

PATHOGENESIS

Pharmacological Modulation of the Bradykinin-Induced Differentiation of Human Lung Fibroblasts: Effects of Budesonide and Formoterol

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Objective. Bradykinin (BK) induces differentiation of lung fibroblasts into myofibroblasts, which play an important role in extracellular matrix remodeling in the airways of asthmatic patients. It is unclear whether this process is affected by antiasthma therapies. Here, we evaluated whether a glucocorticoid, budesonide (BUD), and a long-acting β_2 -agonist, formoterol (FM), either alone or in combination, modified BK-induced lung fibroblast differentiation, and affected the BK-activated intracellular signaling pathways. **Methods.** Human fetal lung fibroblasts were incubated with BUD (0.001–0.1 μ M) and/or FM (0.0001–0.1 μ M) before exposure to BK (0.1 or 1 μ M). Fibroblast differentiation into α -smooth-muscle-actin-positive (α -SMA⁺) myofibroblasts, BK2 receptor (B2R) expression, extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation (p-ERK1/2), intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and p65 nuclear factor kappa B translocation were evaluated. **Results.** BUD (0.1 μ M) and FM (0.1 μ M), either alone or in combination, completely inhibited BK-induced α -SMA protein expression and decreased the numbers of α -SMA⁺ fibroblasts, with a clear reduction in α -SMA stress fibers organization. BUD also completely inhibited the increase of B2R, whereas FM with or without BUD had no effect. BK-induced increases of $[Ca^{2+}]_i$ and p-ERK1/2 were significantly reduced to similar levels by BUD and FM, either alone or in combination, whereas p65 translocation was completely inhibited by all treatments. **Conclusion.** Both BUD and FM, either alone or in combination, effectively inhibited the BK-induced differentiation of fibroblasts into α -SMA⁺ myofibroblasts and the intracellular signaling pathways involved in fibroblast activation. These results suggest that BUD and FM combination therapy has potential to inhibit fibroblast-dependent matrix remodeling in the airways of asthmatic patients.

Keywords α -smooth muscle actin (α -SMA), bradykinin B2 receptor (B2R), human lung fibroblasts, mitogen-activated protein kinases (MAPKs)

INTRODUCTION

Bradykinin (BK), a peptide formed from kininogen precursors following the activation of plasma and tissue kallikreins, is known to mediate multiple pro-inflammatory effects in a variety of disorders (1, 2). Most of the biological actions of BK are mediated via an interaction of the constitutive B2 receptor (B2R), which is widely distributed throughout the human body, while the expression of the inducible B1 receptor (B1R) requires induction by pro-inflammatory cytokines, such as IL-1 β , TNF- α , or by infectious stimuli (2). In asthma, BK, by increasing the cholinergic tone and acting on inflammatory and structural cells, induces bronchial obstruction, vasodilation, plasma extravasation, and stimulation of sensory neurons and increases the synthesis and release of nitric oxide, prostaglandins, leukotrienes, cytokines, eicosanoids, and neuropeptides (3–5). Interestingly, BK is also capable not only of stimulating human lung fibroblast contractility, cytokine release, and proliferation but also of inducing fibroblast differentiation into α -smooth-muscle-actin-positive

(α -SMA⁺) myofibroblasts through the activation of B2R (6–8). Myofibroblasts are the cells predominantly responsible for increased production and deposition of extracellular matrix molecules in the airways of asthmatic patients, contributing to airway wall thickening (e.g. by increasing the thickness of reticular basement membrane), and to the partially reversible airway obstruction that characterizes severe asthma (9).

Fibroblast activation and differentiation are regulated in part by epidermal growth factor (EGF)-like factors and the EGF receptor-dependent downstream signaling pathways, which includes phosphorylation of mitogen-activated protein kinases (MAPKs) (10, 11). In addition to the MAPKs, the involvement of the transcription factor, nuclear factor kappa B (NF κ B), and of a transient elevation of intracellular calcium concentration $[Ca^{2+}]_i$ in (myo)fibroblast activation and differentiation have also been described (11, 12). Of note, previous studies showed that BK-induced myofibroblast differentiation is dependent on ERK1/2 pathway and on intracellular calcium mobilization, while NF κ B is able to modulate B2R expression in fibroblasts (8, 12, 13).

Little information is available regarding the combination of an inhaled corticosteroid (ICS) and a long-acting β_2 -agonist (LABA)—currently the most successful asthma therapy (14)—whether they may counteract activation and

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differentiation of airway fibroblasts in asthma. However, it was shown recently that the combination therapy of the ICS budesonide (BUD) and the LABA formoterol (FM) significantly attenuated the number of submucosal tissue myofibroblasts enhanced after allergen inhalation (15). Furthermore, BUD-FM combination therapy was shown to decrease the thickness of reticular basement membrane and bronchial wall in patients with asthma (16). It is not known whether cellular and intracellular pathways are involved in these effects.

This study was designed to evaluate *in vitro* whether BUD and FM, either alone or in combination, could modify the BK-induced differentiation of fibroblasts into α -SMA⁺ myofibroblasts. In addition, the effects of the two drugs were investigated on the BK-induced B2R expression and on the downstream pathways possibly involved in BK-induced fibroblast activation: ERK1/2 phosphorylation (p-ERK1/2), intracellular Ca²⁺ mobilization, and p65 NF κ B translocation.

MATERIALS AND METHODS

Fibroblast Culture

The human fetal lung fibroblast cell-line (HFL-1) was obtained from the American Type Culture Collection (Manassas, VA, USA) and used in all experiments. Cells were cultured in 75-cm² tissue culture flask with high-glucose Dulbecco's Modified Eagle medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (5000 IU/ml). Fibroblasts were passaged approximately every 7 days after reaching confluence by dissociating the monolayer with 1:1 0.05% trypsin: 0.1% ethylenediamine tetraacetate solution (EDTA) and used in passages 2–10. All cells were mycoplasma negative, checked by the Hoechst staining method (17). In all experiments, the vehicle for BUD dilution was ethanol, whereas dimethyl sulfoxide (DMSO) was used for FM. The final concentration of both vehicles was <0.05% and did not affect cell viability as assessed by trypan blue dye exclusion as well as all cell functions tested (data not shown).

Experimental Design

The following readouts were evaluated in HFL-1 fibroblasts: (1) fibroblast differentiation into α -SMA⁺ myofibroblasts; (2) B2R protein expression; (3) p-ERK1/2; (4) intracellular Ca²⁺ mobilization; and (5) p65 NF κ B translocation. The α -SMA and B2R expression were evaluated after 48-h incubation with/without BK (1.0 μ M) in the presence or absence of BUD (0.001–0.1 μ M) and/or FM (0.0001–0.1 μ M). ERK1/2 MAPK phosphorylation was evaluated in cells preincubated for 30 or 60 min with BUD (0.001–0.1 μ M) and/or FM (0.001–0.1 μ M) and then stimulated for 5 min with BK (0.1 μ M). For the evaluation of Ca²⁺ mobilization, cells were preincubated for 5, 15, 30, or 60 min with BUD (0.1 μ M) and/or FM (0.1 μ M) before exposure to BK (1.0 μ M). For the evaluation of p65 NF κ B translocation, cells were exposed to BK (1.0 μ M) for 180 min with or without BUD (0.1 μ M) and/or FM (0.1 μ M).

Western Blot Analysis

Analysis of α -SMA and B2R expression, and of ERK1/2 and NF κ B activation, was performed by Western blot. Cellular lysates were prepared as previously described (12, 18, 19). Equal amounts of total protein were resolved on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P Millipore Corporation, Billerica, MA, USA). The membranes were blocked with 3% nonfat dry milk (for α -SMA), 5% bovine serum albumin (BSA) (for B2R), or 5% nonfat dry milk (for ERK1/2) in Tris-buffered saline containing 0.05% Tween 20. Subsequently, membranes were incubated with a mouse anti- α -SMA antibody (Dako cytometry, Carpinteria, CA, USA), a mouse monoclonal anti-B2R antibody (BD Biosciences Inc., Milan, Italy), rabbit anti-total ERK1/2, and anti-phosphorylated ERK1/2 antibodies (Cell Signalling, Technology®, Beverly, MA, USA). Appropriate peroxidase-conjugated secondary antibodies were used. Then, specific reactive proteins were visualized using the enhanced chemiluminescence system (SuperSignal West Dura Extended Duration Substrate, Pierce Inc., Rockford, IL, USA). The relevant band intensities were quantified using a Versadoc Imaging System model 3000 (Biorad Laboratories Inc., Hercules, CA, USA). For α -SMA and B2R expression, to ensure equal protein loading, the membranes were stripped and reprobed with anti- β -actin antibody (clone C4) (Boehringer Mannheim Inc., Mannheim, Germany). To quantify the proportion of activated ERK (p-ERK) at the different experimental conditions, the ratio between p-ERK and the total endogenous ERK expression was calculated (20).

For the study of p65 NF κ B translocation, proteins from nuclear fractions were prepared using QProteome cell compartment kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. Sample protein concentrations were determined by Bradford assay, and the samples were stored at -80°C . Nuclear extract was separated by electrophoresis on SDS-PAGE and transferred to PVDF. Membranes were blocked in 5% milk in Tris-buffered saline containing 0.05% Tween 20 and incubated with a rabbit polyclonal anti-NF κ B p65 antibody (SC-372, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with peroxidase-conjugated secondary antibody, the bands were visualized by the enhanced chemiluminescence system and relevant band intensities were quantified using a Versadoc Imaging System model 3000 (Biorad Laboratories Inc., Hercules, CA, USA). To ensure equal loading, the membranes were stripped and reprobed with lamin A/C antibody (BD Biosciences, Milan, Italy) for nuclear extracts (21).

Immunostaining of α -SMA

Organization of α -SMA was evaluated by immunofluorescence using a conventional indirect technique (12). Briefly, fibroblasts were cultured on glass cover slips (0.13×10^6 cells/glass cover slip) and stimulated as described earlier. After incubation, cells were fixed in methanol for 5 min at -20°C and then stained with mouse monoclonal antibody against human α -SMA

(Sigma-Aldrich S.r.l., Milan, Italy) or with isotype-matched control mouse IgG (Immunotech, Westbrook, ME, USA). FITC-conjugate goat anti-mouse IgG (heavy and light chain) (Immunotech, Beckman Coulter Company, Milan, Italy) was used as the secondary antibody. Slides were then cover-slipped using the Vectashield fluoromount (Vector Laboratories Inc., Burlingame, CA, USA) and stored at -20°C until analyzed for fluorescence.

Fluorimetric Determination of the $[\text{Ca}^{2+}]_i$

The fluorescent calcium indicator Fura-2 was used to determine the $[\text{Ca}^{2+}]_i$ (10) in HFL-1 fibroblasts. Fibroblasts ($2.5\text{--}3 \times 10^4$) grown on 20 mm cover slips were incubated with 10 μM Fura-2 acetoxymethyl ester (Fura-2AM; Fluka, Milan, Italy) and Pluronic F-127 (Invitrogen, Milan, Italy) in the medium for 45 min at 37°C . After the addition of known concentrations of BK to the perfusion chamber, the time-course of the cytosolic calcium level was determined as described previously (12). The $[\text{Ca}^{2+}]_i$ was calculated according to the equation:

$$[\text{Ca}^{2+}]_i = \frac{bKD(R - R_{\min})}{R_{\max} - R}$$

where R is $E340/E380$; $R_{\min}$ is $E340/E380$ in zero calcium; $R_{\max}$ is $E340/E380$ in calcium-saturated solution; b is $E380$ in zero calcium/ $E380$ in calcium-saturated solution; and $KD = 140$ nM (the dissociation constant of the dye at room temperature) (12). To obtain the parameter values, after each experiment, cells were incubated in 10 μM 4-bromo-calcium ionophore A23187 for 20–40 min in a zero-calcium bath (0 calcium plus 1-mM EGTA) and then perfused with the saturated Ca^{2+} solution. At the end of this procedure, 5-mM MnCl_2 was added to the bath to quench the fluorescence of the dye and to determine the background values.

Statistical Analysis

Statistical evaluation was performed using the statistical software package GraphPad Prism 3.0 (Graph Pad Software Inc., San Diego, CA, USA). The data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis of multiple treatment groups was performed using one-way analysis of variance and subsequently Dunnett's two-sided comparison as post hoc test. Differences between two groups were compared using Student's unpaired t -test. A p -value of $<.05$ was considered as statistically significant.

RESULTS

BK-Induced α -SMA Expression

Endogenous expression of α -SMA in unstimulated fibroblasts shown by Western blot analysis increased significantly from 1.6 to 2-fold after incubation with BK (1 μM) for 48 h (Figures 1–3). The BK-induced α -SMA expression was completely inhibited (to a level below the constitutive expression) by coincubation with either BUD (0.1 μM) ($p < .001$), FM (0.1 μM) ($p < .001$), or the

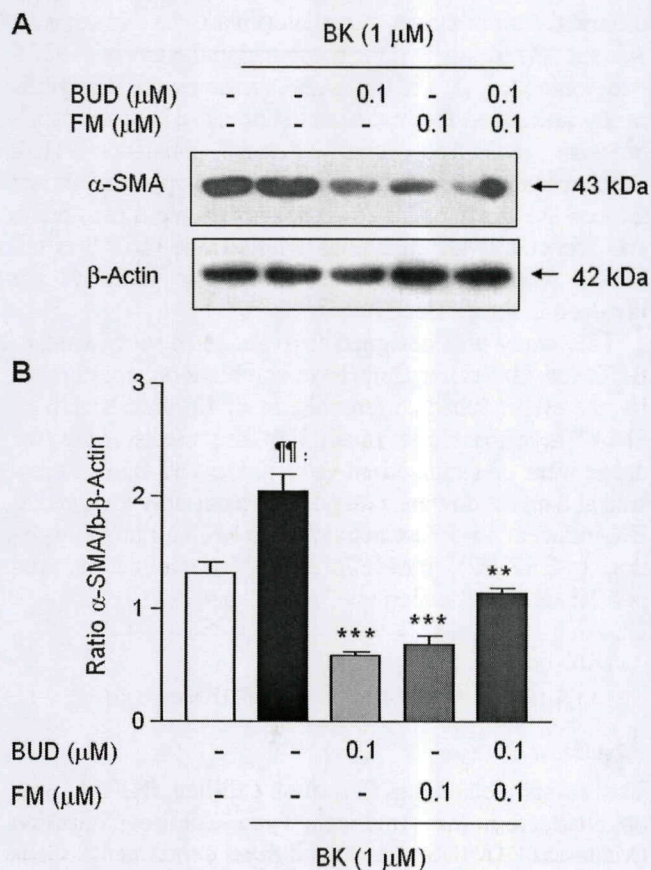


FIGURE 1.—Effects of BUD and/or FM on BK-induced expression of α -SMA protein in HFL-1 fibroblasts. Cells were stimulated for 48 h with BK 1 μM in the presence or absence of BUD and/or FM (both 0.1 μM), and α -SMA protein expression was then evaluated by immunoblot analysis. (A) Representative blottings for α -SMA and the house-keeping protein β -actin; arrows indicate the molecular size of α -SMA (43 kDa) and β -actin (42 kDa) characteristic bands. (B) After densitometric analysis, data were normalized to β -actin and plotted as mean $\pm$ SEM. The different experimental conditions are reported on the abscissa whereas the α -SMA: β -actin ratio is shown on the ordinate. *** $P < .01$ versus unstimulated cells; ** $p < .01$ and *** $p < .001$ versus cells stimulated with BK in the absence of the drugs.

combination of the two drugs ($p < .01$) (Figure 1A and B). In contrast, BUD and FM had no effect on constitutive α -SMA expression in unstimulated fibroblasts (data not shown). When fibroblasts were treated with lower concentrations of the drugs and at a ratio BUD:FM = 10:1 (approximate to that used in Symbicort[®]), a significant and concentration-dependent reduction of the BK-induced α -SMA expression was observed (Figure 2).

Immunofluorescence analysis demonstrated that the enhanced α -SMA expression induced by BK was the result of an increase in the numbers of α -SMA⁺ fibroblasts as well as an increase in polymerization of α -SMA filaments (Figure 3). In BK-stimulated fibroblasts, BUD and FM, either alone or in combination, decreased the numbers of α -SMA⁺ cells, and a clear reduction in the organization of α -SMA stress fibers was also observed (Figure 3). In contrast, no such effects of the two drugs were observed in unstimulated fibroblasts (data not shown).

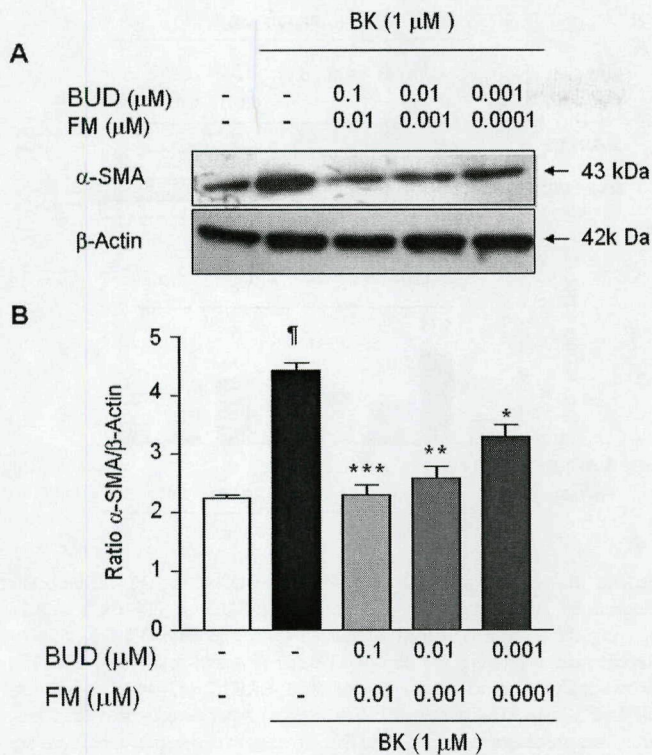


FIGURE 2.—Effects of BUD and FM, in combination, on BK-induced expression of α -SMA protein in HFL-1 fibroblasts. Cells were stimulated for 48 h with BK 1 μ M in the presence or absence of BUD (0.001–0.1 μ M) and FM (0.0001–0.01 μ M) at BUD:FM dose ratio of 10:1 (approximate to that used in Symbicort[®]). (A) Representative blotting for α -SMA and the house-keeping protein β -actin; arrows indicate the molecular size of the α -SMA (43 kDa) and β -actin (42 kDa) characteristic bands. (B) After densitometric analysis, data were normalized to β -actin and plotted as mean $\pm$ SEM. The different experimental conditions are reported on the abscissa, whereas the α -SMA: β -actin ratio is shown on the ordinate. $^{\dagger}p < .05$ versus unstimulated cells; $*p < .05$, $**p < .01$, and $***p < .001$ versus cells stimulated with BK in the absence of the drugs.

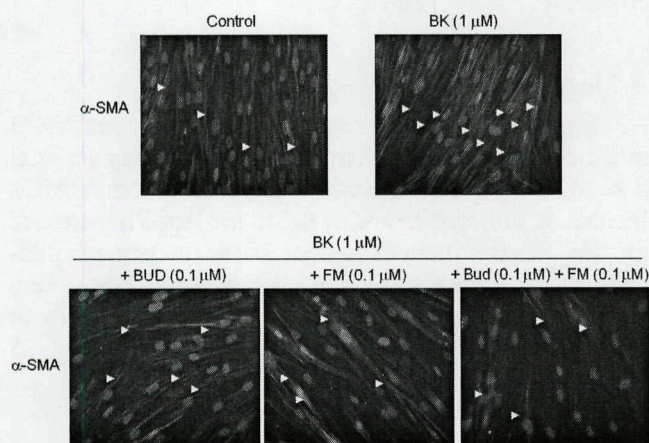


FIGURE 3.—Representative photomicrographs obtained by confocal microscopy after immunofluorescence staining, showing the effect of BUD and/or FM on the BK-induced α -SMA cytoskeletal organization in HFL-1 fibroblasts. Cells were stimulated for 48 h with BK 1 μ M (or vehicle) in the presence or absence of BUD and/or FM (both 0.1 μ M). Arrows indicate the polymerization of α -SMA⁺ cytoskeletal fibers. Magnification 20 $\times$.

BK-Induced B2R Expression

The constitutive expression of B2R protein in fibroblasts was upregulated by 45% after incubation with BK (1 μ M) for 48 h ($p < .05$) as shown by the Western blot analysis (Figure 4). Coincubation with BUD (0.1 μ M) completely abolished the BK-induced increase in B2R expression ($p < .001$), resulting in a level below that of the constitutive expression. In contrast, FM alone (0.1 μ M) or in combination with BUD (0.1 μ M) had no significant effect on B2R expression (Figure 4). No effects of the drugs on constitutive B2R expression were observed in unstimulated fibroblasts (data not shown).

BK-Induced p-ERK1/2

Activation of ERK1/2 occurs through phosphorylation of threonine (Thr) 202 and tyrosine (Tyr) 204 by a single upstream MAP kinase (MEK). Exposure of fibroblasts to BK (0.1 μ M) for 5 min resulted in a fourfold increase ($p < .001$) of p-ERK1/2 compared with the baseline conditions as shown by Western blot analysis using a phospho-specific polyclonal antibody recognizing p-ERK1/2 MAPK (Figure 5). Preincubation for 30 min

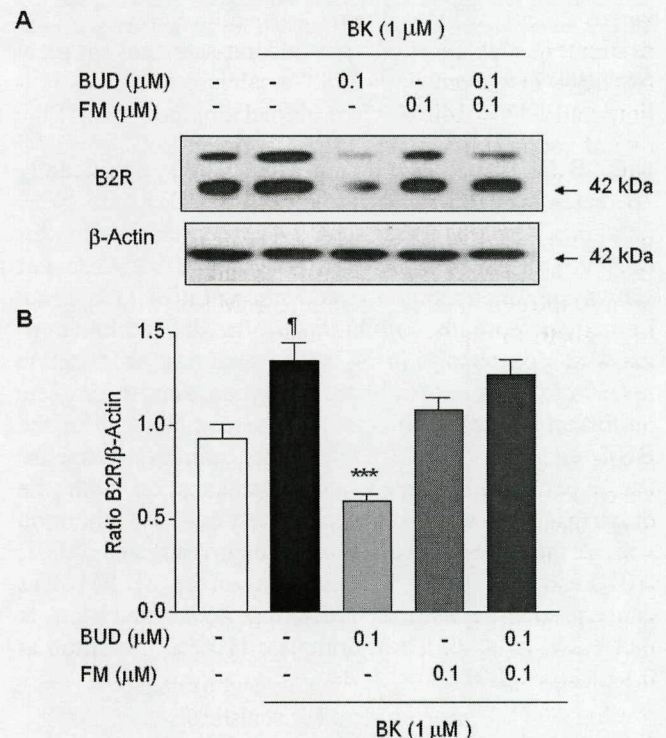


FIGURE 4.—Effects of BUD and/or FM on BK-induced B2R expression in HFL-1 fibroblasts. Cells were stimulated for 48 h with BK 1 μ M (or vehicle) in the presence or absence of BUD and/or FM (both 0.1 μ M), and B2R protein expression was then evaluated by immunoblot analysis. (A) Representative blotting for B2R and the house-keeping protein β -actin; arrows indicate the molecular size of the B2R (42 kDa), and β -actin (42 kDa) characteristic bands. (B) After densitometric analysis, data were normalized to β -actin and plotted as mean $\pm$ SEM. The different experimental conditions are reported on the abscissa whereas the B2R: β -actin ratio is shown on the ordinate. $^{\dagger}p < .05$ versus unstimulated cells; $***p < .001$ versus cells stimulated with BK in the absence of the drugs.

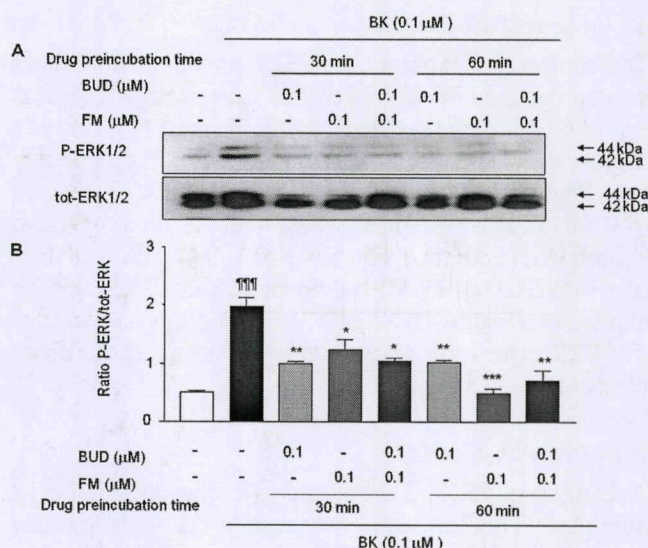


FIGURE 5.—Effects of BUD and FM on ERK1/2 activation in HFL-1 fibroblasts induced by BK. Cells were pretreated with BUD and/or FM (both 0.1 μ M) for 30 or 60 min, and then stimulated for 5 min with BK 0.1 μ M. (A) Representative blotting for phosphorylated (P-) and total (tot-) ERK1/2; arrows indicate the molecular size of the P-ERK1/2 (42–44 kDa) and tot-ERK1/2 (42–44 kDa) characteristic bands. (B) After densitometric analysis, data were plotted as mean $\pm$ SEM. The different experimental conditions are reported on the abscissa whereas the P-ERK1/2 and tot-ERK1/2 ratios on the ordinate. $***p < .001$ versus unstimulated cells; $*p < .05$, $**p < .01$, and $***p < .001$ versus cells stimulated with BK in the absence of the drugs.

with BUD (0.1 μ M) or FM (0.1 μ M) significantly decreased p-ERK1/2 by 67% ($p < .01$) and 49% ($p < .05$), respectively (Figure 5), while preincubation for only 15 min with either drug had no effect (data not shown). Preincubation for 60 min with BUD did not further increase the inhibition while FM inhibited p-ERK1/2 completely ($p < .001$) declining to baseline level. After 60 min of drug preincubation, almost complete inhibition was also obtained by the combination of the BUD and FM (Figure 5). The concentration–response study performed after 60-min preincubation with the drugs (Figure 6) showed approximately 60–70% inhibition with all the three BUD concentrations investigated (0.001, 0.01, and 0.1 μ M), whereas the effect of FM was concentration-dependent, producing 40% inhibition at 0.001 μ M ($p < .05$), and complete (100%) inhibition at 0.1 μ M ($p < .001$).

BK-Induced Ca^{2+} Mobilization

Fluorimetric analysis demonstrated that in the presence of extracellular Ca^{2+} ($CaCl_2$ 1.8 mM), the addition of BK (1 μ M) to the Fura-2-loaded fibroblasts resulted in a rapid increase in $[Ca^{2+}]_i$ that reached a peak (360 ± 16 nM) within 15 s and declined to nearly resting level within 10 min (Figure 7A). The approximate 15-fold elevation of $[Ca^{2+}]_i$ by BK was significantly reduced by about 40% when fibroblasts were preincubated with BUD (0.1 μ M) and/or FM (0.1 μ M) for 60 min before exposure to BK ($p < .01$, for each; Figure 7B). A significant reduction was also observed when the cells were preincubated with either

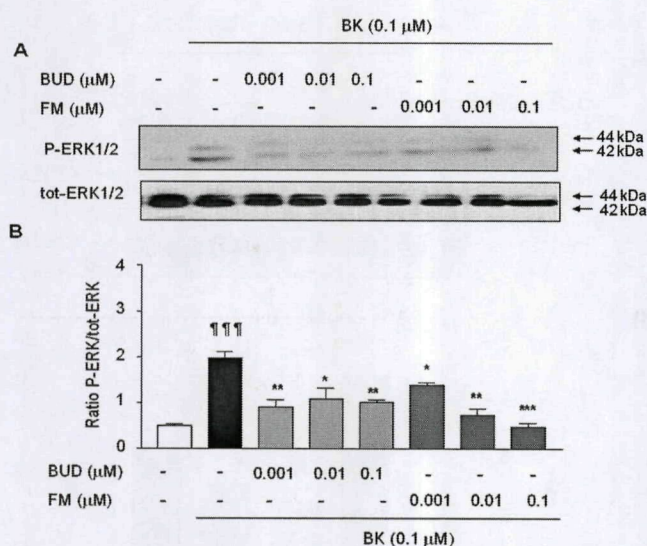


FIGURE 6.—Effects of BUD and FM on p-ERK1/2 in HFL-1 fibroblasts induced by BK. Cells were pretreated with BUD or FM (both 0.001–0.1 μ M) for 60 min, and then stimulated for 5 min with BK 0.1 μ M. (A) Representative blotting for phosphorylated (P-) and total (tot-) ERK1/2; arrows indicate the molecular size of the P-ERK1/2 (42–44 kDa) and tot-ERK1/2 (42–44 kDa) characteristic bands. (B) After densitometric analysis, data were plotted as mean $\pm$ SEM. The different experimental conditions are reported on the abscissa, whereas the P-ERK1/2 and tot-ERK1/2 ratios on the ordinate. $***p < .001$ versus unstimulated cells; $*p < .05$, $**p < .01$, and $***p < .001$ versus cells stimulated with BK in the absence of the drugs.

drug for 5, 15, or 30 min (Figure 7C). Both BUD and FM achieved maximal effects—approximately 70% reduction ($p < 0.001$)—after 30 min preincubation. For FM, the time-dependency of the effect was evident; with only 10% and 31% reduction achieved after 5 and 15 min, respectively. For BUD, the time-dependency of the effect was less obvious and the 56% reduction that was achieved after only a 5-min incubation period suggests a rapid, possibly nongenomic mechanism of action of BUD on BK-induced $[Ca^{2+}]_i$ elevation.

BK-Induced p65 NF κ B Translocation

NF κ B activation was evaluated by quantifying p65 NF κ B in the cell nuclei using an immunoblotting assay (in relation to the nuclear house-keeping protein lamin A/C). Incubation of fibroblasts with BK (1 μ M) for 3 h increased the p65 NF κ B protein expression in the nuclear compartment by 63% ($p < .05$). This increase was completely prevented when the cells were preincubated for 1 h with BUD (0.1 μ M) or FM (0.1 μ M) or the combination ($p < .05$ for all treatments; Figure 8).

DISCUSSION

This study, performed in HFL-1 fibroblasts, showed that both BUD and FM, either alone or in combination, are capable of totally blocking BK-induced differentiation into myofibroblasts, preventing the BK-induced enhancement of α -SMA expression, the increase in the numbers of α -SMA $^+$ cells, as well as the polymerization of α -SMA

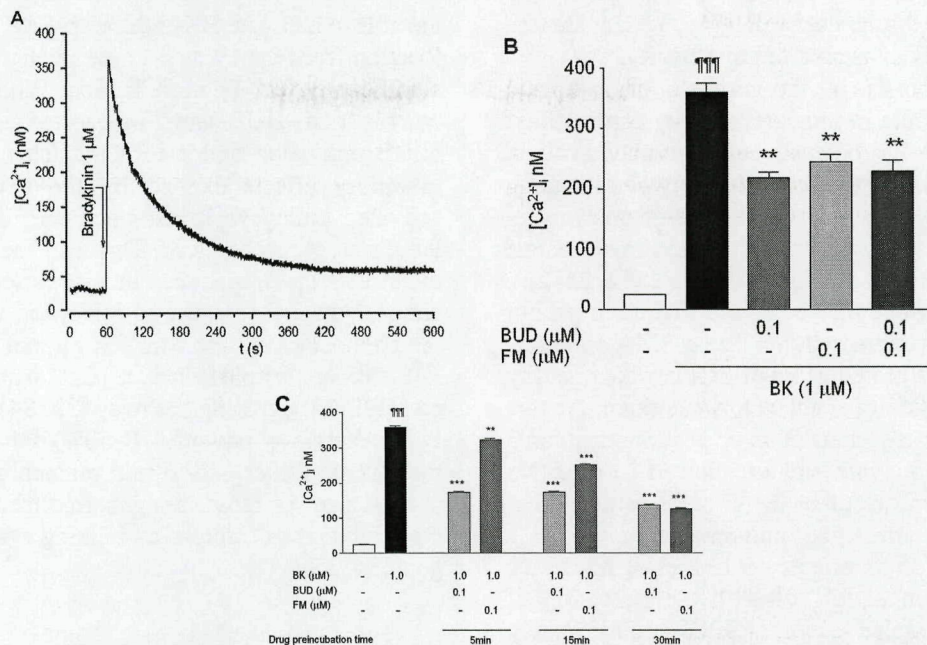


FIGURE 7.—Effects of BUD and/or FM on BK-induced increase in $[Ca^{2+}]_i$ in HFL-1 fibroblasts. (A) BK-induced (1 μ M) rapid increase in $[Ca^{2+}]_i$. (B) Cells were cultured in the presence of BUD and/or FM (both 0.1 μ M) for 60 min, and then exposed to BK 1 μ M. (C) Time-dependent effects of BUD and FM; cells were preincubated with BUD or FM (both 0.1 μ M) for 5, 15, and 30 min before exposure to BK 1 μ M, and $[Ca^{2+}]_i$ levels were measured by fluorimetric analysis. The different experimental conditions are reported on the abscissa and the $[Ca^{2+}]_i$ on the ordinate. Data are presented as means $\pm$ SEM. $^{***}P < .001$ versus unstimulated cells; $^{**}P < .01$ and $^{***}P < .001$ versus cells stimulated with BK in the absence of the drugs.

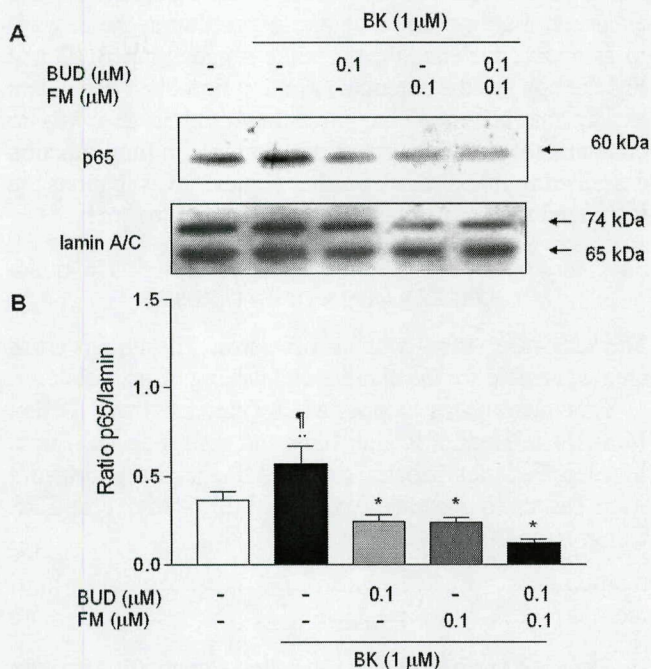


FIGURE 8.—Effects of BUD and/or FM on p65 NF κ B translocation into the nucleus induced by BK in HFL-1 fibroblasts. Cells were pretreated for 1 h with BUD and/or FM (both 0.1 μ M) before stimulation with BK 1 μ M for 3 h. (A) Representative blotting, showing p65 NF κ B and the house-keeping protein lamin A/C levels in the nuclear compartment; arrows indicate the molecular size of p65 (60 kDa) and lamin A/C (65–74 kDa) characteristic bands. (B) After densitometric analysis data were normalized to lamin and shown as anti-p65/anti-lamin ratio. The different experimental conditions are reported on the abscissa and the anti-p65/anti-lamin ratio on the ordinate, and shown as mean $\pm$ SEM. $^{\dagger}P < .05$ versus unstimulated cells; $^{*}P < .05$ versus cells stimulated with BK in the absence of the drugs.

filaments. BUD also completely inhibited the BK-induced upregulation of B2R expression while FM either alone or in combination with BUD had no effects. These results suggest that other mechanisms other than the downregulation of B2R expression are involved in blocking the BK-induced differentiation of fibroblasts into α -SMA⁺ myofibroblasts by the combination of BUD and FM. The mechanisms that are most likely to be involved are BK-activated intracellular pathways such as p-ERK1/2, Ca^{2+} mobilization, and NF κ B translocation, all of which were effectively downregulated by the two drugs. Importantly, the inhibitory effects of BUD and FM demonstrated in vitro here were obtained at nanomolar concentrations that are considered therapeutically relevant (22, 23).

The number of myofibroblasts is increased in the airways of asthmatic patients; myofibroblasts are a rich source of tissue matrix proteins and in the airways they play a key role in tissue matrix remodeling leading to an increased thickness of the subepithelial basement membrane (24). The demonstration here that the combination of BUD and FM counteracts BK-induced fibroblast differentiation into α -SMA⁺ myofibroblasts in vitro is in agreement with the recent findings that inhaled BUD and FM combination therapy significantly attenuated the allergen-induced increase in the number of submucosal tissue myofibroblasts in asthmatic patients (15). However, in that clinical study, only the combination therapy had this effect; it was not observed with BUD monotherapy. Another clinical study has also shown that BUD and FM inhalation therapy in asthmatic patients reversed the increased thickness of the subepithelial basement

membrane and the bronchial wall (16), which are the fundamental features of asthma of any severity.

Subepithelial fibrosis in the airways of asthmatic patients may contribute to increased airway contractility. Importantly, α -SMA has been suggested to play a role in the production of contractile force during wound healing and fibrocontractive diseases, exerting tension on cytoskeletal components, such as microtubules and intermediate filaments, organized as a tensegrity structure (12, 25, 26). This study demonstrated that BUD and FM inhibited not only α -SMA protein expression but also α -SMA organization, a condition that promotes myofibroblast contractility (12). Although we did not evaluate to what extent the two drugs influenced protein translation or posttranscriptional processing of protein synthesis, we showed an effective decrease in the numbers of α -SMA⁺ cells with a clear reduction in α -SMA stress fiber polymerization.

BK exerts most of its effects on fibroblast functions, including cell differentiation, through the activation of B2R, a G protein-coupled receptor (GPCR) constitutively expressed on most cell types and that can be up- or down-regulated by a variety of molecules (2, 27). Physiologically, regulation of the human B2R occurs at the protein level with receptor-mediated ligand internalization following BK binding and being accompanied by a transient loss of surface expression (27, 28). In agreement with a previous report showing that the expression of B2R (and B1R) induced by TNF- α or IL-1 β in lung fibroblasts can be inhibited at the posttranscriptional level by dexamethasone (29), we have also demonstrated in this study that the BK-induced B2R upregulation was completely abolished at the protein level by BUD. In contrast, despite the demonstration that the LABA may downregulate the TNF- α -mediated induction of cell-surface proteins on lung fibroblasts (30), in this study we did not observe any significant inhibitory activity of FM alone on BK-induced B2R expression and, on the other hand, we also found a counteracting effect of FM on BUD inhibition of B2R expression. We may suggest that FM, acting through the cyclic adenosine monophosphate (cAMP)-mediated intracellular pathways (31) able to regulate B2R transcriptional genes (32), has the capability to functionally antagonize the molecular mechanisms induced by BUD in downregulating B2R expression.

The intracellular pathways that regulate the expression of the B2R gene and those which are activated by B2R stimulation in human cells are only partially known (27, 29). In human osteoblastic cell line and gingival fibroblasts, TNF- α -induced B2R gene expression involves NF κ B and p38 MAPK pathways (13). Stimulation of B2R engages a number of intracellular events and pathways, including phosphorylation of MAPK, activation of the transcription factor NF κ B, and mobilization of [Ca²⁺]_i, all of which are reported to promote fibroblast differentiation and activation (8, 11–13), and have been the focus of this study. Indeed, as also demonstrated in this study, BK induces p-ERK1/2, elevation of [Ca²⁺]_i levels, and NF κ B activation in human lung fibroblasts. We have showed here that BUD and FM, either alone or in combination,

are able to abrogate BK-induced p-ERK1/2 and p65 translocation from the cytosol to the nucleus, and to decrease significantly [Ca²⁺]_i mobilization. When administered in combination, BUD and FM exerted additive effects on NF κ B activation and p-ERK1/2. This suggests that the inhibitory effects exerted by the two drugs on BK-activated pathways investigated are likely mediated by the different mechanisms. This also seems to be valid for the effects on BK-induced increases with [Ca²⁺]_i mobilization, where the degree of inhibition was dependent on the preincubation time with FM but not with BUD. While FM is probably able to reduce [Ca²⁺]_i mobilization via the cAMP/PKA signaling pathway (33, 34). Our finding that BUD exerted a powerful (>50%) inhibition of [Ca²⁺]_i mobilization after only 5-min preincubation suggests the involvement of rapid, nongenomic mechanisms, such as the inhibition of calcium-mobilizing cytoplasmic messengers (34, 35).

CONCLUSION

In summary, both BUD and FM, either alone or in combination, at therapeutically relevant concentrations, effectively inhibited the BK-induced differentiation of fibroblasts into α -SMA⁺ myofibroblasts and the intracellular signaling pathways involved in fibroblast activation. Since myofibroblasts are the key cells responsible for enhanced matrix production and deposition in the airways of asthmatic patients, these results suggest that BUD and FM therapy has the potential to inhibit fibroblast-dependent airway matrix remodeling in asthma and in this way to counteract with the longterm deterioration in lung function described in this disease. Further clinical investigations are warranted to confirm the results of this in vitro study.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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REFERENCES

1. Kaplan AP, Joseph K, Silverberg M. Pathways for bradykinin formation and inflammatory disease. *J Allergy Clin Immunol* 2002; 109:195–209.
2. Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, Zuraw BL. International Union of Pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol Rev* 2005; 57:27–77.
3. Proud D. The kinin system in rhinitis and asthma. *Clin Rev Allergy Immunol* 1998; 16(4):351–364.
4. Ricciardolo FLM, Nadel JA, Yoshihara S, Geppetti P. Evidence for reduction of bradykinin-induced bronchoconstriction in guinea-pigs by release of nitric oxide. *Br J Pharmacol* 1994; 113:1147–1152.
5. Ricciardolo FLM, Nadel JA, Graf PD, Bertrand C, Yoshihara S, Geppetti P. Role of kinins in anaphylactic-induced bronchoconstriction mediated by tachykinins in guinea-pigs. *Br J Pharmacol* 1994; 113:508–512.

6. Mio T, Liu X, Toews M, Adachi Y, Romberger D, Spurzem J, Rennard SI. Bradykinin augments fibroblast-mediated contraction of released collagen gels. *Am J Physiol Lung Cell Mol Physiol* 2001; 281(1):L164–L171.
7. Hayashi R, Yamashita N, Matsui S, Fujita T, Araya J, Sassa K, Arai N, Yoshida Y, Kashii T, Maruyama M, Sugiyama E, Kobayashi M. Bradykinin stimulates IL-6 and IL-8 production by human lung fibroblasts through ERK- and p38 MAPK-dependent mechanisms. *Eur Respir J* 2000; 16(3):452–458.
8. Vancheri C, Gili E, Failla M, Mastruzzo C, Salinaro E, Lofurno D, Pistorio MP, La Rosa C, Caruso M, Crimi N. Bradykinin differentiates human lung fibroblasts to a myofibroblast phenotype via the B2 receptor. *J Allergy Clin Immunol* 2005; 116(6):1242–1248.
9. Holgate ST, Arshad HS, Roberts GC, Howarth PH, Thurner P, Davies DE. A new look at the pathogenesis of asthma. *Clin Sci* 2010; 118:439–450.
10. Boxall C, Holgate ST, Davies DE. The contribution of transforming growth factor- β and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur Respir J* 2006; 27:208–229.
11. Chetty A, Cao GJ, Nielsen HC. Insulin-like Growth Factor-I signaling mechanisms, type I collagen and alpha smooth muscle actin in human fetal lung fibroblasts. *Pediatr Res* 2006; 60(4):389–394.
12. Petecchia L, Sabatini F, Usai C, Carnevali S, Ognibene M, Vanni C, Eva A, Fabbri LM, Rossi GA, Ricciardolo FL. Mechanisms of bradykinin-induced contraction in human fetal lung fibroblasts. *Eur Respir J* 2010; 36(3):655–664.
13. Brechter AB, Persson E, Lundgren I, Lerner UH. Kinin B1 and B2 receptor expression in osteoblasts and fibroblasts is enhanced by interleukin-1 and tumour necrosis factor- α . Effects dependent on activation of NF- κ B and MAP kinases. *Bone* 2008; 43(1):72–83.
14. Edwards SJ, von Maltzahn R, Naya IP, Harrison T. Budesonide/formoterol for maintenance and reliever therapy of asthma: a meta analysis of randomised controlled trials. *Int J Clin Pract* 2010; 64(5):619–627.
15. Kelly MM, O'Connor TM, Leigh R, Otis J, Gwozd C, Gauvreau GM, Gauldie J, O'Byrne PM. Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma. *J Allergy Clin Immunol* 2010; 125(2):349–56.e13.
16. Capraz F, Kunter E, Cermik H, Ilvan A, Pocan S. The effect of inhaled budesonide and formoterol on bronchial remodeling and HRCT features in young asthmatics. *Lung* 2007; 185(2):89–96.
17. Battaglia M, Pozzi D, Grimaldi S, Parasassi T. Hoechst 33258 staining for detecting mycoplasma contamination in cell cultures: a method for reducing fluorescence photobleaching. *Biotech Histochem* 1994; 69(3):152–156.
18. Aarbiou J, Verhoosel RM, Van Wetering S, De Boer WI, Van Krieken JH, Litvinov SV, Rabe KF, Hiemstra PS. Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression in vitro. *Am J Respir Cell Mol Biol* 2004; 30(2):193–201.
19. Kim J, Jain D, Tliba O, Yang B, Jester WJ, Panettieri RJ, Amrani Y, Puré E. TGF- β potentiates airway smooth muscle responsiveness to bradykinin. *Am J Physiol Lung Cell Mol Physiol* 2005; 289(4):L511–L520.
20. Pat B, Yang T, Kong C, Watters D, Johnson DW, Gobe G. Activation of ERK in renal fibrosis after unilateral ureteral obstruction: modulation by antioxidants. *Kidney Int* 2005; 67(3):931–943.
21. Brandenburg LO, Kipp M, Lucius R, Pufe T, Wruck CJ. Sulforaphane suppresses LPS-induced inflammation in primary rat microglia. *Inflamm Res* 2010; 59(6):443–450.
22. Van den Bosch JM, Westermann CJ, Aumann J, Edsbäcker S, Tönnesson M, Selroos O. Relationship between lung tissue and blood plasma concentrations of inhaled budesonide. *Biopharm Drug Dispos* 1993; 14(5):455–459.
23. Campestrini J, Lecaillon JB, Godbillon J. Automated and sensitive method for the determination of formoterol in human plasma by high-performance liquid chromatography and electrochemical detection. *J Chromatogr B Biomed Sci Appl* 1997; 704(1–2):221–229.
24. Brewster CE, Howarth PH, Djukanovic R, Wilson J, Holgate ST, Roche WR. Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol* 1990; 3(5):507–511.
25. Darby I, Skalli O, Gabbiani G. Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest* 1990; 63(1):21–29.
26. Hinz B, Celetta G, Tomasek JJ, Gabbiani G, Chaponnier C. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Mol Biol Cell* 2001; 12(9):2730–2741.
27. Prado GN, Taylor L, Zhou X, Ricupero D, Mierke DF, Polgar P. Mechanisms regulating the expression, self-maintenance, and signaling-function of the bradykinin B2 and B1 receptors. *J Cell Physiol* 2002; 193(3):275–286.
28. Saifudeen Z, Du H, Dipp S, El-Dahr SS. The bradykinin type 2 receptor is a target for p53-mediated transcriptional activation. *J Biol Chem* 2000; 275(20):15557–15562.
29. Haddad E, Fox A, Rousell J, Burgess G, McIntyre P, Barnes P, Chung KF. Post-transcriptional regulation of bradykinin B1 and B2 receptor gene expression in human lung fibroblasts by tumor necrosis factor- α : modulation by dexamethasone. *Mol Pharmacol* 2000; 57(6):1123–1131.
30. Silvestri M, Fregonese L, Sabatini F, Dasic G, Rossi GA. Fluticasone and salmeterol downregulate in vitro, fibroblast proliferation and ICAM-1 or H-CAM expression. *Eur Respir J* 2001; 18(1):139–145.
31. Johnson M. Molecular mechanisms of β_2 -adrenergic receptor function, response, and regulation. *J Allergy Clin Immunol* 2006; 117:18–24.
32. Marin Castano ME, Schanstra JP, Hirtz C, Pesquero JB, Pecher C, Girolami J-P, Bascands JL. B₂ kinin receptor upregulation by cAMP is associated with BK-induced PGE₂ production in rat mesangial cells. *Am J Physiol Renal Physiol* 1998; 274:F532–F540.
33. Ay B, Iyanoye A, Sieck GC, Prakash YS, Pabelick CM. Cyclic nucleotide regulation of store-operated Ca²⁺ influx in airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2006; 290(2):L278–L283.
34. Gao YD, Zou JJ, Zheng JW, Shang M, Chen X, Geng S, Yang J. Promoting effects of IL-13 on Ca²⁺ release and store-operated Ca²⁺ entry in airway smooth muscle cells. *Pulm Pharmacol Ther* 2010; 23(3):182–189.
35. Horvath G, Vasas S, Wanner A. Inhaled corticosteroids reduce asthma-associated airway hyperperfusion through genomic and nongenomic mechanisms. *Pulm Pharmacol Ther* 2007; 20(2):157–162.

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