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Bradykinin stimulates interleukin-8 production by human lung fibroblasts

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SUMMARY

Bradykinin (BK) is a potent inflammatory mediator that is generated from kininogens by the actions of plasma and tissue kallikreins. Lung fibroblasts have the potential to participate in the inflammatory responses by releasing proinflammatory cytokines in response to a variety of stimuli. We postulated that human lung fibroblasts might produce interleukin-8 (IL-8) in response to BK stimulation. The present study showed that BK stimulated human lung fibroblasts to produce IL-8 in a dose- and time-dependent manner. Furthermore, Northern blot analysis showed that BK increased IL-8 mRNA expression. The stimulatory effect of BK on IL-8 production was detected at the concentration of 10 nM, and the maximal stimulation was achieved with 100 to 1000 nM. Phorbol 12-myristate 13-acetate pretreatment diminished the ability of BK to stimulate IL-8 production. In addition, GF109203X, a selective protein kinase C inhibitor, blocked BK-induced IL-8 production. These observations suggest that the stimulatory effect of BK on IL-8 production by lung fibroblasts is, at least partially, mediated through protein kinase C. These data suggest that BK may be involved in the inflammatory reaction leading to interstitial lung disorders through stimulating IL-8 production by lung fibroblasts.

INTRODUCTION

Bradykinin (BK) is a nonapeptide that influences the cardinal features of inflammation. Its actions include vasodilatation and vascular leakage.[1] BK is thought to be a potent inflammatory mediator that may be involved in asthma, sepsis and inflammatory joint disease.[1–3] Recent studies have also shown that BK plays a significant role in the pathogenesis of acute (adult) respiratory distress syndrome (ARDS) and sepsis-induced acute lung injury.[4,5] These findings suggest a pivotal role of BK and related kinins in the generation and continuation of tissue injury and inflammation of the lung. BK was found to stimulate synthesis of prostaglandins and leukotrienes via the activation of phospholipase A_2 in endothelial cells and fibroblasts.[1] The interactions between BK and other inflammatory mediators, such as proinflammatory cytokines, have also been reported.[6–9]

Interleukin-8 (IL-8) is one of the C-X-C chemokines, and has the ability to attract and activate neutrophils and other leucocytes.[10] IL-8 is produced by monocytes, alveolar macrophages, endothelial cells, epithelial cells and fibroblasts.[11–15] Elevated concentrations of IL-8 in the bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis (IPF) or pulmonary sarcoidosis may contribute to the influx of neutrophils into the pulmonary interstitium.[16,17] Extremely high levels of IL-8 are found in ARDS,[18,19] in which IL-8 and neutrophil numbers were correlated with mortality.[19] In this regard, IL-8 may play an important role in the pathogenesis of various inflammatory lung diseases including ARDS and IPF.

In the alveolar interstitium, the principal cell type is the fibroblast, which plays an integral role in repairing damaged lung tissue. Fibroblasts have the potential to participate in the inflammatory responses in the alveolar interstitium by releasing proinflammatory cytokines in response to a variety of stimuli.[20–22] To clarify further the role of BK in pulmonary biology, we characterized the effect of BK on IL-8 production by lung fibroblasts. In this report, we demonstrate for the first time that BK stimulates human lung fibroblasts to produce IL-8 by increasing its gene expression. Our findings also demonstrate that the stimulatory effect of BK on IL-8 production is mediated, at least in part, via the protein kinase C (PKC)-dependent activation pathway. These data support the notion that BK may be involved in the control of the inflammatory reaction associated with interstitial lung disorders through IL-8 production by lung fibroblasts.

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MATERIALS AND METHODS

Reagents

BK and GF109203X were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Interleukin-1β (IL-1β) and

interleukin-1 receptor antagonist (IL-1Ra) were purchased from Pepro Tech EC Ltd. (London, UK). Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Nissui Co. (Tokyo, Japan).

Cell culture

Human lung tissues were obtained from the lung of patients who were undergoing lobectomy because of lung cancer. Normal parts of the tissue were washed with PBS, and pleural tissue and bronchi were removed after resection. Specimens were cut into small pieces and digested with 0·5–1 μg/ml of clostridium collegenase (Wako) and 5–10 mg/ml of deoxyribonuclease 1 (Sigma) for 2 hr. After digestion, the single cells were filtered through sterile gauze, washed three times with DMEM and filtered through nylon mesh. The cells were resuspended in complete medium [DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS; ICN Biomedicals, Seven Hills, Australia), 100 U/ml penicillin G, 50 μg/ml streptomycin (Gibco, Grand Island, NY), and 2 mM L-glutamine (ICN)]. The cells were cultured overnight to allow them to adhere to the plastic dish. The dish was washed to remove non-adherent cells. Fibroblasts after three to seven passages were used as human lung fibroblasts, and grown to confluence at 37° in humidified 5% CO_2 in complete medium.

Measurement of IL-8

Immunoreactive IL-8 was measured using an enzyme-linked immunosorbent assay (ELISA) kit purchased from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). Lung fibroblasts were treated with trypsin and plated at a density of 1×10^5 cells/well in 24-well flat-bottom plates. The response of lung fibroblasts to BK was assessed by adding various concentrations of BK in serum-free DMEM. Cell-free culture supernatants were collected and stored at −20° until IL-8 assay. The supernatants were assayed for IL-8 according to the manufacturer's instructions. The assay sensitivity of this kit was approximately 1 pg/ml.

Northern blot analysis

Lung fibroblasts were seeded into culture dishes and stimulated for 4 hr with varying concentrations of BK. Total cellular RNA in the treated cells was extracted by an acid guanidium thiocyanate–phenol–chloroform method using ISOGEN (Nippon Gene, Tokyo, Japan) and Northern blot analysis was carried out as previously described.[23] Twenty micrograms of total RNA was size-fractionated by electrophoresis through 1% agarose/17% formaldehyde gels, and transferred to nylon membranes (Ammersham, Oakville, Canada). The 750 base pair cDNA of human IL-8 and 1·0 kilobase cDNA of human GAPDH inserted into pBluescript II SK^+ were used as probes.[24] The probes were labelled with digoxigenin dUTP by using a non-radioactive DNA-labelling and detection kit (Boehringer Mannheim, Mannheim, Germany). The intensity of the band was analysed by National Institute of Health Image 1.55 program.

Statistical analysis

All assays were performed in triplicate. The data were expressed as mean ± standard error of the mean. Differences between two groups were compared by Student's *t*-test. A P value less than 0·05 was considered as statistically significant.

RESULTS

Effect of BK on IL-8 production by lung fibroblasts

We examined whether treatment of human lung fibroblasts with BK modulated the production of immunoreactive IL-8. Lung fibroblasts were incubated for 24 hr with increasing amounts of BK, and IL-8 was measured in the supernatants. As shown in Fig. 1, lung fibroblasts produced trace amounts of IL-8 without stimuli. BK stimulated lung fibroblasts to produce IL-8 in a dose-dependent manner. The effect of BK was observed at the low concentration of 10 nM, and the maximal stimulation was achieved with 100–1000 nM (Fig. 1). The time–course of IL-8 production by lung fibroblasts was examined. Lung fibroblasts were stimulated with 100 nM BK and cultured for 24 hr. The amounts of IL-8 production in response to BK increased in a time-dependent manner, and reached a maximum at 24 hr (Fig. 2).

Northern blot analysis of IL-8 gene expression in BK-stimulated lung fibroblasts

We examined the IL-8 mRNA expression of BK-stimulated lung fibroblasts. Lung fibroblasts were stimulated for 4 hr with varying concentrations of BK. Unstimulated lung fibroblasts did not contain detectable levels of IL-8 mRNA, while a significant increase in IL-8 mRNA levels was observed upon stimulation with 10–1000 nM BK (Fig. 3). We examined time-dependent increases in IL-8 mRNA levels. An increase of IL-8 mRNA levels was detectable 30 min after BK stimulation, reached a maximum in 2–4 hr, and decreased thereafter (Fig. 4).

Effect of an IL-1Ra on BK-induced IL-8 production

Since it has been reported that BK stimulates IL-1β gene expression in cultured human fibroblasts,[29] it is possible that

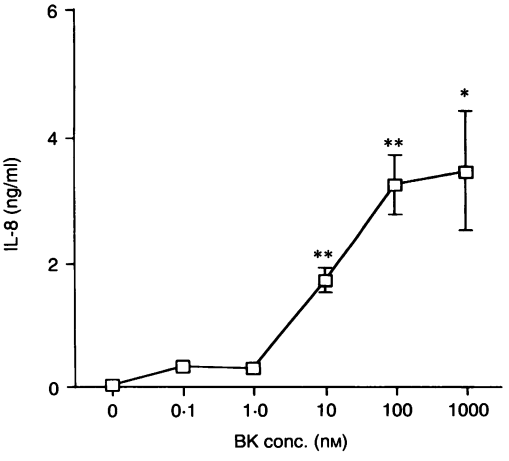

Figure 1. Effect of BK on IL-8 production by human lung fibroblasts. Cells were incubated for 24 hr with various concentrations of BK. IL-8 was determined by ELISA in the supernatants. Results are expressed as mean ± SEM of three separate experiments; $*P<0·05$; $**P<0·01$.

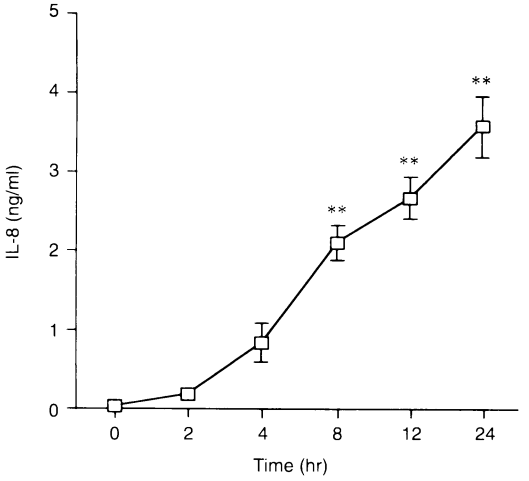

Figure 2. Time course of IL-8 production by human lung fibroblasts stimulated with BK. Cells were incubated with 100 nM BK for the time indicated, and IL-8 was determined by ELISA in the supernatants. Results are expressed as mean ± SEM of three separate experiments; $^{**}P<0{\cdot}01$.

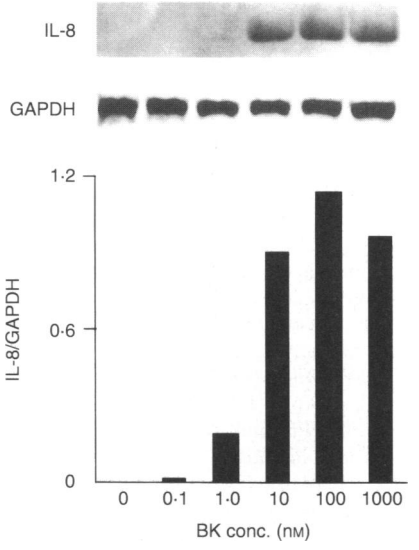

Figure 3. Effect of BK on IL-8 mRNA accumulation in human lung fibroblasts. Cells were incubated for 4 hr in the presence of various concentrations of BK. Total cellular RNA was extracted, and the levels of IL-8 mRNA were evaluated by Northern blot analysis. Densitometric ratios of IL-8 to GAPDH are shown in the lower panel. The data shown are representative of three identical experiments.

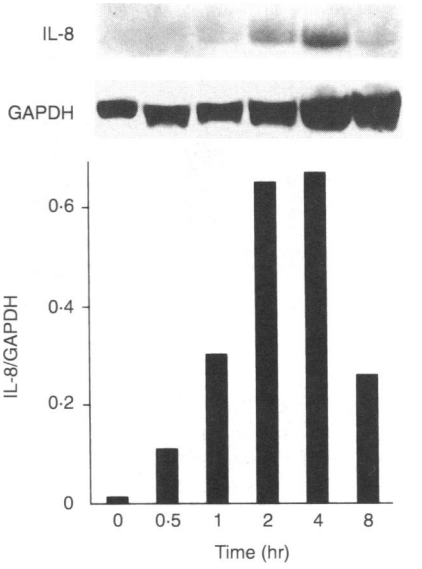

Figure 4. Time-course of BK-stimulated IL-8 mRNA expression in human lung fibroblasts. Cells were incubated with 100 nM BK. At the time indicated total cellular RNA was extracted, and the levels of IL-8 mRNA were evaluated by Northern blot analysis. Densitometric ratios of IL-8 to GAPDH are shown in the lower panel. The data shown are representative of three identical experiments.

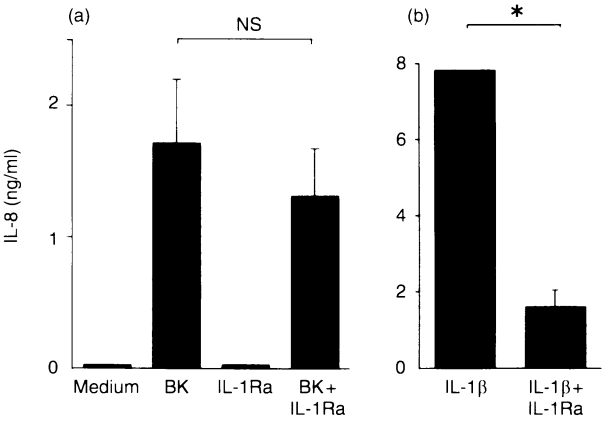

Figure 5. Effect of an IL-1 receptor antagonist (IL-1Ra) on BK-induced IL-8 production. Cells were incubated with 100 nM BK (a) or 10 pg/ml IL-1β (b) in the presence or absence of 20 ng/ml IL-1Ra for 24 hr. IL-8 was determined by ELISA in the supernatants. Results are expressed as mean ± SEM of three separate experiments; NS, not significant; $^{*}P<0{\cdot}001$.

BK-induced IL-8 production may require autocrine IL-1 production by BK-stimulated lung fibroblasts. We used a specific IL-1Ra in order to ascertain whether endogenous IL-1 production was involved in BK-induced IL-8 production. Figure 5 shows that IL-1Ra, at concentrations that block IL-1β-induced IL-8 production, did not significantly alter BK-induced IL-8 production.

PKC involvement in BK-induced IL-8 production

To investigate whether PKC was involved in BK-induced IL-8 production, we evaluated the effect of a modulator or an inhibitor of PKC. Since prolonged exposure to PMA lead to the inactivation of PKC in fibroblasts (Fig. 6a), we pretreated lung fibroblasts with PMA for 36 hr. As shown in Fig. 6(a), PMA pretreatment partially diminished BK-induced IL-8 production by lung fibroblasts. Furthermore, GF109203X, a specific inhibitor of PKC, markedly inhibited BK-induced IL-8 production by lung fibroblasts (Fig. 6b).

DISCUSSION

A variety of factors, including tissue damage, allergic reactions, viral infections and other inflammatory events, activate a series

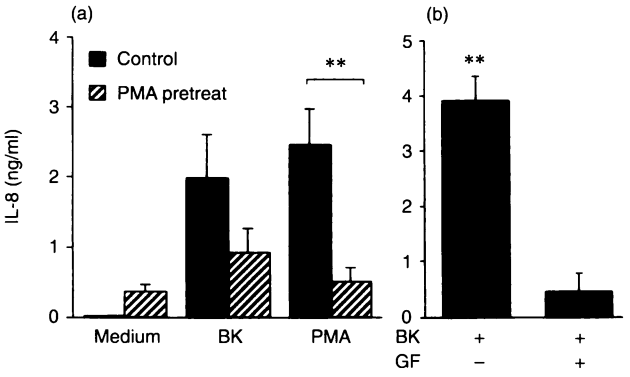

Figure 6. Protein kinase C (PKC) involvement in BK-induced IL-8 production. (a) Cells were incubated in the presence or absence of 100 nM PMA for 36 hr. Cells were subsequently stimulated with 100 nM BK or 10 nM PMA. (b) Cells were incubated with 100 nM BK in the presence or absence of GF109203X, a specific inhibitor of PKC, for 24 hr. IL-8 was determined by ELISA in the supernatants. Results are expressed as mean ± SEM of three separate experiments; $**P<0.01$.

of proteolytic reactions that generate BK in the tissues.[25] The pharmacological properties of BK have led to the suggestion that it may be an important mediator of inflammation.[26] In addition, BK is clearly capable of stimulating the release of other inflammatory mediators, such as prostaglandins, platelet-activating factor and proinflammatory cytokines.[6–9,27,28] Therefore, the roles of BK in inflammation and vascular permeability are an important consideration in the pathophysiology of pulmonary disorders such as asthma and acute lung injury.

Since lung fibroblasts at the sites of inflammation are likely to be exposed to BK,[1,26] we examined whether BK affected IL-8 production by lung fibroblasts. The present study clearly showed that BK stimulated human lung fibroblasts to produce IL-8 in a dose- and time-dependent manner along with increased IL-8 mRNA.

The mechanisms of the stimulatory effect of BK on IL-8 production remain to be clarified. Since BK was reported to augment IL-1β production by embryonal lung fibroblasts (WI-38),[29] it is possible that IL-8 production of lung fibroblasts may require endogenous IL-1 production by BK-stimulated fibroblasts. However, BK did not stimulate lung fibroblasts to release immunoreactive IL-1β in this system (data not shown). The reason for this differential response is not known. In our study, the response of lung fibroblasts to BK was assessed in serum-free medium. There may be differences in BK sensitivity for fibroblasts of similar tissue origin (e.g. lung). We examined the effect of an IL-1Ra on BK-induced IL-8 production by lung fibroblasts. An IL-1Ra did not significantly alter BK-induced IL-8 production. In addition, IL-8 mRNA accumulation was detectable as early as 30 min after BK stimulation. These observations suggest that the stimulatory effect of BK on IL-8 production may not require autocrine IL-1 production by lung fibroblasts.

BK is well known to activate PKC through the BK receptor that is coupled to G-protein.[30] To determine whether PKC was involved in BK-induced IL-8 production, we examined the effect of a modulator or an inhibitor of PKC in our culture system. PMA, a stimulator of PKC, stimulated IL-8 production by lung fibroblasts. We took advantage of the fact that prolonged exposure to PMA lead to the inactivation of PKC in fibroblasts.[31] In our experiments, PMA pretreatment partially diminished the ability of BK to induce IL-8 production. GF109203X, a selective PKC inhibitor, used at concentrations that effectively inhibited the PKC activity of lung fibroblasts, blocked BK-induced IL-8 production. These observations suggest that the stimulatory effect of BK on IL-8 production by lung fibroblasts is, at least partially, mediated through PKC. Studies are in progress to delineate mechanisms that are involved in BK-induced IL-8 production.

In conclusion, we have demonstrated for the first time that BK significantly stimulated the production of IL-8 by human lung fibroblasts, and that the stimulatory effect of BK on IL-8 production may be mediated via the PKC-dependent activation pathway. IL-8 may play a role in recruitment of neutrophils and other leucocytes from the vascular space into the alveolar interstitium in a number of interstitial pulmonary disorders.[16–19] Our findings suggest that the generation of BK in inflammatory pulmonary lesions, such as ARDS and IPF, may stimulate other inflammatory responses by increasing IL-8 production from lung fibroblasts.

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