC TTC TGC-3′), the NF-κB site (5′-TGC TAA TTC CTC TTC TGC-3′) and the Nuclear Factor-κB (NF-κB) site (5′-TGG TAA TTC CTC TTC TGC-3′) were labeled using γ32P-ATP (Amersham Biosciences) and T4 polynucleotide kinase (Promega). Fifteen micrograms of nuclear fractions, 40,000 counts/min labeled double-stranded probe, and 2 μl of 5× binding buffer (20% glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM dithiothreitol, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25 mg/ml poly(dI-dC)poly(dI-dC)) were mixed in a total volume of 10 μl. In competition assays, 100× unlabeled competitors were added at the same time of probe addition. In supershift analyses, 2 μl (2 μg/ml) of antibodies such as anti-p65, anti-p50, anti-c-Fos, and anti-NF-IL-6 were added to the reaction. The mixture was incubated at room temperature for 30 min, then loaded on a 4% polyacrylamide gel in 0.5× TBE buffer, and subjected to electrophoresis for 1.5 h. The gel was dried and exposed for autoradiography on Kodak XAR film at −70 °C for 1–2 days.

Statistical Analysis—Results were expressed as the mean ± S.E. from n determinations. Student two-tailed t tests were used to determine the significant differences between the means. Values of p < 0.05 were accepted as statistically significant.
RESULTS

BK Causes Release of IL-8 Protein and an Increase in IL-8 mRNA—As previously reported by our group (23), HASM cells release IL-8 after BK stimulation. We chose a concentration of 10 μM of BK for our present experiments as our previous studies showed that this concentration causes maximal IL-8 release. In time course experiments, IL-8 was detected at a low level (25 pg/ml) as early as 4 h after BK and reached a peak (237 pg/ml) at 16 h (Fig. 1A). IL-8 mRNA was increased 5-fold 2 h after BK, 7-fold at 4 h, reduced to 2.5-fold after 6 h, and returned to basal levels after 8 h (Fig. 1B). To reveal whether the induction resulting from IL-8 gene transcription or post-transcriptional stabilization of IL-8 mRNA, we carried out transcriptional blockade studies. The RNA polymerase II inhibitor actinomycin D (10 μg/ml) was added to HASM cell culture 2 h after BK stimulation. The IL-8 mRNA level was analyzed by RT-PCR after 1, 2, 3, and 4 h of actinomycin D treatment and compared. After addition of actinomycin D for 2 h, only half of IL-8 mRNA (2.2-fold to basal level) was left (Fig. 1C). In contrast, IL-8 mRNA increased progressively to its peak level (7-fold) if no actinomycin D was added. In studies where no BK was added prior to actinomycin D treatment the half-life of IL-8 mRNA was similar (Fig. 1D), suggesting that BK is not acting by post-transcriptional modification of IL-8 transcripts.

Mutations in the NF-κB, AP-1, and NF-IL-6 Binding Sites in the IL-8 Promoter Reduce BK-stimulated Luciferase Activity—To determine whether BK was acting transcriptionally and to probe the transcription elements involved in IL-8 induction, vectors containing the wild-type IL-8 promoter or mutations of binding sites for either AP-1, NF-IL-6, or NF-κB in the IL-8 promoter were co-transfected into HASM cells with the
control vector pRL-TK. There was no significant difference in basal promoter activity. After BK stimulation, luciferase activity was increased 2.7-fold in cells transfected with the wild-type IL-8 promoter, suggesting that BK was acting transcriptionally. In contrast, luciferase activities were only increased to 2.1 (22% reduction compared with wt, \( p < 0.05 \)), 2.0 (26% reduction, \( p < 0.05 \)), and 1.4 (49% reduction, \( p < 0.05 \))-fold when the cells were transfected by vectors containing site mutations of AP-1, NF-IL-6, and NF-\( \kappa \)B, respectively (Fig. 2). This suggests that all three factors are necessary for maximum IL-8 induction by BK.

Nuclear Translocation of Transcription Factors—To detect NF-\( \kappa \)B nuclear translocation, cytoplasmic and nuclear fractions were isolated and separated by SDS-PAGE gel and the membrane was probed with anti-NF-\( \kappa \)B antibody (p65). We found that under resting condition NF-\( \kappa \)B was located in the cytoplasmic fraction, but was translocated to the nucleus after BK treatment for 30 min (Fig. 3A). In parallel IxB, an inhibitor of NF-\( \kappa \)B, was degraded in the cytoplasm as shown in Fig. 3A. NF-\( \kappa \)B translocation was also confirmed by immunofluorescence.
In contrast, AP-1 and NF-IL-6 were located in the nuclei under resting conditions (data not shown). This is consistent with other studies showing that NF-IL-6 and AP-1 can be activated by phosphorylation in the nucleus rather than translocation.

**BK Increases NF-κB, AP-1, and NF-IL-6 Binding to the IL-8 Promoter**—We next used EMSA to examine whether the transcription factors bind to the IL-8 promoter after BK stimulation. We found that NF-κB started to bind to the IL-8 promoter at 20 min after BK stimulation and reached a peak at 60 min (Fig. 4A). The binding was completely abolished by adding a 100-fold excess of cold NF-κB oligonucleotides to the reaction, but not by adding a 100-fold excess of cold nonspecific oligonucleotides (AP-1) (Fig. 4C). Supershift analyses were performed with nuclear extracts by adding 4 µg of specific antibodies against p65 and p50. Both antibodies effectively supershifted the protein-DNA complex (Fig. 4B). These results indicated that the binding was specific. AP-1 and NF-IL-6 also strongly bound to the IL-8 promoter at 60 min after BK stimulation (Fig. 4, D and E). Similarly, competition experiments with cold oligo-
gonucleotides and supershifts showed that the binding was specific.

Dexamethasone Inhibits IL-8 Induction by BK—Glucocorticoids are major regulators of inflammatory processes that act by regulating inflammatory gene transcription. We performed experiments to determine whether glucocorticoids would inhibit BK induced IL-8 release. We found that pretreatment with the glucocorticoid dexamethasone, at a concentration of 1 μM for 30 min before BK, completely abolished BK-induced IL-8 release (p < 0.001) (Fig. 5A). We then examined the mechanism of the inhibition by dexamethasone using EMSA. We found that dexamethasone prevented the binding of all three transcription factors (NF-κB, AP-1, and NF-IL-6) to the IL-8 promoter (Fig. 5B). We further detected that NF-κB was reduced significantly in the nucleus, and IκB-α was induced with dexamethasone pretreatment (Fig. 5C). Dexamethasone also abolished the increased luciferase activity induced by BK when HASM cells were transfected by wild-type IL-8 promoter (p < 0.01).

Exogenous PGE2 Causes Activation of the IL-8 Promoter—As we have previously shown that autocrine PGE2 release plays a role as an intermediary factor in BK-induced IL-8 generation, we first measured IL-8 by ELISA after HASM cells treated with exogenous PGE2. IL-8 was detected at significant levels.
FIG. 6. PGE₂-mediated IL-8 induction by BK. A, exogenous PGE₂ induced IL-8 release in a dose-dependent fashion. HASM cells were incubated with 1 and 10 μM PGE₂ for 16 h. Released IL-8 in the medium was measured by ELISA. Each point represents the mean ± S.E. of three determinations from three independent experiments. B, time course of PGE₂ on IL-8 mRNA induction. HASM cells was incubated with 10 μM PGE₂ for 0, 2, 4, and 6 h. IL-8 and GAPDH (as an internal control) mRNA was measured by RT-PCR. Data representative of three experiments are shown. C, the effect of AP-1, NF-IL-6, and NF-κB binding site mutations on the IL-8 promoter activity in response to PGE₂. These vectors were co-transfected with the control vector pRL-TK into HASM cells for 4 h, then followed by further 16-h culture and 1-h treatment with PGE₂ (10 μM) (solid bars) or without PGE₂ (open bars). Normalized luciferase activity was calculated by dividing firefly by Renilla. The graph showed the fold increase of luciferase activity in cells treated with PGE₂ by comparing with those without PGE₂ treatment. Each bar is the mean ± S.E. of four determinations from two independent experiments. *, p < 0.05 compared with the effect of BK on the wild-type IL-8 promoter. D, HASM cells were pretreated either with PGE₂ for 30 min before 60-min treatment with BK. The nuclear extracts were incubated with labeled NF-κB, AP-1, or NF-IL-6 probes to detect the effect of PGE₂ on protein-DNA binding. Data representative of three experiments are shown.
The HASM cells were pretreated with indomethacin at a concentration of 0.01, 0.1, 1.0, and 10 μM for 30 min before BK stimulation for 24 h. IL-8 was measured by ELISA. The cells were also pretreated with both PGE2 (10 μM) and indomethacin for 30 min before BK treatment for 24 h. IL-8 was measured by ELISA. HASM cells were pretreated either with indomethacin or combination of PGE2 and indomethacin for 30 min before 60-min treatment with BK. The nuclear extracts were incubated with labeled NF-κB, AP-1, or NF-IL-6 probes to detect the effect of these two chemicals on protein-DNA binding. The cytoplasmic and nuclear fractions were extracted and separated by SDS-PAGE and probed with anti-NF-κB antibody. D, indomethacin and dexamethasone markedly reduced the increased luciferase activity induced by BK when HASM cells were transfected by wild-type IL-8 promoter, but this was rescued by exogenous PGE2. **, p < 0.01 compared with control; ++, p < 0.01 compared with BK; #, p < 0.05 compared with dexamethasone and indomethacin.
(200 and 290 pg/ml) when the cells were treated by exogenous PGE$_2$ at concentrations of 1 and 10 mM (Fig. 6A). We also determined whether exogenous PGE$_2$ could increase in IL-8 transcription. RT-PCR showed IL-8 mRNA induction when HASM cells were stimulated by exogenous PGE$_2$. IL-8 mRNA was increased 6-fold 2 h after PGE$_2$ treatment (10 mM), 5-fold at 4 h, and reduced to 3-fold after 6 h (Fig. 6B). Furthermore, exogenous PGE$_2$ increased the activity of IL-8 promoter constructs when vectors containing the wild-type IL-8 promoter or mutations of each binding site were transfected into HASM cells followed by PGE$_2$ stimulation, luciferase activity was increased to 2.8-fold in cells transfected with the wild-type IL-8 promoter. In contrast, luciferase activities were only increased to 1.5 (46% reduction compared with wild-type, p < 0.05), 2.1 (25% reduction, p < 0.05), and 1.4 (50% reduction, p < 0.05)-fold when the cells were transfected by vectors containing site mutations of AP-1, NF-IL-6, and NF-κB, respectively (Fig. 6C). A protein–DNA binding study by EMSA showed that AP-1, NF-IL-6, and NF-κB also bound to IL-8 promoter after PGE$_2$ stimulation (Fig. 6D).

**Indomethacin Inhibits IL-8 Induction by BK**—To probe the role of exogenous PGE$_2$ on BK induced IL-8 transcription, we next studied the effect of indomethacin, a COX inhibitor, on IL-8 release and transcription factor binding to the IL-8 promoter in response to BK. Indomethacin partially inhibited the BK-induced IL-8 release at a concentration of 1 mM of indomethacin, IL-8 release was reduced to 46% (p < 0.01). At a higher concentration (10 mM), IL-8 release was reduced to 26% (p < 0.01). However, this reduction was rescued by adding exogenous PGE$_2$ (p < 0.01) (Fig. 7A). In EMSA studies, indomethacin (10 mM) interrupted the binding of AP-1 and NF-IL-6. However, the interruption was reversed after exogenous PGE$_2$ was added. Interestingly, indomethacin did not affect NF-κB binding (Fig. 7B). Indomethacin (10 mM) also markedly reduced the increased luciferase activity induced by BK when HASM cells were transfected by wild-type IL-8 promoter (p < 0.01), but the effect was rescued by exogenous PGE$_2$ (10 mM) (p < 0.05) (Fig. 7D). A similar result was obtained when the cells were treated by dexamethasone consistent with the effect of dexamethasone being partially due to inhibition of PGE$_2$ production. These results indicated that exogenous PGE$_2$ was acting in the main via AP-1 and NF-IL-6. We further detected that NF-κB was not reduced in the nucleus, and IκB-α was not induced with indomethacin pretreatment (Fig. 7C).

**DISCUSSION**

The major finding of this study was that BK induced IL-8 release transcriptionally through the combined activation of NF-κB, AP-1, and NF-IL-6 and that interaction between prostanoid-dependent and -independent processes played a key role in this process. Mutation studies of the IL-8 promoter showed that all three transcription factor binding sites were required for optimal promoter activity. Furthermore, EMSA studies showed that all three transcription factors bound to the IL-8 promoter after BK stimulation. Binding of AP-1 and NF-IL-6 to the IL-8 promoter by BK was inhibited by indomethacin and mimicked by PGE$_2$ suggests that it was prostanoid dependent. Indomethacin was not effective at inhibiting NF-κB translocation and did not influence NF-κB binding to the IL-8 promoter as assessed by EMSA, suggesting that NF-κB is activated mainly through prostanoid independent mechanisms in response to BK. Dexamethasone, the anti-inflammatory glucocorticoid, abolished both IL-8 release in response to BK and the binding of the three transcription factors to the IL-8 promoter. There are several novel aspects to these studies. They are the first to show that these transcriptional factors are involved in IL-8 release to bradykinin. We show for the first time that PGE$_2$ acts as an autocrine intermediary factor in activating AP-1 and NF-IL-6 in response to BK.

IL-8 protein levels accumulated to a peak at 16 h with no further increase at 24 h. This is consistent with the increase in IL-8 mRNA, which was maximal at 4 h, reflecting the delay in protein synthesis. The fact that mRNA was reducing after 4 h but protein did not show a drop reflects the stability of IL-8 protein. IL-8 mRNA was reduced by actinomycin D, suggesting it was mainly dependent on gene transcription. Consistent with this, transfection studies with wild-type IL-8 promoter luciferase reporter constructs showed that BK increased luciferase activity. We subsequently defined the main transcription factors involved. We found that BK induced NF-κB translocation to the nucleus, that NF-κB binding to the IL-8 promoter was increased by BK, and that IL-8 promoter constructs with the NF-κB site mutated had negligible promoter activity. This suggests that NF-κB is the major transcription factor involved in BK stimulated IL-8 release. Other studies have shown that BK can induce NF-κB activation in the lung fibroblast cell line WI-38 and in cultured human epithelial cells (8, 27), but the downstream targets of NF-κB activation have not been extensively studied. We have therefore extended these studies to show that NF-κB binds to the IL-8 promoter and regulates IL-8 release in HASM cells. We demonstrated translocation of NF-κB using both Western blotting and immunofluorescence. Consistent with this we found evidence of IκBα degradation in response to BK. IκBα is the inhibitory cytosolic protein bound to NF-κB, and its degradation is a key step in NF-κB activation. This process is initiated through signal-induced phosphorylation of two serines (Ser$^{32}$ and Ser$^{36}$) on the IκBα molecule. The phosphorylation event in turn induces polyubiquitination of IκBα on lysines 21 and 22. Phosphorylated and ubiquitinated IκBα can be rapidly recognized and degraded by proteasome, a multiprotease complex (28). Our results showed that IκBα was nearly completely degraded at 30 min after BK stimulation. NF-κB could thus translocate to the nucleus and activate IL-8 gene transcription.

Our transfection studies with mutant AP-1 and NF-IL-6 IL-8 promoter constructs in HASM cells showed that BK-induced luciferase activities were reduced significantly compared with wild type vector for both mAP-1 and mNF-IL-6, suggesting that both of these transcription factors are involved in IL-8 transcription by BK. This was supported by the EMSA results where BK induced strong AP-1 and NF-IL-6 binding to the IL-8 promoter.

Although BK has previously been shown to activate NF-κB, ours are the first studies to show that NF-IL-6 can be activated by BK.
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by BK. BK has previously been shown to activate AP-1 in HEK cells (29), but as with NF-κB, the downstream targets have not been identified. Our work extends these studies to show that IL-8 is a major downstream target.

As we have previously shown that BK-induced IL-8 release is partially dependent on the generation of endogenous PGE₂, we performed studies to determine the role of PGE₂ in the transcriptional regulation of IL-8 by BK. We found that indomethacin, a COX inhibitor, caused a concentration-dependent inhibition of IL-8 release. We have previously shown that BK increases PGE₂ levels and that this concentration of indomethacin abolishes it (31). Furthermore, indomethacin abolished the binding of NF-IL-6 and AP-1 to the IL-8 promoter in EMSA studies, suggesting that BK-induced activation of NF-IL-6 and AP-1 is prostanoïd-dependent. Consistent with this, exogenous PGE₂ is the major COX metabolite of HASM cells increased binding of NF-IL-6 and AP-1 to the IL-8 promoter. Furthermore, PGE₂ increased the activity of exogenous wild-type IL-8 promoter reporters, which was reduced in AP-1 and NF-IL-6 mutations. These assertions were further supported by rescue experiments using exogenous PGE₂. Although exogenous PGE₂ also activated NF-κB and NF-κB mutation reduced PGE₂-induced IL-8 promoter reporter activity, indomethacin did not reduce NF-κB binding to the IL-8 promoter or nuclear translocation of NF-κB, suggesting that BK-induced NF-κB activation can occur through both prostanoïd-dependent and -independent mechanisms and that when endogenous PGE₂ is blocked by indomethacin, prostanoïd-independent actions can still produce maximal NF-κB binding to the IL-8 promoter. We then went on to study the effect of the anti-inflammatory glucocorticoid dexamethasone on BK-induced IL-8 release and transcription factor binding. We found that dexamethasone suppressed BK-induced IL-8 production and inhibited binding of NF-κB, AP-1, and NF-IL-6 to the IL-8 promoter, suggesting that the effect was mediated through inhibition of NF-κB, AP-1, and NF-IL-6. These results might explain why dexamethasone completely abolished the IL-8 induction by BK. The effect of dexamethasone on NF-κB appeared to be via inhibition of its translocation to the nucleus. This is similar to studies in osteoarthritic synovial tissue in which dexamethasone reduced TNF-stimulated NF-κB translocation and its binding (30). As we have previously shown that dexamethasone markedly inhibits COX-2 mediated PGE₂ generation by BK (31), it is likely that the effects of dexamethasone are partly mediated by inhibition of PGE₂ generation and partly by inhibition of NF-κB translocation, and consistent with this, exogenous PGE₂ partially rescued the reduction in wild-type IL-8 luciferase activity caused by dexamethasone.

There are two types of BK receptors, B₁ and B₂, which are members of the seven-transmembrane GPCR superfamily. We have previously shown that BK induces IL-8 release via the B₂ receptor (23). Recently studies showed that signaling via a variety of GPCRs can lead to activation of NF-κB. BK-mediated NF-κB activation proceeds from the receptor through Gₛ which leads to phosphoinositide 3-kinase and Akt activation, and ultimately to the 1αB (9). Furthermore PYK2 activation can lead to NF-κB activation and, through phosphoinositide 3-kinase, Akt, and 1αB to NF-κB activation (32). Further studies are required to identify the complete signaling pathway linking the B₂ receptor to other transcription factors such as NF-IL-6 and AP-1.

In conclusion, we have delineated the transcription factors responsible for IL-8 release by HASM in response to BK. Our studies suggest that NF-κB is the major transcription factor involved but that AP-1 and NF-IL-6 also contribute. PGE₂ acts as an autocrine intermediary factor to active AP-1 and NF-IL-6, whereas NF-κB is activated via both prostanoïd-dependent and -independent mechanisms. These studies increase our understanding of the mechanisms whereby seven-transmembrane GPCR ligands such as BK contribute to inflammatory diseases.

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Transcriptional Regulation of Interleukin (IL)-8 by Bradykinin in Human Airway Smooth Muscle Cells Involves Prostanoid-dependent Activation of AP-1 and Nuclear Factor (NF)-IL-6 and Prostanoid-independent Activation of NF-κB

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