Bradykinin increases IL-8 generation in airway epithelial cells via COX-2-derived prostanoids

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Rodgers, Helen C., Linhua Pang, Elaine Holland, Lisa Corbett, Simon Range, and Alan J. Knox. Bradykinin increases IL-8 generation in airway epithelial cells via COX-2-derived prostanoids. Am J Physiol Lung Cell Mol Physiol 283: L612–L618, 2002. First published April 12, 2002; 10.1152/ajplung.00483.2001.—Interleukin (IL)-8, the C-X-C chemokine, is a potent neutrophil chemoattractant that has been implicated in a number of inflammatory airway diseases such as cystic fibrosis. Here we tested the hypothesis that bradykinin, an inflammatory mediator and chloride secretagogue, would increase IL-8 generation in airway epithelial cells through autocrine generation of endogenous prostanoids. Bradykinin increased IL-8 generation in both a non-cystic fibrosis (A549) and cystic fibrosis epithelial cell line (CFTE290) that was inhibited by the nonselective cyclooxygenase (COX) inhibitor indomethacin and the COX-2 selective inhibitor NS-398. COX-2 was the only isoform of COX expressed in both cell lines. Furthermore, the COX substrate arachidonic acid and exogenous prostaglandin E2 both increased IL-8 release in A549 cells. These results suggest that bradykinin may contribute to neutrophilic inflammation in the airway by generation of IL-8 from airway epithelial cells. The dependence of this response on endogenous production of prostanoids by COX-2 suggests that selective COX-2 inhibitors may have a role in the treatment of airway diseases characterized by neutrophilic inflammation such as cystic fibrosis or chronic obstructive pulmonary disease.

interleukin-8; lung; chemokines; inflammation; cystic fibrosis; cyclooxygenase-2

INTERLEUKIN (IL)-8, the C-X-C chemokine, is a potent chemotactant for neutrophils (37) that has been implicated in a number of inflammatory diseases, such as cystic fibrosis (CF) (21), adult respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), and asthma (19). There are a number of sources of IL-8 in the airway, including structural cells such as airway epithelial cells (32). As airway epithelial cells form a barrier against invading microorganisms, production of IL-8 by airway epithelial cells is likely to contribute to host defense by promoting neutrophil chemotaxis and airway inflammation. Although this is a useful response in acute inflammatory responses, an exaggerated inflammatory response can contribute to pathogenesis in chronic disease. Neutrophil-driven lung destruction is particularly important in CF, where the inflammatory response occurs early in the disease (1, 4, 14) and is severe and sustained. Several agents increase IL-8 release by airway epithelial cells, particularly cytokines [IL-1β], tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and bacterial products (6, 10, 20). No information is available on whether proinflammatory mediators such as bradykinin (BK) also increase IL-8 and whether there are differences between CF epithelial cells and non-CF epithelial cells in the regulatory mechanisms.

BK is a nine-amino acid peptide that is formed locally in body fluids and tissues from the plasma precursor kininogen during inflammatory processes (25, 31, 33). Increased kallikrein levels have been reported in saliva in CF (18). BK is also a chloride secretagogue in several epithelial systems, including the airways (9, 26, 29). This may be of relevance in CF, where epithelial chloride secretion is impaired (28). BK increases IL-8 production in human lung fibroblasts (11), cultured human decidua-derived cells (5), and human airway smooth muscle cells (23), but not corneal epithelial cells (35), suggesting that the response to BK is tissue specific. The effect of BK on IL-8 release has not been studied in airway epithelial cells.

Previously, we reported that BK-induced IL-8 release is critically dependent on endogenous prostanoids in airway smooth muscle (23), although the cyclooxygenase (COX) isoform responsible was not defined, as airway smooth muscle expresses both COX-1 and COX-2 after BK treatment. It is not known whether BK-induced IL-8 release is COX dependent in other cell types. This might be particularly important in airway epithelial cells, which form a barrier to protect against microorganisms and generate several neutrophil chemoattractants. Prostanoid synthesis is mediated by two COX isoforms (36), constitutive COX-1 (24) and COX-2, which is inducible in most cells but is constitutively expressed in epithelial cells (2, 27). BK can release prostanoids in most cell systems by mobilizing arachidonic acid (AA) via phospholipase A2 (3, 34) and in some via induction of COX-2 (22). Here

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we tested the hypothesis that BK would induce IL-8 release by airway epithelial cells and determined the prostanoid dependence. Furthermore, we compared a non-CF airway epithelial cell line (A549) with a ΔF508-expressing human CF airway epithelial cell line (CFTE29ΔΔ) to determine whether prostanoid release and IL-8 generation were similarly regulated in CF epithelial cells. Because A549 cells are known to express solely COX-2 (2, 27), they provide a useful model system to determine whether prostanooids derived from COX-2 can stimulate IL-8 release. We found that BK induces IL-8 release in both cell lines and that this is dependent on COX-2-derived endogenous prostanooids. Our results suggest that COX-2 inhibitors may have a role in diseases characterized by neutrophilic inflammation such as CF and COPD.

MATERIALS AND METHODS

Cell culture. A549 cells were purchased from the European Collection of Animal Cell Cultures (ECACC no: 86012804; Salisbury, Wiltshire, UK). Aliquots of cells frozen in 10% dimethyl sulfoxide (dimethyl sulfoxide)-90% fetal calf serum (FCS) were thawed and suspended in culture medium comprising 90% DMEM-10% FCS containing 2 mM L-glutamine, 100 μg/ml penicillin G; and 100 μg/ml streptomycin; and 2.5 μg/ml amphotericin. 

CFTEΔΔ epithelial cells, derived from a human transformed tracheal epithelial cell line, homozygous for the ΔF508 CF mutation, were kindly provided by Professor D. C. Gruenert (University of California, San Francisco, CA). Aliquots of cells frozen in 10% DMSO-90% FCS were thawed and suspended in culture medium comprising 90% MEM-10% FCS containing 2 mM L-glutamine, 100 μg/ml penicillin G; and 100 μg/ml streptomycin.

Experiment protocols. Aliquots of cells frozen in 10% DMSO-90% FCS were thawed and centrifuged at 100 g. Cells were resuspended in 10% FCS (Seralab, Crawley Down, UK)-DMEM (A549 cells) or MEM (CFTE29ΔΔ cells) (both Sigma, Poole, UK) and plated in 24-well culture plates at a seeding density of 1 × 10^4 cells/cm^2 and grown in 95% air-5% CO2 at 37°C. Culture medium was replaced on alternate days until the cells were confluent. Cells were growth arrested for 24 h in media without FCS before all experiments.

In time course experiments cells were incubated with BK (10 μM) for 1 to 24 h, whereas in concentration response experiments, cells were incubated with 0.01–100 μM BK. At the indicated times, the culture media were harvested and stored at −20°C until the RIA for prostaglandin (PG) E2 content, as a representation of prostanoid generation (28), and/or an ELISA for IL-8 were performed.

To test the inhibition of various drugs on the effect of BK, we preincubated cells for 0.5 h with the nonselective COX inhibitor indomethacin (IND) or the selective COX-2 inhibitor NS-398 before incubation with BK. IND, purchased from Sigma, was dissolved in ethanol. NS-398 was purchased from Sigma (Sigma) and dissolved in distilled water. The vehicles did not alter PGE2 or IL-8 release (data not shown).

IL-8 assay. The concentration of the IL-8 in the culture medium was determined by ELISA (CLB, Amsterdam, The Netherlands) according to the manufacturer’s instructions. We have described this in detail elsewhere (23).

PGE2 assay. PGE2 concentration was determined by RIA as previously described (22). We have previously validated this assay and shown it to have a low cross-reactivity with other metabolites of AA. Each sample was analyzed for PGE2 in duplicate.

Western blot analysis. COX-1 and COX-2 expression were assessed by Western blotting (22). The protein concentration of cell extracts was determined with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hemel Hempstead, UK). Sufficient aliquots of sample (30 μg protein/track) were mixed 1:1 with sample buffer [20 mM Tris-HCl, pH 6.8, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, and 0.025% bromphenol blue; all from Sigma] and boiled for 5 min before electrophoresis.

Eletrophoresis was performed on a 20 × 20-cm 7.5% SDS-polyacrylamide gel (45 μA, 5 h). The separated proteins were then electrobotted (150 V, 3 h) to pure nitrocellulose membrane (Gelman Sciences, Northampton, UK). The blot was blocked for 2 h at 4°C in blocking reagent [8% fat-free dried milk powder in PBS, pH 7.4, with 0.3% Tween 20 (PBS-T)], incubated with primary monoclonal anti-human COX-2 antibody (1:2,000 in blocking reagent; Cayman Chemical) for 2 h at room temperature before being washed with PBS-T, and incubated with rabbit anti-mouse IgG coupled with horseradish peroxidase (HRP, 1:2,000 in blocking reagent; Sigma) for 1 h at room temperature. Semi quantitative staining was achieved by using enhanced chemiluminescence detection. We performed this detection by washing the blot with PBS-T, incubating it with the SuperSignal CL-HRP substrate system (Pierce, Rockford, IL) for 1 min, and finally exposing it to Hyperfilm ECL (Amersham Life Science, Little Chalfont, UK). The positions and molecular masses of COX-2 and COX-1 were validated by reference to rainbow-colored molecular mass markers (Amersham Life Science). We carried out reprobing of COX-1 by incubating the membrane in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl) at 50°C for 30 min with occasional agitation, washing the membrane in a large volume of PBS-T, blocking the membrane for 2 h in blocking reagent, and then following the steps described above to detect COX-1 with monoclonal anti-ovine COX-1 antibody (with cross-reactivity to human COX-1, 1:2,000 in blocking reagent; Cayman Chemical).

Statistical analysis. Data were expressed as means ± SE from n determinations. The statistical analysis was performed with the software program PRISM (Graphpad, San Diego, CA). A one-way ANOVA and/or an unpaired two-tailed Student’s t-test were used to determine the significant differences between means. P values <0.05 were accepted as statistically significant.

RESULTS

BK stimulates IL-8 release in A549 cells. To investigate the time course of IL-8 production, we cultured A549 cells in the presence or absence of BK (10 μM) as shown in Fig. 1A. There was a time-dependent increase in IL-8 production after stimulation with BK (88.9 ± 15.3 pg/ml at 1 h, 1,018 ± 104 at 20 h, 1,249 ± 60 at 24 h), which was significant compared with controls at all time points, showing a maximal twofold increase at 20–24 h (20 h, P < 0.01; 24 h, P < 0.001). A549 cells cultured with BK (0.001–100 μM) showed a concentration-dependent rise in IL-8 (Fig. 1B) compared with control after 8 h of incubation (1 μM, P < 0.001; 10 μM, P < 0.001; 100 μM, P < 0.001).

BK stimulates PGE2 release in A549 cells. Treatment of A549 cells with BK (10 μM) caused a time-dependent accumulation of PGE2 (Fig. 2A). The increase was...
There was a threefold increase in PGE$_2$ production, reaching a peak between 2 and 4 h (2 h, $P < 0.001$; 4 h, $P < 0.01$), which declined at 8 h ($P < 0.05$) and was not significant at 16 h. The effect was concentration dependent as seen in Fig. 2B, with a significant effect seen at a concentration of 10 µM ($P < 0.001$).

**COX isoform responsible for PGE$_2$ release in A549 cells.** Western blot showed that COX-2 was expressed under resting conditions but that COX-1 was not (Fig. 3). There was no effect of BK on COX-2 protein expression (Fig. 3), suggesting that the increase in PGE$_2$ produced by BK is likely to be mediated by AA release and prostanoïd production from existing COX-2 rather than further induction of COX-2.

**Effect of COX inhibitors on BK-induced IL-8 and PGE$_2$ production in A549 cells.** To investigate the role of endogenous PGs in BK-induced IL-8 and PGE$_2$ generation, we studied the effect of the nonselective COX inhibitor IND and the selective COX-2 inhibitor NS-398 (both at concentrations of 1 µM) on BK-induced IL-8 and PGE$_2$ release. BK-induced IL-8 production was significantly inhibited by both NS-398 ($P < 0.01$) and IND ($P < 0.001$), as shown in Fig. 4A. BK-induced PGE$_2$ release was also inhibited by IND and NS-398 (Fig. 4B; $P < 0.001$ for IND, $P < 0.01$ for NS-398). These findings suggest that BK-induced IL-8 production is partly mediated by endogenous COX products.

**Effect of exogenous COX substrate AA or PGE$_2$ on IL-8 production in A549 cells.** Furthermore, we examined whether exogenously applied COX substrate AA or PGE$_2$ would increase IL-8 release. A549 cells were cultured in the presence of AA (0.1–10 µM) or PGE$_2$ (0.1–10 µM), and cell culture supernatants were collected at 16 h. Both AA and PGE$_2$ significantly increased IL-8 release in a concentration-dependent manner, with values increasing from 95 ± 5 pg/ml in control cells to 168 ± 10 pg/ml after 10 µM PGE$_2$ and 128 ± 1 pg/ml after 10 µM AA (both $P < 0.001$, Fig. 5).

**BK stimulates PGE$_2$ release in CFTE290$^+$ epithelial cells.** BK (10 µM) caused a time-dependent PGE$_2$ accumulation (Fig. 6A), which reached a peak between 1 and 2 h (1 h, $P < 0.001$; 2 h, $P < 0.001$) and declined rapidly. The rise was smaller and less sustained than in the A549 cells. Lower concentrations of BK had no effect (Fig. 6B).

**COX isoform responsible for PGE$_2$ generation in CFTE290$^+$ epithelial cells.** As in A549 cells, we found that the only isoform of COX expressed in CFTE290$^+$ cells was COX-2 (Fig. 3). COX-2 was not induced by BK (Fig. 3), again suggesting that BK-induced PGE$_2$ release was likely mediated by AA release from phospholipase A$_2$ with subsequent generation of prostanoïds from existing COX-2 rather than further induction of COX-2.

**Fig. 1.** A: time-dependent stimulation of interleukin (IL)-8 release by 10 µM bradykinin (BK) in A549 cells. Cell culture supernatants were collected at times indicated in stimulated cells. B: concentration-dependent stimulation of IL-8 release by BK in A549 cells at 8 h of incubation. Each point represents the mean ± SE of 9 determinants from 3 experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, unpaired t-test.

**Fig. 2.** A: time-dependent release of prostaglandin (PG) E$_2$ by 10 µM BK in A549 cells at 0, 1, 2, 4, 8, and 16 h. B: concentration response of the effect of BK (0.01–10 µM) on PGE$_2$ production in A549 cells treated for 2 h. Each point represents mean ± SE of 9 determinations from 3 independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, unpaired t-test.
Effect of COX inhibitors on BK-induced PGE₂ and IL-8 production in CFTE29ο/H11002 epithelial cells. Because our results in A549 cells suggested that IL-8 release in these cells was related to COX products, we performed selected experiments to determine whether BK would induce IL-8 release in a prostanoid-dependent manner in the CF epithelial cell line CFTE29ο. BK caused IL-8 release that was significantly reduced by IND (P < 0.04) or NS-398 (P < 0.001), suggesting that endogenous COX products are involved (Fig. 7A). Consistent
Fig. 7. A: the effect of COX inhibitors on BK-induced IL-8 release in CFTE29o cells. Growth-arrested cells were incubated with 1 μM IND or 1 μM NS-398 for 30 min before treatment with BK (10 μM) for 20 h. Each time point represents the mean ± SE of 6 determinants from 2 independent experiments. B: the effect of various inhibitors on BK-induced PGE2 release in CFTE29o cells. Growth-arrested cells were incubated with 1 μM IND or NS-398 for 30 min before treatment with BK (10 μM) for 1 h. Each time point represents the mean ± SE of 9 determinants from 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, unpaired t-test.

DISCUSSION

The aim of this study was to test the hypothesis that BK would induce IL-8 release in airway epithelial cells and to determine the prostanoid dependence of the effect. There are several novel findings in our study. It is the first to show that BK increases IL-8 production in A549 and CFTE29o cells and to show that BK-induced IL-8 release is mediated in part through COX-2-mediated prostanoid generation in any epithelial cell line. The implication of these results is that COX-2 inhibitors may have a role in the treatment of lung diseases associated with neutrophilic inflammation.

We found that BK caused IL-8 release in both cell lines. Previous studies have shown that BK induces the release of neutrophil chemotactic activity in A549 cells and bronchial epithelial cells (15, 16), although the factors responsible have not been identified. Our results suggest that IL-8 may be a component of the neutrophil chemotactant activity produced by BK in airway epithelial cells. The IL-8 response of epithelial cells to BK appears to be tissue specific, as BK did not increase IL-8 release by corneal epithelial cells despite the presence of BK receptors (35).

CF is characterized by neutrophilic inflammation in the airways driven by IL-8 (12). IL-8 production is increased in bronchoalveolar lavage fluid and in cultured airway epithelial cells by a number of stimuli, including cytokines (IL-1β, TNF-α, IFN-γ), Pseudomonas aeruginosa, and respiratory syncytial virus (6, 10, 20). We found no evidence of increased IL-8 production by CF cells compared with A549 cells. This is consistent with the results of another study showing increased RANTES but not IL-8 production by CF epithelial cells (30). These findings do, however, contrast with a study by DiMango and colleagues (7), who show increased IL-8 production in CF IB3 cells, a ΔF508-expressing cell line, compared with wild-type cells due to increased activation of nuclear factor-κB.

In our studies there was also no evidence of greater PGE2 production by CFTE29o cells. Indeed the magnitude and duration of PGE2 production in response to BK were less in CFTE29o than in A549 cells. This contrasts with a study that showed a threefold increase in AA production and increased PGE2 production in response to BK in transformed human respiratory epithelial cells derived from a CF patient homozygous for ΔF508 compared with control cells (17). Subsequent studies suggested the increase in AA production in response to BK was due to increased phospholipase A2 activity (34). The disparity between our results and theirs is likely to reflect differences in the cell lines studied or the experimental protocols involved. CF is a heterogeneous condition with over 800 genotypes, and there may be differences in AA metabolism between genotypes. We used A549 cells because we and others have shown they are a good model system for the study of airway prostanoid production (2, 27).

Both A549 and CFTE29o cells exclusively expressed the inducible form of cyclooxygenase, COX-2, by Western blotting. This finding is consistent with our own previous work in A549 cells and other studies suggesting that COX-2 is the dominant COX isoform in airway epithelial cells. Others have shown that A549 cells also have absent COX-1 mRNA by Northern blotting (2). The constitutive expression of COX-2 in epithelial cells may reflect their unique situation where they act as a barrier against invading microorganisms and environmental insults. Ours is the first study to characterize the isoforms of COX present in any CF airway epithelial cell line. Because we found no evidence of further COX-2 induction in response to BK in either of the two cell lines we studied, BK-induced PGE2 production is likely to reflect AA release via phospholipase A2 in these cells, which then stimulates production of PGE2 from constitutive COX-2. Because BK has previously been shown to increase phospholipase A2 activity and AA release in A549 cells (34), we did not measure it here.

We found that BK-induced IL-8 production was inhibited by the nonselective COX inhibitor IND, suggesting a role for endogenous prostanoids in IL-8 generation. Furthermore, the COX-2 selective inhibitor NS-398 had a similar effect, suggesting that endogenous prostanoids generated by constitutively expressed...
COX-2 are responsible for BK-induced IL-8 generation by airway epithelial cells. Because these agents are structurally dissimilar, this effect is likely to be a COX-related effect. The results with NS-398 are consistent with our Western blot results showing that COX-2 is the only COX isoform expressed by these cells and the fact that exogenous PGE_2 stimulated IL-8 release. Unfortunately, there are no highly selective COX-1 inhibitors that can be used in whole cell systems. We have previously shown that both IND and NS-398 reduce COX-2-mediated PGE_2 production by >90% at these concentrations and that NS-398 is COX-2 selective (27).

Our assertion that BK increases IL-8 release via prostanoid generation is further strengthened by experiments with exogenous AA and PGE_2, both of which significantly increased IL-8 release in A549 cells. The effect of exogenous AA was relatively small, which probably reflects the fact that COX-2 is located at a perinuclear site; therefore, endogenous AA release is likely to be more effective in generating prostanoids, which can regulate gene transcription. We performed selected experiments in CPT29o cells, where BK-induced IL-8 production was inhibited by COX inhibitors, suggesting similar prostanoid dependence. The smaller increase in IL-8 generation in response to BK in the cells is likely to be due to their smaller, less-sustained prostanoid generation. The reason that transient increase in PGE_2 release can result in a sustained increase in IL-8 levels is that, as we have previously shown, IL-8 is stable over time under our culture conditions and therefore is accumulated (23).

We have considered the possible therapeutic implication of our studies. The fact that COX inhibitors can significantly reduce BK-induced IL-8 release in CF cells could provide an explanation for the beneficial effects reported with high doses of ibuprofen, a nonselective COX inhibitor on the rate of decline of lung function in children with CF (13). Further studies looking at the effect of selective COX-2 inhibitors in CF would therefore be of interest. COX-2 inhibitors could also have a role in other airway diseases characterized by neutrophilic inflammation, such as COPD or adult respiratory distress syndrome. It could be argued that effects of BK on IL-8 are relatively small and studies to determine whether such changes could alter neutrophil trafficking is vivo are required. BK does stimulate neutrophil chemotactic activity in epithelial cells in vitro, however, and it is likely that IL-8 is contributing to this. It would also be useful to determine whether COX inhibitors alter the IL-8 response to cytokines and bacterial agents.

In conclusion, we have shown that BK releases IL-8 by airway epithelial cells through generations of prostanoids from constitutively expressed COX-2. This suggests that there may be a role for selective COX-2 inhibitors in the treatment of lung diseases characterized by neutrophilic inflammation.

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