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Bradykinin B2 Receptor Mediates NF-κB Activation and Cyclooxygenase-2 Expression via the Ras/Raf-1/ERK Pathway in Human Airway Epithelial Cells[1]

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In this study, we investigated the signaling pathways involved in bradykinin (BK)-induced NF-κB activation and cyclooxygenase-2 (COX-2) expression in human airway epithelial cells (A549). BK caused concentration- and time-dependent increase in COX-2 expression, which was attenuated by a selective B2 BK receptor antagonist (HOE140), a Ras inhibitor (manumycin A), a Raf-1 inhibitor (GW 5074), a MEK inhibitor (PD 098059), an NF-κB inhibitor (pyrrolidine dithiocarbate), and an IκB protease inhibitor (L-1-tosylamido-2-phenylethyl chloromethyl ketone). The B1 BK receptor antagonist (Lys-(Leu^8)des-Arg^9-BK) had no effect on COX-2 induction by BK. BK-induced increase in COX-2-luciferase activity was inhibited by cells transfected with the κB site deletion of COX-2 construct. BK-induced Ras activation was inhibited by manumycin A. Raf-1 phosphorylation at Ser^{338} by BK was inhibited by manumycin A and GW 5074. BK-induced ERK activation was inhibited by HOE140, manumycin A, GW 5074, and PD 098059. Stimulation of cells with BK activated IκB kinase αβ (IKKαβ), IκBα phosphorylation, IκBα degradation, p65 and p50 translocation from the cytosol to the nucleus, the formation of an NF-κB-specific DNA-protein complex, and κB-luciferase activity. BK-mediated increase in IKKαβ activity and formation of the NF-κB-specific DNA-protein complex were inhibited by HOE140, a Ras dominant-negative mutant (RasN17), manumycin A, GW 5074, and PD 098059. Our results demonstrated for the first time that BK, acting through B2 BK receptor, induces activation of the Ras/Raf-1/ERK pathway, which in turn initiates IKKαβ and NF-κB activation, and ultimately induces COX-2 expression in human airway epithelial cell line (A549). ***The Journal of Immunology,*** **2004, 173: 5219–5228.**

Bradykinin (BK)[3] is rapidly generated following inflammation or injury (1). The release of BK is known to mediate multiple proinflammatory effects including smooth muscle contraction, vasodilation, increased vascular permeability, eicosanoid synthesis, and neuropeptide release (1, 2). Furthermore, BK has been shown to play a critical role in the development of airway hyperresponsiveness in experimental models of airway inflammation (3–5). To elucidate the role of BK in this process, we assessed the effect of BK on cyclooxygenase-2 (COX-2) expression in human airway epithelial cells.

COX is the key enzyme to synthesize PGs and thromboxane from arachidonic acid (6). Two COX isozymes, COX-1 and COX-2, have been cloned and identified to have 60% homology in humans (7, 8). COX-1, which is constitutively expressed in most tissues, mediates physiological responses and regulates renal and vascular homeostasis. The second COX isoform, COX-2, is considered to be an inducible immediate-early gene product whose synthesis in cells can be up-regulated by mitogenic or inflammatory stimuli including TNF-α, IL-1β, lipoteichoic acid, and LPS (9–12). COX-2 is thought to be responsible for the production of proinflammatory PGs in various models of inflammation (13). Previous studies have shown that BK can induce COX-2 expression in human airway smooth muscle cells and fibroblasts (14, 15). However, the expression of COX-2 induced by BK in human airway epithelial cell has not been determined, and the signal transduction events, especially the Ras/Raf-1/ERK pathway, leading to the expression of COX-2 by BK are unclear.

Several G protein-couple receptors, including the B2 BK receptor, have been shown to mediate NF-κB activation and cytokine gene transcription in human epithelial cells and fibroblasts (16, 17). BK-stimulated NF-κB activation has been demonstrated to be mediated through B2 BK receptors coupled to the G_i/G_o class of heterotrimeric G proteins (16). Activation of the small GTP-binding protein, Rho A, and PI3K are involved in BK-stimulated NF-κB activation in A549 cells (17, 18). BK has also been reported to activate the small GTP-binding proteins, Cdc42 and Rac1, stimulating the formation of peripheral actin microspikes and membrane ruffling in Swiss 3T3 fibroblasts (19). Moreover, BK was shown to activate Ras in PC-12 rat adrenal pheochromocytoma cells and inner medullary collecting duct cells (20, 21). However, little information is available about the role of Ras in the regulation of NF-κB activation and COX-2 expression following BK stimulation.

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[3] Abbreviations used in this paper: BK, bradykinin; COX-2, cyclooxygenase-2; IKKβ, IκB kinase β; PDTC, pyrrolidine dithiocarbate; Raf-1 RBD, Ras-binding domain for Raf-1; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

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Ras has been found to couple with multiple effector systems to activate distinct physiological and pathological responses such as cell proliferation and proinflammatory mediator release (22, 23). An important class of Ras effectors is the MAPK family. The classic Ras-mediated pathway involves the binding of Raf-1 and subsequent phosphorylation of Raf-1 at Ser^{338} by many kinases (24, 25), which in turn activates ERKs (26), and consequently phosphorylates many target proteins including transcription factors and protein kinases (27). A role for Ras in COX-2 induction has been implied in many cell types (21, 23). However, the role of the Ras/Raf-1/ERK pathway in BK-induced COX-2 expression has not been investigated in human airway epithelial cells (A549). This study was intended to identify the role of the Ras/Raf-1/ERK pathway in BK-mediated NF-κB activation and COX-2 expression in A549 cells. Our hypothesis is that BK might activate the Ras/Raf-1/ERK pathway, which in turn induces IκB kinase $\alpha\beta$ (IKK$\alpha\beta$) and NF-κB activation, and ultimately causes COX-2 expression in A549 cells.

Materials and Methods

Materials

Manumycin A, PD 098059, and SB 203580 were obtained from Calbiochem (San Diego, CA). DMEM/Ham's F-12, FCS, penicillin/streptomycin, and Lipofectamine plus were purchased from Invitrogen Life Technologies (Gaithersburg, MD). Specific Abs for α-tubulin and COX-2 were purchased from Transduction Laboratories (Lexington, KY). Protein A/G beads, IκBα protein (aa 1~317), specific Abs for ERK2, ERK phosphorylated at Tyr^{204}, IκBα, IκBα phosphorylated at Ser^{32}, IKKα, IKKβ, Raf-1, and anti-mouse and anti-rabbit IgG-conjugated HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A specific Ab for Raf-1 phosphorylated at Ser^{338} was purchased from Cell Signaling and Neuroscience (St. Louis, MO). Anti-mouse and anti-rabbit IgG-conjugated alkaline phosphatases were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). pGL2-ELAM-Luc (which is under the control of one NF-κB binding site) and pBK-CMV-Lac Z were kindly provided by Dr. W.-W. Lin (National Taiwan University, Taipei, Taiwan). A Ras dominant-negative mutant (RasN17) and a Ras activity assay kit were purchased from Upstate Biotechnology (Lake Placid, NY). [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham (Buckinghamshire, U.K.). All materials for SDS-PAGE were purchased from Bio-Rad (Hercules, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Cell culture

A549 cells, a human pulmonary epithelial carcinoma cell line with type II alveolar epithelial cell differentiation, was obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM/Ham's F-12 nutrient mixture containing 10% FCS, 50 U/ml penicillin G, and 100 μg/ml streptomycin in a humidified 37°C incubator. After reaching confluence, cells were seeded onto 10-cm dishes for EMSA, 6-cm dishes for immunoblotting or kinase assays, or 12-well plates for transfection and κB-luciferase assays.

Immunoblot analysis

To determine the expression of COX-2, α-tubulin, ERK phosphorylation at Tyr^{204}, ERK2, IKK$\alpha\beta$, IκBα phosphorylation at Ser^{32}, IκBα, Ras, Raf-1 phosphorylation at Ser^{338}, and Raf-1 in A549 cells, proteins were extracted and Western blot analysis was performed as described previously (12). Briefly, A549 cells were cultured in 6-cm dishes. After reaching confluence, cells were treated with vehicle, BK, or pretreated with specific inhibitors as indicated, followed by BK. After incubation, cells were washed twice in ice-cold PBS and solubilized in extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM PMSF, 5 mM DTT, 0.5% Nonidet P-40, 0.05 mM pepstatin A, and 0.2 mM leupeptin. Samples of equal amounts of protein (60 μg) were placed on SDS-PAGE, transferred onto a PVDF membrane and then incubated in TBST buffer (150 mM NaCl, 20 mM Tris-HCl, 0.02% Tween 20 (pH 7.4)) containing 5% nonfat milk. Proteins were visualized by specific primary Abs and then incubated with HRP- or alkaline phosphatase-conjugated second Abs. Immunoreactivity was detected using ECL or NBT/5-bromo-4-chloro-3-indolyl phosphate following the manufacturer's instructions. Quantitative data were obtained using a computing densitometer with scientific imaging systems (Kodak, Rochester, NY).

Preparation of nuclear extracts and the EMSA

A549 cells were cultured in 10-cm dishes. After reaching confluence, cells were treated with vehicle or 10 nM BK for various time intervals, and then cells were scraped and collected. In some experiments, cells were pretreated with specific inhibitors as indicated or transfected with the Ras dominant-negative mutant (RasN17) for 24 h before BK treatment. The cytosolic and nuclear protein fractions were then separated as described previously (28). Briefly, cells were washed with ice-cold PBS, and pelleted. Cell pellets were resuspended in hypotonic buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.5 mM DTT, 10 mM aprotinin, 10 mM leupeptin, and 20 mM PMSF) for 15 min on ice, and vortexed for 10 s. Nuclei were pelleted by centrifugation at 15,000 $\times$ *g* for 1 min. Supernatants containing cytosolic proteins were collected. A pellet containing nuclei was resuspended in hypertonic buffer (20 mM HEPES (pH 7.6), 25% glycerol, 1.5 mM $MgCl_2$, 4 mM EDTA, 0.05 mM DTT, 10 mM aprotinin, 10 mM leupeptin, and 20 mM PMSF) for 30 min on ice. Supernatants containing nuclear proteins were collected by centrifugation at 15,000 $\times$ *g* for 2 min and then stored at −70°C. The protein levels of p65 and p50 NF-κB in the cytosolic and nuclear fractions, and IκBα phosphorylated at Ser^{32} and IκBα in the cytosolic fractions were determined by Western blot analysis performed as described.

A double-stranded oligonucleotide probe containing NF-κB sequences (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega, Madison, WI) was purchased and end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The nuclear extract (2.5~5 μg) was incubated with 1 ng of a ^{32}P-labeled NF-κB probe (50,000~75,000 cpm) in 10 μl of binding buffer containing 1 μg poly(dI-dC), 15 mM HEPES (pH 7.6), 80 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol at 30°C for 25 min. DNA-nuclear protein complexes were separated from the DNA probe by electrophoresis on 5% polyacrylamide gels. Gels were vacuum-dried and subjected to autoradiography with an intensifying screen at −80°C.

Transfection and κB- or COX-2-luciferase assays

For these assays, 2×10^5 A549 cells were seeded onto 12-well plates, and transfected in the following day using Lipofectamine plus (Invitrogen Life Technologies) with 0.5 μg of pGL2-ELAM-Luc, human COX-2 promoter-luciferase (−327/+59), or the κB site (−223/−214) deletion mutant of COX-2 (κBM), plus 1 μg of pBK-CMV-Lac Z. After 24 h, the medium was aspirated and replaced by fresh DMEM/Ham's F12 containing 10% FBS. Cells were stimulated with 10 nM BK for another 24 h before harvest. Luciferase activity was determined by a luciferase assay system (Promega), and was normalized on the basis of Lac Z expression. The level of induction of luciferase activity was compared as a ratio of cells with and without stimulation.

Immunoprecipitation and IKKαβ activity assay

A549 cells were grown in 6-cm dishes. After reaching confluence, cells were either treated with 10 nM BK for the indicated time intervals, pretreated with specific inhibitors as indicated, or transfected with the Ras dominant-negative mutant (RasN17) for 24 h followed by BK treatment. After incubation, cells were washed twice with ice-cold PBS, lysed in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 125 mM NaCl, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM β-glycerophosphate, 50 mM NaF, and 100 μM sodium orthovanadate, and centrifuged. The supernatant was then immunoprecipitated with polyclonal Abs against IKKα or IKKβ in the presence of A/G-agarose beads overnight. The beads were washed three times with lysis buffer and two times with kinase buffer containing 20 mM HEPES (pH 7.4), 20 mM $MgCl_2$, and 2 mM DTT. The kinase reactions were performed by incubating immunoprecipitated beads with 20 μl of kinase buffer supplemented with 20 μM ATP, 0.5 μg of GST-IκBα protein (aa 1~317), and 3 μCi of [γ-^{32}P]ATP at 30°C for 30 min. The reaction mixtures were analyzed by 12% SDS-PAGE followed by autoradiography.

Ras activity assay

Ras activity was measuring by using a Ras activity assay kit. The assay was performed according to the manufacturer's instructions. Briefly, cells were washed twice with ice-cold PBS, lysed in magnesium lysis buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 5% Igepal CA-630 (Upstate Biotechnology, Lake Placid, NY), 10 mM $MgCl_2$, 5 mM EDTA, 10% glycerol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin), and centrifuged. An equal volume of lysate was incubated with 5 μg of Ras-binding domain for Raf-1 (Raf-1 RBD) at 4°C overnight, and beads were washed three times with magnesium lysis buffer. Bound Ras proteins were then solubilized in 2× Laemmli sample buffer and quantitatively detected by Western blotting

(10% SDS-PAGE) using mouse monoclonal Ras with the ECL system, and by densitometry of corresponding bands using scientific imaging systems.

Statistical analysis

Results are presented as the mean ± SE from at least three independent experiments. One-way ANOVA followed by, when appropriate, Bonferroni's multiple-range test was used to determine the statistical significance of the difference between means. A p-value of <0.05 was considered statistically significant.

Results

BK induces COX-2 expression in A549 cells

Human airway epithelial cells (A549) were chosen to investigate the signal pathways of BK in COX-2 expression, an inflammatory response gene. Treatment with BK (0.1~1000 nM) for 4 h induced COX-2 protein expression in a concentration-dependent manner, with a maximum effect at 10 nM BK treatment (Fig. 1*A*); this induction occurred in a time-dependent manner (Fig. 1*B*). After treatment, COX-2 protein bands began to appear at 2 h, reached a maximum at 4 h, and gradually diminished from 8 to 24 h. After 4 h of treatment with 10 nM BK, the COX-2 protein had increased by ~246 ± 34% (Fig. 1*B*). Two types of BK receptors have been defined and cloned: B1 and B2 BK receptors (29). The receptor specificity of BK-mediated COX-2 expression was therefore tested using specific BK receptor antagonists. Pretreatment of cells with the B2 BK receptor antagonist HOE140 (1~100 nM) inhibited the BK-induced COX-2 expression in a concentration-dependent manner, while the B1 BK receptor antagonist (Lys-(Leu^8)des-Arg^9-BK) (10 and 100 μM) had no effect. When cells were treated with 100 nM HOE140, BK-induced COX-2 expression was inhibited by 58 ± 28% (n = 3) (Fig. 1*C*). Therefore, BK-stimulated COX-2 expression in A549 cells is mediated primarily through B2 BK receptors.

Augmentation of COX-2 expression occurred at the level of transcription

A549 cells were pretreated with either actinomycin D (a transcriptional inhibitor) or cycloheximide (a translational inhibitor) and then treated with 10 nM BK. As a result, the BK-induced elevation of COX-2 expression was inhibited by actinomycin D (1 μM) and cycloheximide (3 μM) by ~91 ± 15% and 72 ± 19%, respectively (Fig. 2*A*). The results suggest that the increase in COX-2 protein in A549 cells responsive to BK may have been due to COX-2 transcriptional expression.

NF-κB is involved in BK-induced COX-2 expression

As previously mentioned, NF-κB activation is necessary for COX-2 induction. Pyrrolidine dithiocarbate (PDTC), an antioxidant, has been shown to inactivate NF-κB in macrophages (30). To examine whether NF-κB activation is involved in the signal transduction pathway leading to COX-2 expression caused by BK, the NF-κB inhibitor, PDTC, was used. Fig. 2*B* shows that BK-induced increase in COX-2 protein levels was inhibited by PDTC (3 and 10 μM). When cells were treated with 10 μM PDTC, BK-induced COX-2 expression was inhibited by 87 ± 10% (n = 3). In parallel with the inhibition by PDTC, an IκB protease inhibitor (L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (TPCK), 3 and 10 μM) (31) also inhibited BK-induced COX-2 protein expression. Pretreatment of cells with 10 μM TPCK completely abolished the BK response (Fig. 2*B*). To further confirm the role of NF-κB in the regulation of COX-2 expression, the human COX-2 promoter (−327/+59) or the κB site (−223/−214) deletion mutant (κBM) luciferase plasmid was transfected into A549 cells. As shown in Fig. 2*C*, 10 nM BK induced a 2.1-fold increase in COX-2 luciferase activity in cells transfected with the human COX-2 construct.

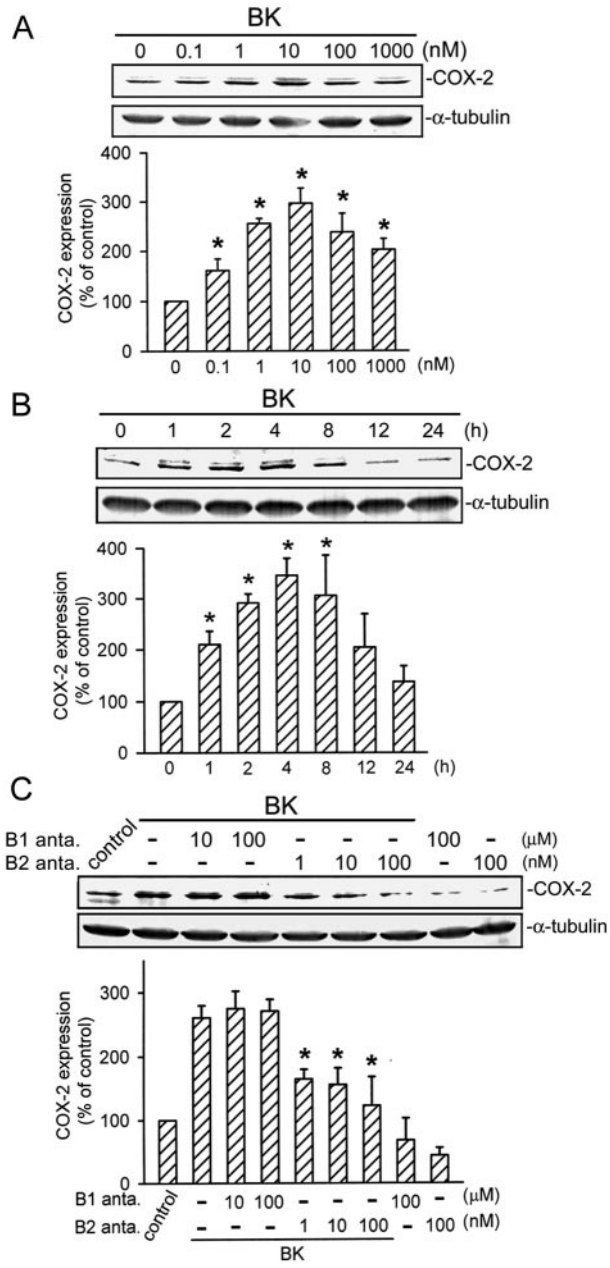

FIGURE 1. BK-induced COX-2 expression is mediated through B2 but not B1 BK receptors in A549 cells. Cells were incubated with various concentrations of BK for 4 h (*A*) or 10 nM BK for the indicated time intervals (*B*), and then COX-2 or α-tubulin protein levels were determined. Equal loading in each lane is shown by the similar intensities of α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SE. *, $p < 0.05$ as compared with the control group. *C*, Cells were pretreated with the B1 BK receptor antagonist (B1 anta.), Lys-(Leu^8)des-Arg^9-BK (10~100 μM), or the B2 BK receptor antagonist (B2 anta.), HOE140 (1~100 nM), for 30 min, and then stimulated with 10 nM for 4 h. Cell were lysed, and then immunoblotted for COX-2 or α-tubulin. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SE. *, $p < 0.05$ as compared with BK treatment.

BK-induced COX-2 luciferase activity was reduced by 67 ± 6% (n = 4) in cells transfected with the κBM construct. The results indicate that NF-κB activation is necessary for BK-induced COX-2 expression in A549 cells.

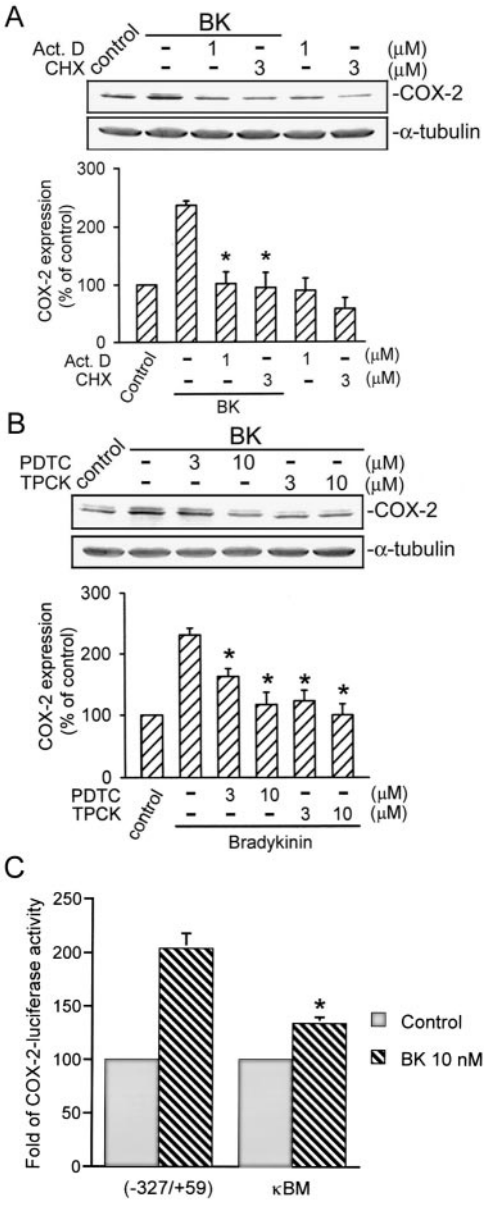

FIGURE 2. NF-κB is involved in BK-mediated COX-2 expression in A549 cells. *A*, BK-induced COX-2 expression involved transcriptional activity. Cells were pretreated for 30 min with 1 μM actinomycin D (Act. D) or 3 μM cycloheximide (CHX), and then stimulated with 10 nM BK for 4 h. Cells were lysed, and then immunoblotted for COX-2 or α-tubulin. *B*, The NF-κB signal pathway was necessary for BK-induced COX-2 expression. Cells were pretreated with various concentrations of PDTC or TPCK for 30 min, and then stimulated with 10 nM BK for 4 h. Cells were lysed, and then immunoblotted for COX-2 or α-tubulin. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. Traces represent results from three independent experiments, which are presented as the mean ± SE. *, $p < 0.05$ as compared with BK treatment. *C*, Cells were transfected with the COX-2 promoter (−327/+59) or the κB site (−223/−214) deletion mutant (κBM) luciferase expression vector, and then treated with 10 nM BK for 24 h. Luciferase activities were determined as described in *Materials and Methods*. The level of induction of luciferase activity was compared with that of cells without BK treatment. Data represent the mean ± SE. *, $p < 0.05$ as compared with BK on cells transfected with the COX-2 promoter (−327/+57).

BK induces NF-κB activation

NF-κB activation was directly evaluated by the translocation of NF-κB from the cytosol to the nucleus and a gel shift DNA-binding assay. Treatment of cells with 10 nM BK resulted in marked

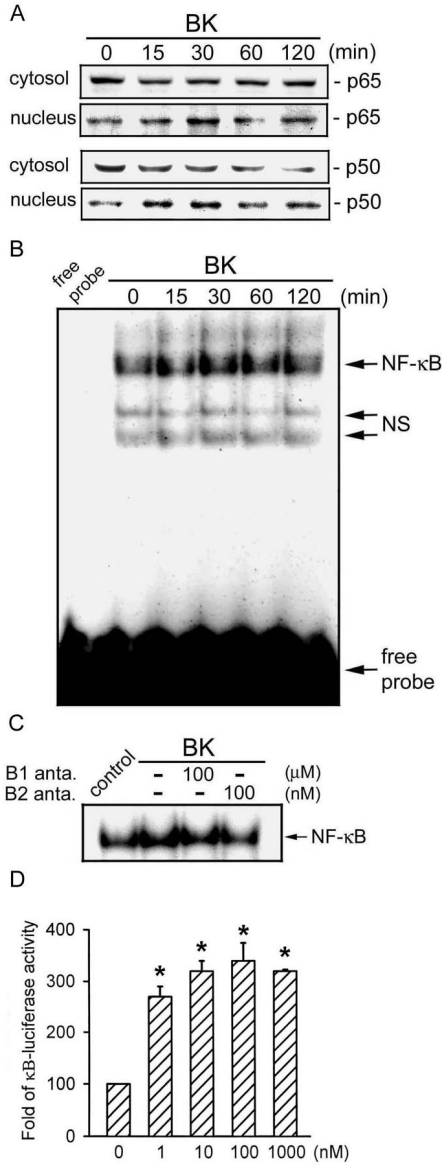

FIGURE 3. BK induced increases in p65 and p50 translocation, NF-κB-specific DNA-protein complex formation, and κB-luciferase activity in A549 cells. *A*, Time-dependent translocation of p65 and p50 by BK. Cells were treated with 10 nM BK for 0∼120 min, and then cytosol and nucleus fractions were prepared as described in *Materials and Methods*. Levels of cytosolic and nuclear p65 and p50 were determined by immunoblotting with p65- and p50-specific Abs, respectively. Typical traces are representative of three experiments with similar results. *B*, Time-dependent activation of NF-κB by BK. A549 cells were incubated with 10 nM BK for 0∼120 min. Following incubation, a nuclear extract was prepared, and EMSA was performed as described in *Materials and Methods*. The top band represents NF-κB. NS, Nonspecific binding. *C*, BK-mediated NF-κB activation is mediated by the B2 BK receptor. A549 cells were pretreated with the B1 BK receptor antagonist (B1 anta.), Lys-(Leu^8)des-Arg^9-BK (100 μM), or the B2 BK receptor antagonist (B2 anta.), HOE140 (100 nM), for 30 min followed by stimulation with 10 nM BK for another 30 min. Nuclear extracts were prepared for determination of NF-κB-specific DNA protein-binding activity by EMSA. *D*, Concentration-dependent-induced κB-luciferase activity by BK. Cells were transiently transfected with 0.5 μg of pGL2-ELAM-Luc and 1 μg of pBK-CMV-Lac Z for 24 h, and then cells were incubated with 1∼1000 nM BK for another 24 h. Luciferase activities were determined as described in *Materials and Methods*. The level of induction of luciferase activity was compared with that of cells without BK treatment. Data represent the mean ± SE of three experiments performed in duplicate. *, $p < 0.05$ as compared with the control without BK treatment.

translocation of p65 and p50 NF-κB from the cytosol to the nucleus which began at 15 min, peaked at 30 min, and then had declined after 60 min of treatment (Fig. 3*A*). In nuclear extracts of unstimulated cells, a slight intensity of NF-κB-specific DNA-protein complex formation was observed. Stimulation of cells with 10 nM BK resulted in time-dependent activation of NF-κB-specific DNA-protein complex formation, with a maximum effect after 30 min of treatment. However, after 60 min of treatment with BK, the intensities of these DNA-protein complexes had decreased (Fig. 3*B*). Pretreatment of cells for 30 min with the B2 BK receptor antagonist HOE140 (100 nM) markedly attenuated BK-induced formation of NF-κB-specific DNA-protein complexes, while the B1 BK receptor antagonist, (Lys-(Leu^8)des-Arg^9-BK) (100 μM), had no effect (Fig. 3*C*). Therefore, BK-stimulated NF-κB activation in A549 cells is mediated primarily through the B2 BK receptor. To directly determine NF-κB activation after BK treatment, A549 cells were transiently transfected with pGL2-ELAM-κB-luciferase as an indicator of NF-κB activation. As shown in Fig. 3*D*, cells treated with BK (1∼1000 nM) for 24 h caused a concentration-dependent increase in κB-luciferase activity with about a 230 ± 18% ($n = 3$) increase after 10 nM BK treatment.

BK causes increases in IKKαβ phosphorylation, IKKαβ activity, IκBα phosphorylation, and IκBα degradation

We further determined the upstream molecules of NF-κB in BK-induced NF-κB activation. Stimulation of cells with 10 nM BK induced an increase in IKKαβ phosphorylation and IKKαβ activity in time-dependent manners, reaching a maximum after 5 and 10 min of treatment, respectively (Fig. 4*A* and *B*). In parallel with IKKαβ phosphorylation and IKKαβ activity, IκBα phosphorylation was apparent after 10 min of treatment with 10 nM BK, and it reached a maximum effect after 20 min of treatment (Fig. 4*C*). Furthermore, it also caused IκBα degradation after 20 min of treatment with 10 nM BK, and the IκBα protein had been resynthesized after 60 min of treatment (Fig. 4*D*).

Ras is involved in BK-induced COX-2 expression

To explore whether Ras might mediate BK-induced COX-2 expression, manumycin A, a Ras inhibitor (32) was used. As shown in Fig. 5*A*, pretreatment of A549 cells with manumycin A (0.3∼3 μM) inhibited BK-induced COX-2 expression in a concentration-dependent manner. When cells were treated with 3 μM manumycin A, BK-induced COX-2 expression was inhibited by 63 ± 11% ($n = 3$) (Fig. 5*A*). Next, we directly measured the Ras activity in response to BK. Fig. 5*B* shows that treatment of A549 cells with 10 nM BK induced an increase in Ras activity in a time-dependent manner, as assessed by immunoblotting samples for Ras immunoprecipitated from lysates using Raf-1 RBD. Maximal activation was detected after 5∼10 min of stimulation, and the response continued until 30 min of stimulation (Fig. 5*B*). The BK-induced increase in Ras activity was markedly inhibited by pretreatment of cells for 30 min with manumycin A (1 and 3 μM) in a concentration-dependent manner (Fig. 5*C*). Taken together, these results imply that Ras activation is involved in BK-induced COX-2 expression.

Raf-1 is involved in BK-induced COX-2 expression

To examine whether Raf-1, a target protein for Ras, might play a crucial role in BK-induced COX-2 expression, the Raf-1 inhibitor,

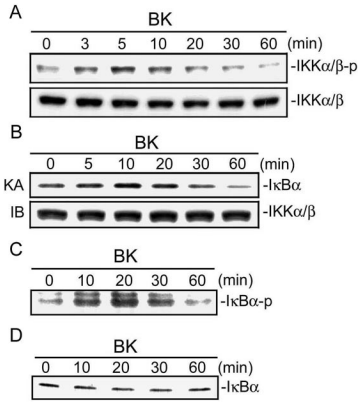

FIGURE 4. BK induced increases in IKKαβ phosphorylation, IKKαβ activity, IκBα phosphorylation, and IκBα degradation in A549 cells. *A*, Cells were incubated with 10 nM BK for the indicated time intervals. Whole cell lysates were prepared, and then immunoblotted with Abs for phospho-IKKαβ or -IKKαβ, respectively. *B*, A549 cells were incubated with 10 nM BK for 0∼60 min, and then cell lysates were immunoprecipitated with Abs specific for IKKα and IKKβ. One set of immunoprecipitates was subjected to the kinase assay (KA) using the GST-IκBα fusion protein as a substrate (*top panel*). The other set of immunoprecipitates was subjected to 10% SDS-PAGE and analyzed by immunoblotting (IB) with anti-IKKαβ Ab (*bottom panel*). Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for IKKαβ. *C* and *D*, Following incubation for 0∼120 min with 10 nM BK, IκBα phosphorylation (*C*) and IκBα degradation (*D*) were determined by immunoblotting using phospho-IκBα- and IκBα-specific Abs, respectively. Typical traces are representative of three experiments with similar results.

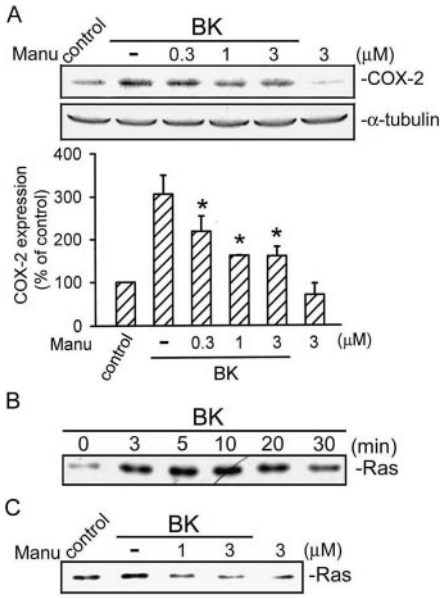

FIGURE 5. Effects of manumycin A on BK-induced COX-2 expression and Ras activation in A549 cells. *A*, Cells were pretreated with vehicle and manumycin A (0.3∼3 μM) for 30 min, followed by stimulation with 10 nM BK for another 4 h, and COX-2 expression was determined by immunoblotting with an Ab specific for COX-2. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. Typical traces are representative of two experiments with similar results, which are presented as the mean ± SE. *, $p < 0.05$ as compared with BK treatment. *B*, A549 cells were incubated with 10 nM BK for 0∼30 min, and then cell lysates were immunoprecipitated with an Ab specific for Raf-1 RBD. The Ras activity assay is described in *Materials and Methods*. Typical traces represent two experiments with similar results. *C*, Cells were pretreated with manumycin A (1 and 3 μM) for 30 min, and then treated with 10 nM BK for another 5 min. Cells were then lysed for the Ras activity assay as described above. Typical traces represent two experiments with similar results.

GW 5074 (33), was used. As shown in Fig. 6*A*, pretreatment of A549 cells with GW 5074 (0.3~3 nM) concentration-dependently inhibited BK-induced COX-2 expression. Treatment of cells with 3 nM GW 5074 slightly affected the basal COX-2 level, but it almost completely inhibited BK-induced COX-2 expression. Raf-1 is associated with Ras-GTP, and then by additional modifications such as phosphorylation at Ser^{338}, becomes the active form (25). The activated Raf-1 then triggers sequential activation of downstream molecules. Thus, phosphorylation of Raf-1 at Ser^{338} is a critical step in Raf-1 activation. Next, we further examined Raf-1 Ser^{338} phosphorylation by BK stimulation in A549 cells using the anti-phospho-Raf-1 Ab at Ser^{338}. When cells were treated with 10 nM BK for various time intervals, Raf-1 Ser^{338} phosphorylation increased at 3 min and peaked at 5~10 min. After 30 min of treatment, the BK-induced Raf-1 Ser^{338} phosphorylation had declined (Fig. 6*B*). In addition, BK-induced Raf-1 Ser^{338} phosphorylation was inhibited by treatment with 1 μM manumycin A and 1 nM GW 5074 (Fig. 6*C*). The results indicate that Raf-1 is a downstream molecule of Ras and is involved in BK-mediated COX-2 protein expression.

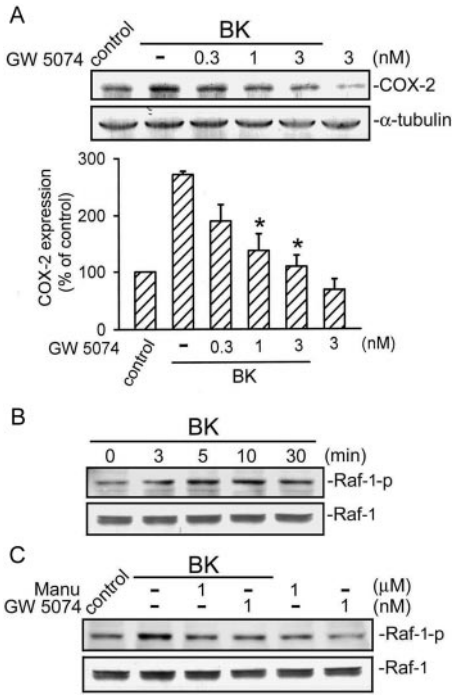

FIGURE 6. Effects of GW 5074 on BK-induced COX-2 expression and Raf-1 phosphorylation in A549 cells. *A*, Cells were pretreated with vehicle and GW 5074 (0.3~3 nM) for 30 min, followed by stimulation with 10 nM BK for another 4 h, and COX-2 expression was determined by immunoblotting with a specific COX-2 Ab. Equal loading in each lane is demonstrated by the similar intensities of α-tubulin. Typical traces represent three experiments with similar results, which are presented as the mean ± SE. ∗, $p < 0.05$ as compared with BK treatment. *B*, A549 cells were incubated with 10 nM BK for 0~30 min, and then Raf-1 phosphorylation (*upper panel*) and Raf-1 (*bottom panel*) protein levels were determined. The presence of equal loading in each lane is shown by the similar intensities of Raf-1. Traces represent results from three independent experiments. *C*, Cells were pretreated with manumycin A (1 μM) or GW 5074 (1 nM) for 30 min, and then treated with 10 nM BK for another 5 min. Cells were then lysed for Raf-1 phosphorylation (*upper panel*) and Raf-1 (*bottom panel*) protein levels as described above. Typical traces represent two experiments with similar results.

ERK is involved in BK-induced COX-2 expression

We next wished to determine whether BK is able to activate ERK, a critical downstream target of Raf-1 (34), which has been shown to induce gene expression (35). We tested the role of ERK in BK-induced COX-2 expression by using the specific MEK inhibitor, PD 098059. As shown in Fig. 7*A*, BK-induced COX-2 expression was markedly attenuated by pretreatment of cells with PD 098059 (3~30 μM) in a concentration-dependent manner. Pretreatment of cells with 30 μM PD 098059 inhibited BK-induced COX-2 expression by 77 ± 14% ($n = 3$). To directly confirm the crucial role of ERK in COX-2 expression, we determined ERK

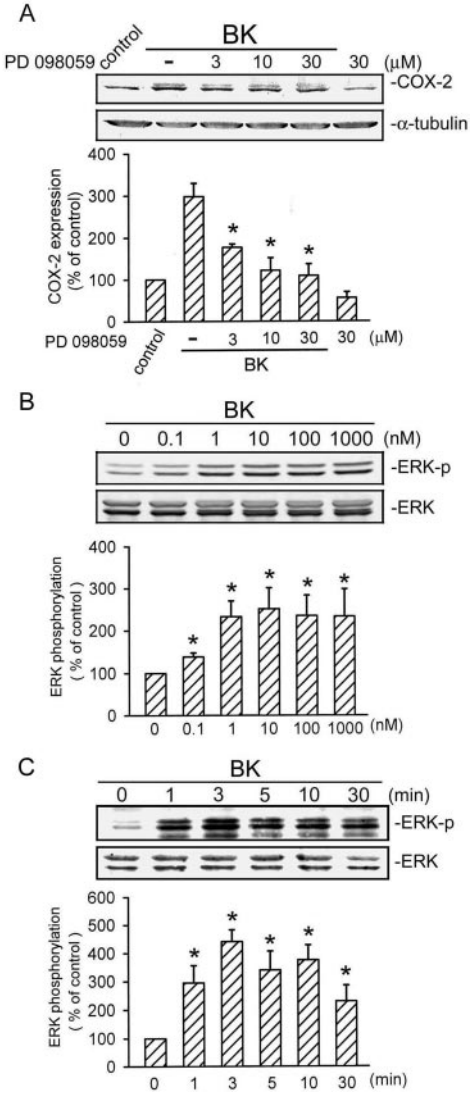

FIGURE 7. ERK is involved in BK-mediated COX-2 expression in A549 cells. *A*, Cells were pretreated with vehicle and PD 098059 (3~30 μM) for 30 min before treatment with 10 nM BK for another 4 h, and COX-2 expression was determined by immunoblotting with a specific COX-2 Ab. Equal loading in each lane is shown by the similar intensities of α-tubulin. Typical traces represent three experiments with similar results, which are presented as the mean ± SE. ∗, $p < 0.05$ as compared with BK treatment. *B* and *C*, Cells were treated with various concentrations of BK for 3 min (*B*) or 10 nM BK for different time intervals (*C*). ERK phosphorylation was shown by immunoblotting with an Ab specific for phosphorylated ERK (p-ERK) (*upper panel*). Equal loading in each lane is shown by the similar intensities of ERK2 (*bottom panel*). Traces represent results from three independent experiments, which are presented as the mean ± SE. ∗, $p < 0.05$ as compared with the control group.

phosphorylation in response to BK. As shown in Fig. 7*B*, treatment with BK (0.1~1000 nM) for 3 min induced ERK phosphorylation in a concentration-dependent manner, with a maximum effect at 10 nM BK treatment. Stimulation of cells with 10 nM BK resulted in time-dependent phosphorylation of ERK. ERK phosphorylation began at 1 min, peaked at 3 min, and then had declined after 5 min of BK treatment (Fig. 7*C*). The protein level of ERK2 was not affected by BK treatment (Fig. 7, *B* and *C*, *bottom panel*). Pretreatment of cells with HOE140 (1~100 nM) inhibited BK-induced ERK activation in a concentration-dependent manner, while (Lys-(Leu^8)des-Arg^9-BK) (10 and 100 μM) had no effect (Fig. 8*A*). None of these treatments had any effect on ERK expression (Fig. 8*A*, *bottom panel*). Therefore, BK-stimulated ERK activation is mediated through the B2 BK receptor. We further examined the relationships among Ras, Raf-1, MEK, and ERK in the BK-mediated signaling pathway. When cells were pretreated for 30 min with PD 098059 (3~30 μM) or the p38 MAPK inhibitor, SB 203580 (0.01~1 μM), BK-induced ERK activation was markedly inhibited by PD 098059 in a concentration-dependent manner, but not by SB 203580 at concentrations of up to 1 μM (Fig. 8*B*). Pretreatment of A549 cells for 30 min with 3 μM manumycin A or 3 nM GW 5074 markedly inhibited BK-induced ERK phosphorylation (Fig. 8*B*). None of these treatments had any effect on ERK expression (Fig. 8 *B* and *C*, *bottom panel*). Based on these results, we suggest that activations of Ras, Raf-1, and MEK occur upstream of ERK in the BK-induced signaling pathway.

Ras, Raf-1, and ERK mediate BK-induced IKK$\alpha\beta$ and NF-κB activation

We further examined whether activation of IKK$\alpha\beta$ and NF-κB occurs through the Ras/Raf-1/ERK signaling pathway. As shown in Fig. 9*A*, pretreatment of cells for 30 min with 3 μM manumycin A, 3 nM GW 5074, and 30 μM PD 098059 markedly attenuated the BK-induced increase in IKK$\alpha\beta$ activity. To further confirm the role of Ras in BK-mediated IKK$\alpha\beta$ activation, a Ras dominant-negative mutant (RasN17) was tested. Transfection of A549 cells with 1 μg of RasN17 inhibited the BK-induced increase in IKK$\alpha\beta$ activity (Fig. 9*A*). Similarly, when cells were pretreated for 30 min with 3 μM manumycin A, 3 nM GW 5074, and 30 μM PD 098059, BK-induced formation of NF-κB-specific DNA-protein complexes was markedly inhibited by manumycin A, GW 5074, and PD 098059 (Fig. 9*B*). Moreover, transfection of A549 cells with 1 μg RasN17 also inhibited the BK-induced effects (Fig. 9*B*). Taken together, these data suggest that activation of the Ras/Raf-1/ERK pathway is also required for BK-induced IKK$\alpha\beta$ and NF-κB activation in A549 cells.

Discussion

BK is recognized to play an important role in asthma. Several studies have shown that BK is generated in human airways within minutes of an allergen challenge (36). Allergen challenge studies have also established that BK plays a pivotal role in the initiation of chronic airway inflammation (37). B2 BK receptor antagonists effectively block the development of the late-phase airway response as well as the development of bronchial hyperresponsiveness following allergen challenge in a number of different experimental animal models (3, 4). To explore the mechanism by which BK promotes airway inflammation, we examined the ability of BK to regulate COX-2 expression in airway epithelial cells. We found that BK can induce COX-2 expression in airway epithelial cells. Similar results were also seen in human airway smooth muscle cells and fibroblasts (14, 15). Because asthmatic patients have elevated levels of kinin concentrations in nasal fluid and bronchoalveolar fluid (38, 39),

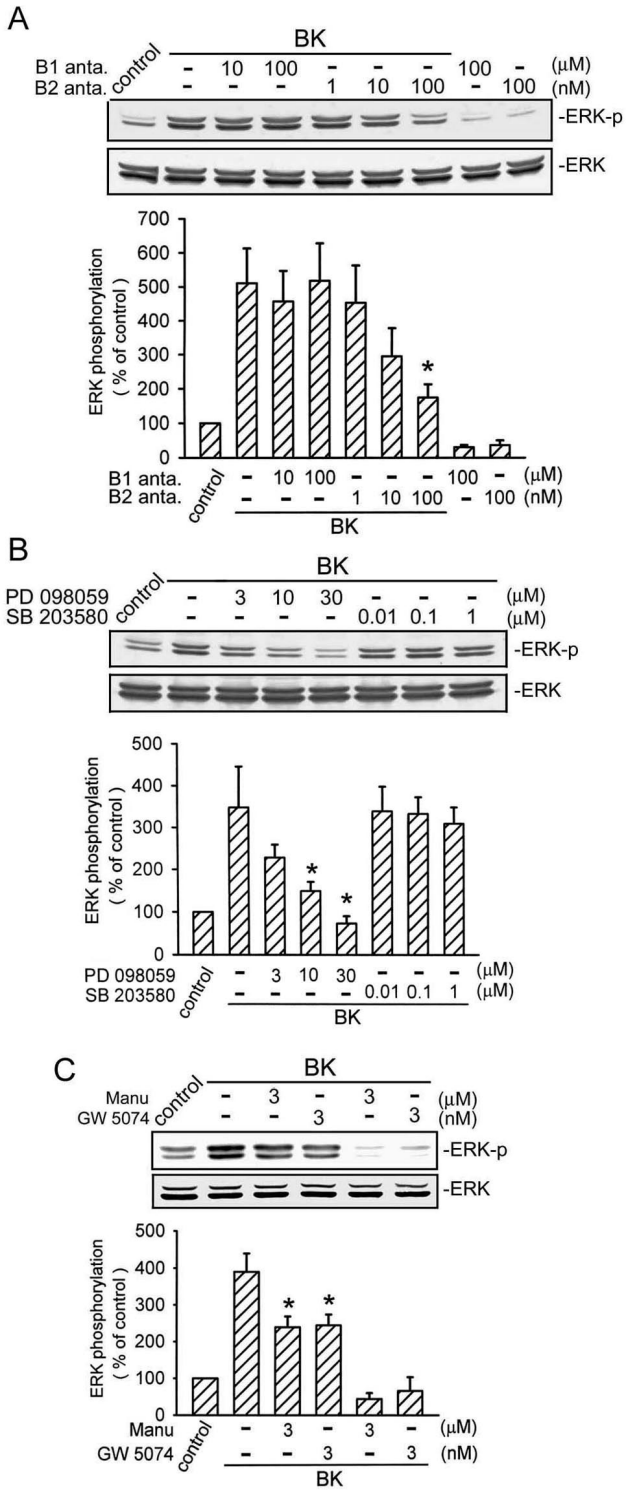

FIGURE 8. Mediation of BK-induced ERK activation through B2 BK receptors and involvement of the Ras/Raf-1/MEK signal pathway in BK-mediated ERK activation in A549 cells. *A*, Cells were pretreated with the B1 BK receptor antagonist (B1 anta.), Lys-(Leu^8)des-Arg^9-BK (10~100 μM), or the B2 BK receptor antagonist (B2 anta.), HOE140 (1~100 nM), for 30 min, and then stimulated with 10 nM BK for 3 min. *B* and *C*, Cells were pretreated with various concentrations of PD 098059 or SB 203580 (*B*), manumycin A or GW 5074 (*C*) for 30 min, and then incubated with 10 nM BK for another 3 min. Whole cell lysates were prepared and subjected to immunoblotting analysis using Abs specific for phosphorylated ERK (ERK-p) (*upper panel*) or nonphosphorylated ERK (ERK) (*bottom panel*). The extent of ERK activation was quantified using a densitometer with Image-Pro plus software Media Cybernetics (Silver Spring, MD). Traces represent results from three independent experiments, which are presented as the mean ± SE. *, $p < 0.05$ as compared with BK treatment.

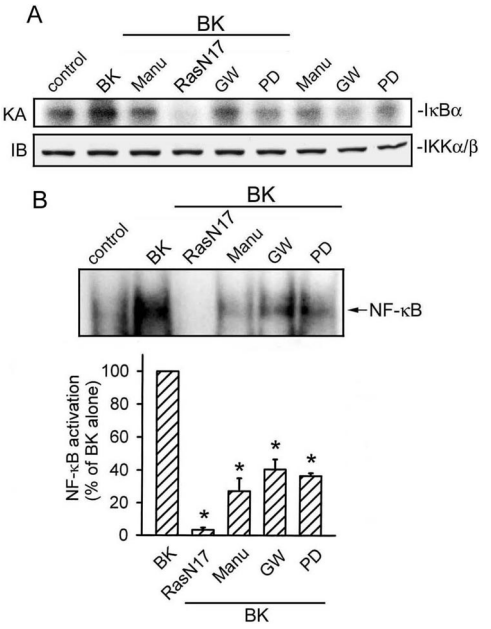

FIGURE 9. Involvement of the Ras/Raf-1/ERK signal pathway in BK-mediated increases in IKK$\alpha\beta$ activity and NF-κB activation in A549 cells. *A*, Cells were pretreated with vehicle, 3 μM manumycin A, 3 nM GW 5074, and 30 μM PD 098059 for 30 min, followed by stimulation with 10 nM BK for another 30 min. Whole cell lysates were prepared, and then immunoprecipitated with Abs specific to IKKα and IKKβ. One set of immunoprecipitates was subjected to the kinase assay (KA) using the GST-IκBα fusion protein as a substrate (*top panel*). The other set of immunoprecipitates was subjected to 10% SDS-PAGE and quantified by immunoblotting (IB) with anti-IKK$\alpha\beta$ Ab (*bottom panel*). Equal amounts of the immunoprecipitated kinase complex present in each kinase assay were confirmed by immunoblotting for IKK$\alpha\beta$. *B*, Cells were transiently transfected with 1 μg of the Ras dominant-negative mutant (RasN17) for 24 h or pretreated with 3 μM manumycin A, 3 nM GW 5074, or 30 μM PD 098059 for 30 min followed by stimulation with 10 nM BK for another 30 min. Nuclear extracts were prepared for determination of NF-κB-specific DNA protein-binding activity by EMSA as described in *Materials and Methods*. The extent of NF-κB activation was quantitated using a densitometer with Image-Pro plus software. Traces represent results from three independent experiments, which are presented as the mean $\pm$ SE. $*$, $p < 0.05$ as compared with BK treatment.

our results may be of importance in understanding the role of BK in the development of airway inflammatory diseases such as asthma.

COX-2 is an important inducible gene in inflammatory and airway diseases. A number of cytokines are known to induce COX-2 expression through both transcriptional and posttranscriptional mechanisms (13). Previous studies have shown that BK is capable of inducing COX-2 protein expression in airway smooth muscle cells, fibroblasts, and epithelial cells, but the mechanisms involved have not been extensively studied (40). A greater understanding of these mechanisms may provide important information to aid our understanding of how BK acts in airway inflammatory diseases. There are several binding sites for a number of transcription factors including NF-κB, NF-IL-6/C/EBP, AP-1, and cAMP response element in the 5′ region of the *COX-2* gene (41). Recent studies of the human COX-2 promoter have demonstrated that COX-2 induction by several transcription factors occurs in a highly stimulus-specific or cell-specific manner. For example, NF-κB has been shown to control the induced transcription of COX-2 in human lung epithelial cells and airway myocytes (42–44). In foreskin fibroblasts, COX-2 induction by IL-1β used the C/EBP-binding domain (45). In airway smooth muscle cells, BK-induced COX-2 expression was mediated by CCAAT/enhancer protein but not by NF-κB or C/EBP, whereas NF-κB was involved in IL-1β-induced COX-2 expression (46). The results of this study showed that NF-κB activation contributed to BK-induced COX-2 induction in A549 cells, and that the inhibitors of the NF-κB-dependent signaling pathway, including PDTC and TPCK, inhibited BK-induced COX-2 expression. Furthermore, BK induced IKK$\alpha\beta$ activation, IκBα phosphorylation, IκBα degradation, p65 and p50 translocation from the cytosol to the nucleus, and formation of an NF-κB-specific DNA-protein complex, as well as an increase in κB-luciferase activity. Moreover, BK-induced COX-2-luciferase activity was attenuated by transfection with the NF-κB mutant of COX-2 construct. Therefore, NF-κB activation is required to induce COX-2 transcription by BK in A549 cells. The results of this study also showed that Ras, Raf-1, and ERK are involved in NF-κB activation through an increase in IKK$\alpha\beta$ activity. A previous report showed that NF-κB activation is mediated via the Ras-dependent signaling pathway, which induces COX-2 transcriptional gene expression in lung epithelial cells (47). Another previous report also showed that in transformed liver epithelial cells, Ras and Raf lead to constitutive activation of NF-κB through the IKK$\alpha\beta$ complex (48). These pathways may mediate BK effect. As shown in Fig. 9, manumycin A, GW 5074, and PD 098059 blocked BK-induced IKK$\alpha\beta$ activation. In addition, RasN17 and these inhibitors attenuated the BK-mediated formation of the NF-κB-specific DNA-protein complex or COX-2 expression, implying a role in the pathway of NF-κB activation following BK stimulation.

Ras, an oncogenic protein, plays a critical role in inducing COX-2 expression (49, 50). Ras might activate a number of signal pathways, including the Raf-1/MEK/ERK pathway and the PI3K/Akt/NF-κB pathway (26, 51, 52). In murine fibroblasts, activation of the Ras/Raf-1/ERK1/2 signal pathway is required for COX-2 induction (50). In this study, we found that treatment of A549 cells with BK caused subsequent activation of Ras, Raf-1, and ERK, and that manumycin A, GW 5074, and PD 098059 all inhibited BK-induced ERK activation and COX-2 expression. These results suggest that the Ras/Raf-1/ERK signal pathway is very important for the induction of COX-2 caused by BK. This suggestion is further supported by our previous report that lipoteichoic acid induces COX-2 expression through the ERK pathway to induce NF-κB activation in human airway epithelial cells (A549) (53). However, the mechanism by which BK activates Ras has not yet been established. The B2 BK receptor is capable of coupling to several heterotrimeric G proteins depending on the cell type, including G_i/G_o, G_s, G_q, G_{11}, and G_{13} (20, 54–56). After ligand binding to G protein-couple receptor, heterotrimeric G proteins undergo GDP-GTP exchange, whereupon the tightly associated heterotrimeric G protein dissociates into α and β_γ subunits. A constitutively active mutant of $G_{i\alpha 2}$ has been shown to mediate Ras activation (57). A previous report also showed that in HEK293T cells, BK-induced ERK2 activation is dependent on G_i-induced Ras activation (58). However, the exact mechanism by which BK activates Ras needs to be further explored.

Two types of BK receptors have been defined and cloned: B1 and B2 BK receptors (29). We found that the B2 BK receptor antagonist HOE140 potently inhibited BK-induced ERK activation, NF-κB activation, and COX-2 expression, while the B1 BK receptor antagonist (Lys-(Leu^8)des-Arg^9-BK) was inactive. Therefore, BK-stimulated ERK activation, NF-κB activation, and COX-2 expression in human airway epithelial cells are mediated through the B2 BK receptor. Our results are in agreement with the findings that the B2 BK receptor is responsible for BK-stimulated NF-κB activation and IL-1β gene expression in human fibroblasts

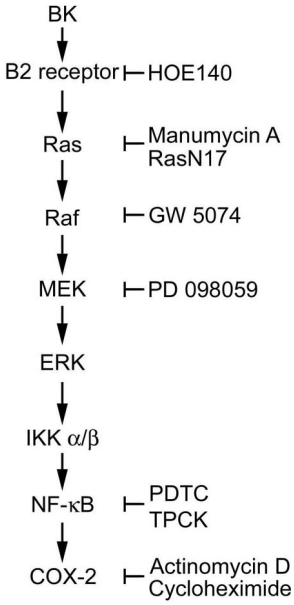

FIGURE 10. Schematic summary of signal transduction by BK induction of COX-2 expression in human airway epithelial cells (A549). BK, acting through the B2 BK receptor, activates the Ras/Raf-1/ERK pathway, which in turn increases IKK$\alpha\beta$ activity, IκBα degradation, and NF-κB activation, and finally induces COX-2 expression in A549 cells.

and epithelial cells (16, 17) and bronchoconstriction in isolated human airways (59, 60).

In summary, we have shown that BK, acting through B2 BK receptors, induces ERK and transcription factor NF-κB activation with a subsequent increase of COX-2 expression in human airway epithelial cells (A549). We have further shown that BK might activate the Ras/Raf-1/ERK pathway, which in turn initiates IKK$\alpha\beta$ and NF-κB activation, and finally induces COX-2 expression in human airway epithelial cells. Fig. 10 is a schematic representation of the signaling pathway of BK-induced COX-2 expression in human airway epithelial cells. These observations provide a novel possible explanation for the critical role of the BK in the transition of acute allergic reactions to chronic airway inflammatory diseases, such as asthma.

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