PGE$_2$ release by bradykinin in human airway smooth muscle cells: involvement of cyclooxygenase-2 induction

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Pang, Linhua, and Alan J. Knox. PGE$_2$ release by bradykinin in human airway smooth muscle cells: involvement of cyclooxygenase-2 induction. Am. J. Physiol. Lung Cell. Mol. Physiol. 17; L1132–L1140, 1997.—Prostanoids may be involved in bradykinin (BK)-induced bronchoconstriction in asthma. We investigated whether cyclooxygenase (COX)-2 induction was involved in prostaglandin (PG) E$_2$ release by BK in cultured human airway smooth muscle (ASM) cells and analyzed the BK receptor subtypes responsible. BK stimulated PGE$_2$ release, COX activity, and COX-2 induction in a concentration- and time-dependent manner. It also time dependently enhanced arachidonic acid release. In short-term (15-min) experiments, BK stimulated PGE$_2$ generation but did not increase COX activity or induce COX-2. In long-term (4-h) experiments, BK enhanced PGE$_2$ release and COX activity and induced COX-2. The long-term responses were inhibited by the protein synthesis inhibitors cycloheximide and actinomycin D and the steroid dexamethasone. The effects of BK were mimicked by the B$_2$-receptor agonist [Tyr(Me)$_2$]BK, whereas the B$_1$ agonist des-Arg$_9$-BK was weakly effective at high concentrations. The B$_2$ antagonist HOE-140 potently inhibited all the effects, but the B$_1$ antagonist des-Arg$_9$-[Leu$_8$]-BK was inactive. This study is the first to demonstrate that BK can induce COX-2. Conversion of increased arachidonic acid release to PGE$_2$ by COX-1 is mainly involved in the short-term effect, whereas B$_2$ receptor-related COX-2 induction is important in the long-term PGE$_2$ release.

prostaglandin E$_2$; airway inflammation; asthma; bradykinin-receptor agonists; bradykinin-receptor antagonists

**BRADYKININ** (BK) is a nine-amino acid peptide that is formed locally in body fluids and tissues from the plasma precursor kininogen during inflammatory processes. It has been reported that asthmatic patients have elevated kinin concentrations in plasma and in nasal and bronchoalveolar lavage fluid (BALF) after allergen challenge (5, 9); BK elicits many features of bronchial asthma such as bronchoconstriction (16, 21). Reports have shown that prostanoids may be involved in BK-induced bronchoconstriction because COX inhibitors such as indomethacin (Indo) significantly attenuate BK-induced bronchoconstriction (16, 21). It is now known that two isoforms of COX mediate prostanoid production (32). COX-1 produces physiological levels of prostanoïds and is constitutively expressed under normal conditions in most tissues (31). COX-2, the inducible isozyme of the enzyme, is induced in many cells under the stimulation of inflammatory mediators such as lipopolysaccharides and cytokines (23, 24). Accumulating evidence suggests that the induction and regulation of COX-2 may be key elements in the pathophysiological process of a number of inflammatory disorders such as asthma. Studies in bovine (10) and guinea pig (13) ASM cells have shown that BK causes PGE$_2$ release, but the same effect has not been demonstrated in cultured human ASM cells and the underlying mechanisms have not been fully investigated. Our group and others have recently shown that interleukin-1$b$ (IL-1$b$) (24) or a mixture of cytokines (6) induces COX-2 in cultured human ASM cells and that this induction is mainly responsible for IL-1$b$-induced prostanoid release (24). Although BK has been reported to cause COX-2 mRNA expression in cultured fibroblasts (29), direct evidence for COX-2 protein induction in response to BK has not been shown in any cell type and the role of COX induction in BK-induced prostanoid generation in ASM cells has not been explored. The aim of this study was, therefore, to investigate the effect of BK on prostanoid (PGE$_2$) production in human ASM cells and to determine the mechanisms responsible, particularly whether COX isoenzyme induction is involved. In addition, we characterized the receptors involved in this BK-induced action by comparing the effect of BK with selective B$_1$- and B$_2$-receptor agonists and by using selective B$_1$- and B$_2$-receptor antagonists to inhibit the responses. We also assessed the effect of the COX inhibitor Indo, the protein synthe-
sis inhibitors cycloheximide (CHX) and actinomycin D (Act), and the anti-inflammatory steroid dexamethasone (Dex) on PGE2 release, COX activity, and the induction of COX-2 isoenzyme after stimulation by BK.

MATERIALS AND METHODS

Cell Culture

Primary cultures of adult human ASM cells were prepared from explants of ASM according to methods previously reported (14, 24). Briefly, human tracheae were obtained from two postmortem individuals (one man aged 44 yr and one woman aged 52 yr with no evidence of airway diseases) within 12 h of death. The trachea was dissected free of epithelium and connective tissue under sterile conditions. Small (2 x 2-mm) explants of ASM were then excised, and ~10 explants were placed in one small petri dish. The explants were incubated in 10% fetal calf serum (FCS)-Dulbecco’s modified Eagle’s medium (DMEM) in humidified 5% CO2-95% air at 37°C, and the medium was changed every 3 days. Smooth muscle cells were usually seen arrested for 24 h in serum-free medium and then incubated 5 days. Smooth muscle cells were usually seen arrested for 24 h in serum-free medium and then incubated 5 days. Smooth muscle cells were usually seen arrested for 24 h in serum-free medium and then incubated 5 days. 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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 at 4°C, and then following the above steps 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 are expressed as means and SE from n determinations. Statistical analysis was performed with the statistical software SPSS (22). Unpaired two-tailed t-test or one-way analysis of variance was used to determine the significant differences between means. The results were adjusted for multiple testing with Bonferroni’s correction. P values < 0.05 were accepted as statistically significant.

**RESULTS**

Time Course of PGE₂ Release, AA Mobilization, COX Activity, and COX Isoform Induction in Response to BK

Treatment of human ASM cells with BK (10 µM) caused a time-dependent accumulation of PGE₂ (Fig. 1A). The increase was significant after only 5 min of incubation (P < 0.001) and was sustained for 16 h of treatment. BK also stimulated [3H]AA release in a time-dependent manner that was significant after 5 min of incubation (P < 0.001; Fig. 1B). The time-dependent enhancement of PGE₂ generation was associated with a time-dependent increase in COX activity (Fig. 1C). The increase was significant as early as 2 h after stimulation (P < 0.01), reached a peak at 4–8 h, and declined gradually at 16 and 24 h. No changes were seen in the time-matched control cells. Direct evidence for a time-dependent induction of COX-2 enzyme protein in human ASM cells after exposure to BK was obtained by Western blotting of cell extracts with a specific antibody that resolves COX-2 from COX-1. As shown in Fig. 2, COX-2 was undetectable in untreated human ASM cells. After treatment, however, COX-2 protein bands started to appear at 2 h, reached a maximum at 4 h, and gradually reduced from 8 to 24 h. Reprobing of the same blot with an antibody specific to COX-1 showed that COX-1 enzyme protein bands existed in both treated and untreated cells and remained virtually unchanged (Fig. 2). Because there was a parallel increase in COX-2 protein and COX activity after a 2- to 4-h incubation with BK, different mechanisms were considered to be involved in the short- and long-term PGE₂ production. Some of the following experiments aimed at investigating the BK receptors responsible for the BK response were therefore conducted in the short term (15 min) and long term (4 h), respectively.

Pharmacological Characterization of BK Receptors Involved Using Selective Agonists

Concentration response of PGE₂ release, COX activity, and COX-2 induction in response to BK and BK-receptor agonists. Treatment of human ASM cells with BK for 15 min (short term) produced a concentration-dependent release of PGE₂. The effect was significant from the lowest concentration tested (10⁻⁸ M; P < 0.001) and reached a peak at 10⁻⁵ M (Fig. 3). Treatment of the cells with the B₂-receptor agonist [Tyr(Me)₈]BK for 15 min also produced a concentration-dependent release of PGE₂ in a pattern similar to that of BK, but the maximum effect was reached at 10⁻⁶ M (Fig. 3). The concentrations of PGE₂ released into the medium were also similar after stimulation with BK and [Tyr(Me)₈]BK. In contrast, PGE₂ production after stimulation with the B₁-receptor agonist des-Arg⁹-BK was much lower than that with BK and [Tyr(Me)₈]BK. The small effect seen was only significant from 10⁻⁶ M
PGE2 PRODUCTION BY BRADYKININ

Fig. 3. Concentration response of effect of BK, B1-receptor agonist des-Arg9-BK, and B2-receptor agonist [Tyr(Me)8]BK on short-term (15-min) PGE2 release in human ASM cells. Each point represents mean ± SE of 12 determinations from 3 independent experiments.

(P < 0.01), and even at a very high concentration (10^-4 M), PGE2 release was still much smaller (Fig. 3). The order of potency of [Tyr(Me)8]BK = BK > des-Arg9-BK suggested that the response was mediated by B2 receptors.

Incubation of these cells with BK, [Tyr(Me)8]BK, and des-Arg9-BK for 4 h (long term) also produced a concentration-dependent release of PGE2 (Fig. 4A), with a similar pattern as short-term incubation. This was accompanied by a concentration-dependent induction of functionally active COX as shown by washing the cells after a 4-h exposure to these stimulators followed by incubation with exogenous AA (Fig. 4B). COX activity was low with the untreated cells and was increased steadily after treatment with BK and [Tyr(Me)8]BK, was significant at 10^-7 and 10^-6 M, respectively. In contrast, des-Arg9-BK was only weakly effective; a significant and much smaller increase was only observed at 10^-4 M (P < 0.001; Fig. 4B). Western blot results showed that untreated human ASM cells contained undetectable levels of COX-2; however, after treatment with BK or BK-receptor agonists, a concentration-related induction of COX-2 isoenzyme was observed (Fig. 5). Again, the potency was different: BK and [Tyr(Me)8]BK were effective from 10^-7 and 10^-8 M, respectively, and des-Arg9-BK was only effective at 10^-4 M, which was in agreement with the COX activity results (Fig. 4B). The order of potency of [Tyr(Me)8]BK = BK > des-Arg9-BK further suggested that these effects are largely mediated by the activation of B2 receptors rather than of B1 receptors.

Full time course of PGE2 release, COX activity, and COX isofrom induction in response to BK-receptor agonists. After the concentration-response relationship in short-term and long-term experiments was characterized, the full time course of the effect of the selective agonists was studied. The B2-receptor agonist [Tyr(Me)8]BK (1 µM) and the B1-receptor agonist des-Arg9-BK (100 µM) both caused time-dependent enhancement in PGE2 release. The enhancement was significant for both agonists after only 5 min of stimulation (63.0 ng/mg protein, P < 0.001 for [Tyr(Me)8]BK and 9.8 ng/mg protein, P < 0.01 for des-Arg9-BK compared with 2.0 and 3.3 ng/mg protein, respectively, for the control cells) and was sustained for 16 h of treatment. The enhancement was accompanied by a time-dependent increase in COX activity that became significant after a 2-h incubation (103.2 ng/mg protein, P < 0.01 for [Tyr(Me)8]BK and 94.2 ng/mg protein, P < 0.01 for des-Arg9-BK compared with 76.0 and 69.3 ng/mg protein, respectively, for the control cells), reached the peak at 8 h (253.0 and 96.1 ng/mg protein, respectively), and declined gradually at 16 and 24 h. COX-2 induction became visible at 1 h and peaked at 4 h for both agonists and gradually reduced from 8 h for [Tyr(Me)8]BK or diminished at 16 h for des-Arg9-BK (blot not shown). No change in the constitutive COX-1 isofrom was observed. The time-course pattern of the B2-receptor agonist [Tyr(Me)8]BK was very similar to that of BK in terms of PGE2 release, COX activity, and
COX-2 induction. The B₁-receptor agonist des-Arg⁹-BK was much less potent than BK and [Tyr(Me)⁸]BK despite being used at a much higher concentration. The results therefore are consistent with activation of B₂ receptors rather than B₁ receptors being involved in BK-induced PGE₂ release, COX activity, and COX-2 induction in human ASM cells.

Pharmacological Characterization of BK Receptors Involved Using Selective Antagonists

Effect of BK-receptor antagonists on PGE₂ release, COX activity, and COX-2 induction in response to BK and BK-receptor agonists. In the short-term experiments, pretreatment of human ASM cells with the B₂-receptor antagonist HOE-140 (1–100 µM) strongly inhibited BK-induced PGE₂ release in a concentration-dependent manner and abolished the effect of BK (10 µM) at 100 µM (Fig. 6), whereas pretreatment with the B₁-receptor antagonist des-Arg⁹,Leu⁸)-BK (1–100 µM) did not show any significant inhibition of PGE₂ release caused by BK (Fig. 6).

In the long-term experiments, HOE-140 exerted a strong and concentration-related inhibition of PGE₂ release, whereas des-Arg⁹,Leu⁸)-BK was ineffective (Fig. 7). Similar results were obtained for COX activity where HOE-140 strongly antagonized the increase in PGE₂ synthesis caused by BK (data not shown). Conversely, the B₁-receptor antagonist des-Arg⁹,Leu⁸)-BK was ineffective. The small responses to high concentrations of the B₁ agonist des-Arg⁹-BK were inhibited by both the B₂-receptor antagonist HOE-140 and the B₁-receptor antagonist des-Arg⁹,Leu⁸)-BK (data not shown). These data suggest that B₂ receptors contribute largely to PGE₂ production and the increase in COX activity after BK stimulation and that B₂-receptor activation is essential in the induction of COX-2 in BK-stimulated human ASM cells.

Effect of Various Inhibitors on BK-Stimulated PGE₂ Synthesis, COX Activity, and COX-2 Induction

The effects of the nonsteroidal anti-inflammatory drug Indomethacin (Indo), the protein synthesis inhibitors CHX (a translation inhibitor) and Act (a transcription inhibitor), and the steroid Dex were also assessed on BK-induced short-term and long-term PGE₂ release and COX activity. In the short-term experiments, Indo (1 µM) completely blocked the BK (10 µM)-induced increase in PGE₂ synthesis (P < 0.001), whereas CHX, Act, and Dex (all 1 µM) were without effect (Fig. 9A). Although BK did not significantly increase short-term COX activity, the basic COX activity (COX-1) was still strongly inhibited by Indo (P < 0.001) but not by CHX, Act, or Dex (Fig. 9A), suggesting that the de novo synthesis of protein and mRNA was not involved in BK-induced short-term PGE₂ production. In the long-term experiments (Fig. 9B), BK-induced enhancement in PGE₂ accumulation was also abolished by Indo (P < 0.001) and markedly inhibited by CHX (P < 0.05), Act (P < 0.001), and Dex (P < 0.001). As observed before, BK caused a significant enhancement in COX activity, and the increase was not only blocked by Indo (P < 0.001) but also strongly suppressed by CHX, Act, and Dex (P < 0.001; Fig. 9B). The effect of the protein synthesis inhibitors and Dex on COX-2 induction was further examined. As detected by Western blotting analysis, CHX strongly inhibited and Act and Dex abolished BK-induced COX-2 induction (blot not shown).
These results provide further and direct evidence that COX-1 is mainly responsible for the accumulation of PGE2 after short-term stimulation and that the exaggerated PGE2 release after long-term incubation with BK in human ASM cells is at least partly composed of de novo synthesis of COX-2 protein that is solely responsible for the enhancement in COX activity.

Cytotoxicity of the Reagents Used in the Study

Cytotoxicity to human ASM cells after 24 h of treatment with all the reagents used in the study was measured by MTT assay, and cell viability was consistently >95% compared with the untreated cells (data not shown). Furthermore, no change in the protein level was observed after any of the treatments (data not shown).

DISCUSSION

Our results showed that BK induced PGE2 production in cultured human ASM cells and that the PGE2 production had a rapid and a sustained phase. We studied the early production at 15 min, and we chose 4 h to study the long-term effect of BK because 4 h was the time of maximum COX-2 induction as determined by Western blotting. In short-term experiments, BK induced a concentration-related increase in PGE2 release but had no effect on COX activity or COX isoenzyme induction. The exaggerated PGE2 release was abolished by the COX inhibitor Indo but was not affected by the protein synthesis inhibitors CHX and Act and the steroid Dex. The results, therefore, indicate that the constitutive COX-1 is responsible for PGE2 release in the short-term experiments. Because BK has been reported to cause AA release via the rise in cytosolic free Ca2+ and activation of the 85-kDa cPLA2 in a number of cultured cells, including ASM cells (30), and because it was found to cause AA release in a time-dependent manner in this study, the short-term increase in PGE2 production by BK in human ASM cells is likely to be due to the increased mobilization of AA and subsequent conversion to PGE2 by the existing COX-1. Increased release of AA is also likely to have contributed to the late increase in PGE2 seen in our long-term experiments. We considered whether the induction of cPLA2 might have contributed to the late increase in PGE2 but found no evidence of cPLA2 induction (data not shown).

In our long-term experiments, we also found, however, that BK stimulated COX activity, and this was accompanied by a corresponding increase in COX-2 enzyme induction that was first apparent at 2 h. In contrast, COX-1 expression was not affected by BK. The late enhancement of PGE2 production and COX activity were abolished by Indo and markedly inhibited by CHX, Act, and Dex. The protein synthesis inhibitors and Dex also strongly suppressed COX-2 induction in response to BK stimulation. The results suggest that the enhanced PGE2 generation in the long-term experi-
ments after BK treatment is largely attributable to the
induction of COX-2, consistent with the hypothesis that
COX-1 is a constitutive isoform, whereas COX-2 is an
inducible isoform responsible for the generation of
prostanoids under inflammatory conditions (31). Al-
though BK has been reported to cause COX-2 mRNA
expression in cultured fibroblasts (29), the present
study is the first that we are aware of to demonstrate
directly that BK causes the induction of COX-2 protein
in any cell type and that the induction forms part of the
BK-induced PGE2 generation. Previous studies of air-
way cells have shown that cytokines (mainly IL-1β) can
increase inflammatory gene expression. Our results
suggest that kinins can also regulate inflammatory
gene expression. Because asthmatic patients have ele-
vated levels of kinin concentrations (5, 9) in nasal fluid
and BALF, our results may be of importance to the
understanding of the airway inflammation in asthma.

Two BK receptor subtypes (B1 and B2) have been
identified (15, 20). B1 and B2 receptor-mediated re-
ponses can be distinguished pharmacologically on
the basis of the relative potencies of agonists or by the
use of receptor-selective antagonists. For the B2 recep-
tor, the order of potency of agonists is [Tyr(Me)8]BK ≈
BK > des-Arg9-BK, whereas for the B1 receptor, the
order is reversed: des-Arg9-BK > BK > [Tyr(Me)8]BK
(11). In our experiments with selective agonists, the
B2-receptor agonist [Tyr(Me)8]BK reproduced all the
effects of BK in a very similar time- and concentration-
dependent pattern, whereas the B1-receptor agonist
des-Arg9-BK was effective only at much higher concen-
trations compared with BK. The PGE2 concentration
and the COX activity in response to des-Arg9-BK stimu-
lation were also considerably lower than those of
BK. The order of potency, therefore, was [Tyr(Me)8]BK ≈
BK > des-Arg9-BK, suggesting that the BK response is
mediated by B2 receptors. The results with selective
agonists were consistent with those obtained with
selective antagonists. The selective B2-receptor antago-
nist HOE-140 concentration dependently and potently
suppressed all the effects of BK, whereas the selective
B1-receptor antagonist des-Arg9,(Leu8)-BK was with-
out effect. These results suggest that all of the actions
of BK are mediated by B2 Receptors. It is unlikely that
the B1 receptors were also involved because the B1
antagonist des-Arg9,(Leu8)-BK was only slightly effec-
tive and the weak effect of the B1-receptor agonist
des-Arg9-BK could be explained by the lack of receptor
selectivity at high concentrations. Our results are in
agreement with the findings that B2 receptors are
responsible for BK-induced bronchoconstriction ob-
erved in isolated human airways (16, 21) and PGE2
production in cultured guinea pig ASM cells (13).

On the basis of the observations that B1- and B2-
receptor antagonists have very weak inhibitory effects
on BK-induced bronchoconstriction in guinea pigs, on
3H]BK binding in guinea pig and sheep airway tissues,
and on the BK-induced efflux of Ca2+ in cultured guinea
pig tracheal smooth muscle cells, a novel BK receptor
subtype, B3, has been proposed (12, 13). All of the effects
of BK in our present study were inhibited by the
selective B2-receptor antagonist HOE-140, which does
not provide any evidence to support the existence of a
novel B3 receptor in cultured human ASM cells, at least
in terms of PG production.

It is not clear whether the consequences of COX-2
induction and PGE2 production by BK in human ASM
would be detrimental or beneficial for airway function
in inflammatory airway diseases. Prostanoids are im-
portant mediators known to influence many aspects of
airway function such as airway tone, mucus secretion,
and cell proliferation. PGE2 is an important anti-
flammatory mediator and has considerable broncho-
protective effects in the airways (25), and because PGI2,
like PGE2, is also coupled to adenosine 3′,5′-cyclic
monophosphate elevation, it could have a similar protec-
tive effect (17). It is possible, therefore, that the exagge-
rated PGE2 production as a result of COX-2 induction is
part of a negative feedback mechanism that is exerting a braking effect on the inflammatory response. The induction of COX-2 itself may also shunt the released AA away from the generation of a potent bronchoconstrictor of the lipoxygenase pathway toward the synthesis of bronchodilators such as PGE₂ and PGI₂ of the COX pathway. However, PGE₂ at higher concentrations also causes ASM contraction (2) due to weak agonism at the Tx receptor (18), and other products of COX, such as PGI₂, TXA₂, and PGD₂, are potent proinflammatory modulators that cause bronchoconstriction via activation of the Tx prostaglandin receptor (2, 18). BK has been shown to generate prostanooids in various airway tissues, and PGE₂ and PGI₂ have been shown to be the dominant COX products (7, 16). In our previous study (24), we also found that after stimulation with IL-1β human ASM cells released large quantities of PGE₂ and PGI₂ and the release of PGE₂, TXA₂, and PGD₂ was much lower. Although we did not measure the spectrum of prostanooids produced after COX-2 induction in the present study, it is likely that it would be similar. Further studies are required to determine whether BK alters the activity of specific PG synthases. Accumulating reports have shown that BK-induced bronchoconstriction is significantly attenuated by COX inhibitors such as Indomethacin (16, 21) and by TXA₂ receptor/synthetase inhibitors (1, 16). BK-induced airway responses may therefore be largely mediated by the release of prostanooids that contract airways via activation of the TXA₂ receptor. However, the overall impact of prostanooids on the airway may then depend on the cell type, the balance of the prostanooids released, and the prostanooid receptor subtype activated.

The fact that BK causes bronchoconstriction in asthmatic subjects but not normal subjects is intriguing. This may be a dose-related phenomenon, and if enough BK was given to normal subjects, they would develop bronchoconstriction. Alternatively, it could be speculated that the spectrum of prostanooids produced may differ between asthmatic and normal subjects or that because COX-2 is already partially induced by cytokines in asthma, BK may be having an additive or synergistic effect. Immunohistochemical studies of asthmatic airways would be useful to determine the relative COX-1 and COX-2 distribution in ASM cells.

In conclusion, our studies have demonstrated that human ASM cells release large quantities of PGE₂ in response to BK stimulation. The underlying mechanisms are different for the short-term and long-term responses. Although both are mediated by B₂ receptors, short-term increases are due to the conversion by existing COX-1 of increased AA release to PGE₂, whereas the long-term increases are mainly due to the induction of COX-2. Because elevated levels of kinin (9) and proinflammatory cytokines (8, 19) have been found in BALF in asthmatic subjects and BK (this study and IL-1β (24) have been shown to cause prostanooid generation and COX-2 induction in human ASM cells, it is tempting to speculate that the coexistence of proinflammatory mediators and cytokines in the airway, and the consequential induction of COX-2 may play an important role in the pathogenesis of airway inflammation in asthma.

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