

Bradykinin differentiates human lung fibroblasts to a myofibroblast phenotype via the B2 receptor

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Background: The identification of factors mediating the transition of lung fibroblasts into myofibroblasts is considered fundamental in the comprehension of abnormal reparative processes. Bradykinin, a mediator known for its proinflammatory action, is able to induce cytokine production and contractility in fibroblast cultures.

Objectives: In this study the ability of bradykinin to drive fibroblast into a myofibroblast phenotype at the cellular and molecular level was evaluated.

Methods: α -Smooth muscle actin (α -SMA) expression and TGF- β in bradykinin stimulated fibroblasts were tested by means of flow cytometry, Western blot, and RT-PCR. Cell proliferation and collagen production were evaluated by the colorimetric methylthiazol tetrazolium assay and sirius red assay, respectively. Which bradykinin receptor mediates the expression of α -SMA was evaluated using selective B1 and B2 blocking agents. Furthermore, the effect of bradykinin on extracellular signal-regulated kinase 1/2 phosphorylation was explored.

Results: Bradykinin caused in lung fibroblasts a significant increase in α -SMA at the cellular and molecular level. The B2 receptor was held responsible for this effect because a specific receptor antagonist had entirely blocked this effect. Bradykinin was able to induce fibroblast proliferation and collagen production. Bradykinin significantly activated mitogen-activated protein kinase pathway by phosphorylating extracellular signal-regulated kinase 1/2, whereas PD98059, a specific inhibitor, was able to block myofibroblast induction. Although bradykinin induced an increase of TGF- β on fibroblasts, the blockage of this cytokine did not alter α -SMA expression.

Conclusion: The data support the hypothesis that bradykinin may be involved in bronchial remodeling and lung fibrosis beyond its well recognized proinflammatory activity, also suggesting a new potential therapeutic strategy to control altered reparatory processes. (*J Allergy Clin Immunol* 2005;116:1242-8.)

Key words: Remodeling, lung fibrosis, α -SMA, MAPK

The appearance of fibroblast-like cells expressing α -smooth muscle actin (α -SMA) named myofibroblasts is considered a critical feature in the bronchial remodeling process seen in bronchial asthma as well as in the abnormal reparative process that characterizes pulmonary fibrosis.^{1,2} These cells are a predominant source of increased extracellular matrix deposition, and their number in the submucosa of subjects with asthma correlates with subepithelial collagen deposition, thus contributing to airway wall thickening and to the irreversible or partially reversible airway obstruction that may characterize these patients.³ Myofibroblasts are also distinctive of the fibrotic process that characterizes lung fibrosis, and their emergence in the lung has been correlated with the reduction of lung compliance.^{4,5} Thus, the identification of factors and the understanding of the mechanisms mediating the transition of fibroblasts into myofibroblasts are considered fundamental in the comprehension of the genesis of the fibrotic process regardless of where it takes place, either in the bronchi as observed in asthma or in lung parenchyma as described in the abnormal repair occurring in pulmonary fibrosis. Among the mediators involved in this process, TGF- β plays a crucial role and is considered by far the most potent inducer of myofibroblast transformation and activation.^{6,7} Another mediator largely generated during inflammation and tissue injury is bradykinin, a peptide generated from kininogens by the action of plasma and tissue kallikreins.⁸ Although bradykinin is best known for its proinflammatory activity, like TGF- β , it is able to elicit fibroblast activation. There is in fact evidence to show that bradykinin stimulates nuclear factor- κ B activation and cytokine release in cultured human lung fibroblasts⁹⁻¹¹ as well as fibroblast contractility,^{12,13} suggesting that this mediator might be involved in airway remodeling and fibrotic processes. This study was undertaken to investigate the role of bradykinin in inducing the transformation of human pulmonary fibroblasts into myofibroblasts. To this end, α -SMA expression, cell proliferation, TGF- β , and collagen production in bradykinin-stimulated fibroblasts were examined. In addition, which bradykinin receptor mediates the expression of α -SMA and the role played by the mitogen-activated protein kinase (MAPK) pathway in the induction of α -SMA expression were evaluated.

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Abbreviations used

α -SMA: α -Smooth muscle actin
ERK: Extracellular signal-regulated kinase
MAPK: Mitogen-activated protein kinase
MFI: Mean fluorescence intensity
MTT: Colorimetric methylthiazol tetrazolium assay

METHODS

Cell culture

Primary lines of normal human lung fibroblasts were established by using an outgrowth from explant following the method described by Jordana et al.² Fibroblast lines were derived from histologically normal areas of lung specimens from patients undergoing resective surgery for cancer. Cultured fibroblasts were always used at a passage earlier than the fifth. The cells were suspended at 1×10^5 cells/mL in supplemented RPMI and cultured at 37°C in a 5% CO₂ atmosphere.

Exposure of human lung fibroblast to bradykinin

After 48 hours, the cells were cultured in a serum free medium in the presence of bradykinin (50 μ mol/L; Sigma-Aldrich, Milano, Italy) at 37°C with 5% CO₂ atmosphere for 72 hours. In some experiments, the bradykinin B1 receptor antagonist Lys-(Des-Arg⁹,Leu⁸)-bradykinin (Sigma) and/or the bradykinin B2 receptor antagonist D-Arg,(Hyp³,Thi⁵,8,D-Phe⁷)-bradykinin (Sigma) were added 60 minutes before the addition of bradykinin and subsequently added every day.

Flow-cytometry analysis

The cells were washed, fixed with paraformaldehyde (PFA) 2%, and permeabilized with Triton 1X (Sigma-Aldrich). The cells were then incubated for 60 minutes with α -SMA-ab (1:200; DAKO, Glostrup, Denmark), anti-hTGF- β 1 (1:200; R&D Systems, Minneapolis, Minn), or anti-Phospho-p44/42 MAPK (1:100; Cell Signaling Technology, Beverly, Mass). Subsequently, the cells were washed once with PBS/BSA 1% and incubated with Goat F(ab')₂ Fragment Anti-Mouse IgG (H+L)-FITC (Beckman Coulter, Milano, Italy). Samples were analyzed by using a Coulter Epics Elite ESP flow cytometer (Coulter Corp, Miami, Fla).

RNA isolation and RT-PCR

Fibroblasts were stimulated with bradykinin (50 μ mol/L), and after 48 hours of incubation, RNA was extracted from cells as previously described.¹⁴ RT-PCR was performed by using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Milano, Italy).

The following specific primer pairs were used: α -SMA sense 5'-GTGTGTGACAATGGCTCTGG-3' and antisense 5'-TGAAGGATGGCTGGAACAGG-3' (775-bp PCR product); β -actin sense 5'-TGA-CGG-GGT-CAC-CCA-CAC-TGT-GCC-CAT-CTA-3' and antisense 5'-CTA-GAA-GCA-TTG-CGG-TGG-ACG-ATG-GAG-GG-3' (661-bp product). PCR was performed for 30 cycles by using a cycling program of 94°C for 5 minutes, 55°C for 59 minutes, and 72°C for 59 minutes; the final extension was at 72°C for 7 minutes. PCR-amplified products were electrophoresed through a 1.8 % agarose gel (Bio-Rad, Larne, United Kingdom) containing 0.5 mg/mL ethidium bromide and compared with DNA reference markers.

Cell proliferation

Cell proliferation was assessed by determining the total number of cells in each culture dish after 72 hours of incubation in the presence

of bradykinin by means of a bright-line hemocytometer (Sigma) and by means of the colorimetric methylthiazol tetrazolium assay (MTT).¹⁵ All experiments were performed in triplicate.

Collagen deposition

Fibroblasts stimulated with bradykinin were scraped into a lysis buffer. Total soluble collagen was measured by the Sircol Soluble Collagen Assay (Biocolor, Newtownabbey, United Kingdom). The collagen-dye complex was precipitated by centrifugation at 10,000g for 10 minutes. The precipitated complex was resuspended in 1 mL alkali reagent. The obtained solution was finally placed in a 96-well flat-bottomed plate and evaluated in a plate reader (absorbance, 540 nm).

α -SMA Western blot analysis

Fibroblasts stimulated with bradykinin in presence or absence of 60 μ mol/L PD98059, a specific MAPK/extracellular signal-regulated kinase (ERK) kinase 1 inhibitor, were analyzed by Western blotting for α -SMA. PD98059 was added 60 minutes before the addition of bradykinin. Cultured cells were scraped and lysed. An aliquot of the samples was processed for protein concentrations following the Bradford method. Electrophoresis was performed in 12% SDS-PAGE by using 60 μ g cell proteins/lane. After separation, proteins were transferred onto a nitrocellulose membrane (Hybond ECL, Amersham Biosciences Europe GmbH, Milan, Italy). After blocking, the membranes were incubated with anti- α -SMA-Ab (1:500) (Dako Cytomation, Glostrup, Denmark) overnight at 4°C. Membranes were then incubated with horseradish peroxidase-conjugated antimouse secondary antibodies (1:5000; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). Specific bands were visualized by using the SuperSignal chemiluminescent detection system (Pierce Chemical Co, Rockford, Ill).

Statistical analysis

Results are expressed as means $\pm$ standard errors of the mean (SEM). α -SMA expression, cell proliferation, collagen production, and TGF- β expression in lung fibroblasts cultured with or without bradykinin were all evaluated by means of unpaired Student *t* test. The effect of specific B1 and B2 receptor antagonists, the expression of phospho-ERK 1/2, and the role of PD98059 on α -SMA were evaluated by means of 1-way ANOVA, followed, when significant differences were found, by the Newman-Keuls posttest. A *P* value of less than .05 was considered significant. Statistical analysis of data was performed by using GraphPad Prism 4.00 (GraphPad Software, San Diego, Calif).

RESULTS

Expression of α -SMA on lung fibroblasts stimulated with bradykinin

In preliminary experiments, the kinetic of α -SMA expression induced by bradykinin was examined. To this end, human lung fibroblasts with 50 μ mol/L bradykinin were stimulated and the culture stopped at 24, 48, and 72 hours. The expression of α -SMA, evaluated by flow cytometry, increased in a time-dependent manner and reached the maximum effect after 72 hours (Fig 1, A). The presence of a dose-dependent effect was investigated by stimulating lung fibroblasts with various concentrations of bradykinin. The expression of α -SMA in bradykinin-stimulated lung fibroblasts increased in a dose-dependent manner, achieving the highest expression with 50 μ mol/L

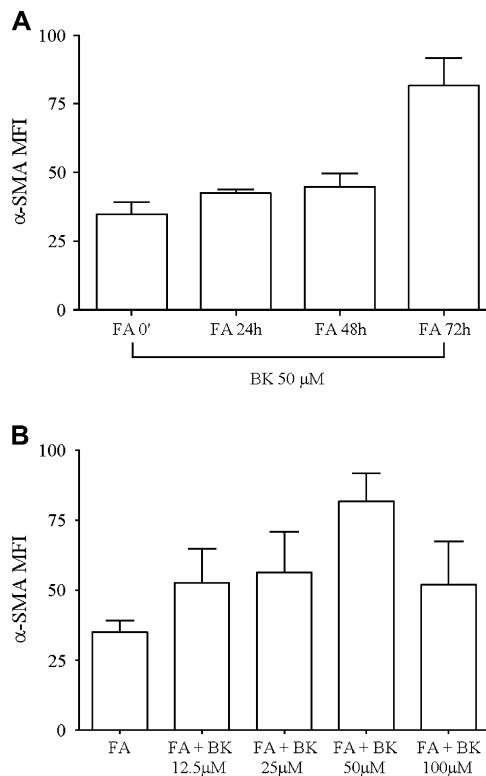


FIG 1. **A**, Time course of α -SMA expression in bradykinin (BK)-stimulated fibroblasts, N = 3. **B**, Dose-response to bradykinin of α -SMA expression in stimulated fibroblasts, N = 3. FA, Fibroblasts alone.

bradykinin (Fig 1, B). Therefore, this particular time point and concentration were used in all of the subsequent experiments.

In lung fibroblasts stimulated with bradykinin at 50 μ mol/L for 72 hours, α -SMA mean fluorescence intensity (MFI) increased significantly from 40.4 ± 6.9 of the unstimulated cells to 89.75 ± 8.4 ($P < .01$; Fig 2, A). Experiments performed on bronchial fibroblasts showed a similar behavior (data not shown).

Accordingly to flow-cytometry analysis, α -SMA protein expression in fibroblast lysates was enhanced by bradykinin as shown by Western blot analysis (Fig 2, B). Furthermore, RT-PCR demonstrated that α -SMA transcription in fibroblasts was also increased after the stimulation with bradykinin (Fig 2, C).

Effect of B1 and B2 receptor antagonists on α -SMA expression

To ascertain which of the bradykinin receptors was involved in the aforementioned results, specific bradykinin B1 and B2 receptor antagonists (ra) were used. The expression of α -SMA was not affected where fibroblast cultures were pretreated with a B1ra (α -SMA MFI, 91.2 ± 7.1 vs 95.2 ± 12.6 of the bradykinin stimulated fibroblasts without B1ra; $P = \text{NS}$). However, the B2ra alone or in combination with the B1ra significantly attenuated the

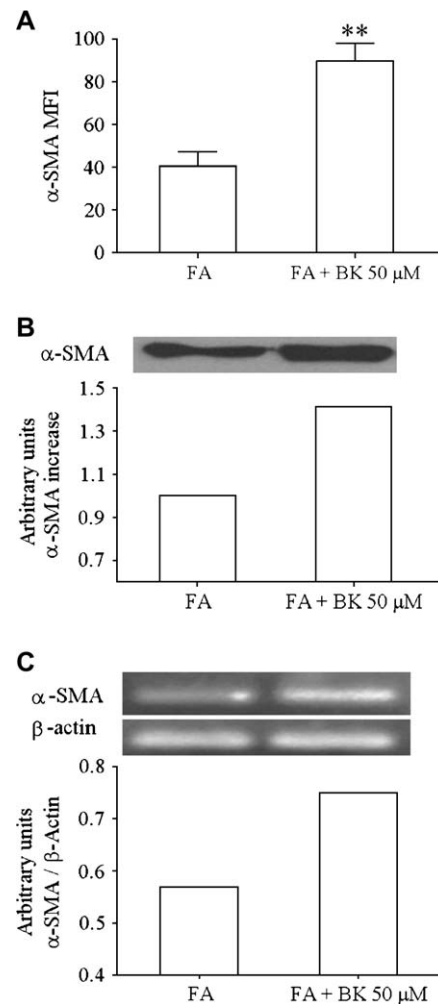


FIG 2. **A**, α -SMA expression in fibroblasts cultured with or without bradykinin (BK) 50 μ mol/L, N = 7. $**P < .01$ vs unstimulated fibroblasts. **B**, α -SMA protein content of the cells. **C**, α -SMA RT-PCR of fibroblasts cultured with or without BK. FA, Fibroblasts alone.

MFI of α -SMA on bradykinin-stimulated fibroblasts to 55.5 ± 2.8 and 53.8 ± 7.2 , respectively ($P < .05$; Fig 3).

Effect of bradykinin on fibroblast proliferation and collagen production

Bradykinin significantly increased the number of fibroblasts from $0.49 \pm 0.02 \times 10^6$ cells/mL to $0.7 \pm 0.02 \times 10^6$ cells/mL ($P < .01$; Fig 4, A). These results were confirmed by the experiments where proliferation was evaluated with the MTT method. Bradykinin enhanced the absorbance of the produced formazan from 0.46 ± 0.03 to 0.59 ± 0.04 nm ($P < .05$; Fig 4, B).

To characterize further the functional activation induced by bradykinin in lung fibroblast, the collagen production in this *in vitro* experimental setup was studied. Using bradykinin at the same dose for all of the experiments (50 μ mol/L), it significantly increased the production of collagen compared with the unstimulated cells, from 20.3 ± 4.3 μ g/mL to 36.8 ± 4.6 μ g/mL ($P < .05$; Fig 5).

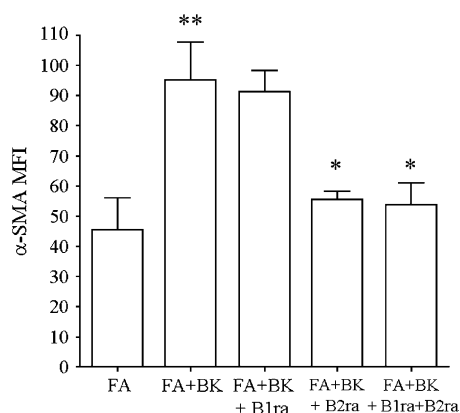


FIG 3. α -SMA expression in fibroblasts cultured with bradykinin (BK) in the presence or absence of specific BK B1 receptor antagonist, B2 receptor antagonist, or both. N = 4. ** $P < .01$ vs unstimulated fibroblasts, * $P < .05$ vs BK stimulated fibroblasts without B1 or B2 receptor antagonists. FA, Fibroblasts alone.

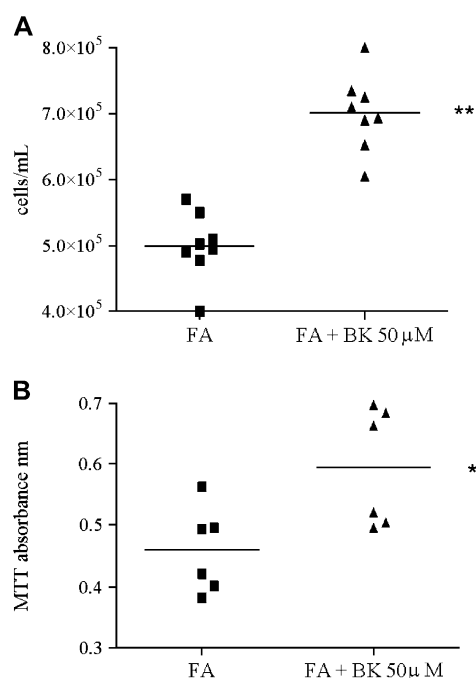


FIG 4. **A**, Fibroblast proliferation with or without bradykinin (BK). N = 8, ** $P < .01$ vs unstimulated cells. **B**, Fibroblast proliferation as determined by the MTT method. N = 6, * $P < .05$ vs unstimulated cells. FA, Fibroblasts alone.

MAPK pathway mediates α -SMA expression

To identify the intracellular signaling pathway involved in bradykinin-induced α -SMA expression by human lung fibroblasts, the role of ERK 1/2 pathway was investigated by flow cytometry and Western blot analysis. Lung fibroblasts were treated with bradykinin for various periods as long as 30 minutes, and phosphorylation of ERK 1/2 was then examined at those time points. As shown in Fig 6, A, stimulation of lung fibroblasts with bradykinin rapidly induced the phosphorylation of ERK 1/2. The

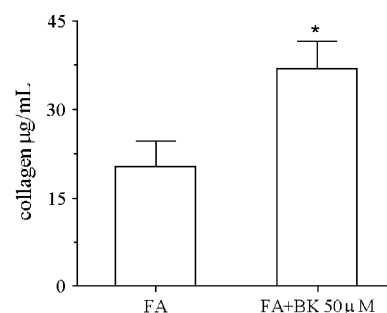


FIG 5. Collagen deposition of fibroblasts stimulated with or without bradykinin. N = 3. * $P < .05$ vs unstimulated fibroblasts. FA, Fibroblasts alone.

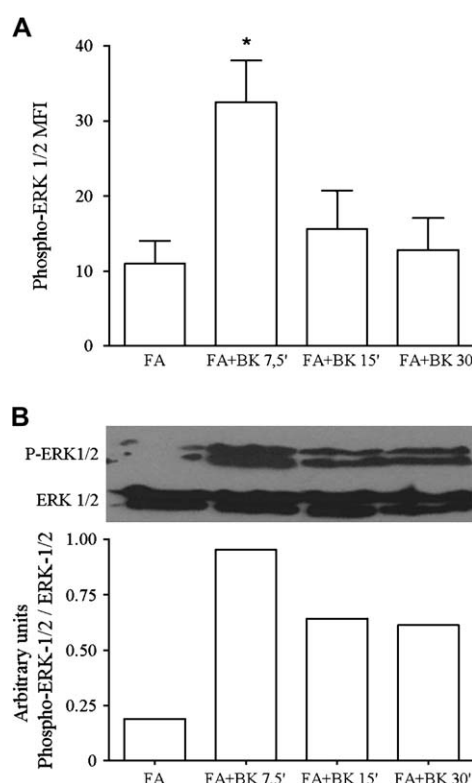


FIG 6. **A**, Phospho-ERK1/2 (P-ERK 1/2) expression in fibroblasts cultured with or without bradykinin (BK), N = 5. * $P < .05$ vs unstimulated fibroblasts. **B**, Phosphorylation of ERK1/2 in BK stimulated and unstimulated cells. FA, Fibroblasts alone.

phosphorylation peak was seen at 7.5 minutes after the bradykinin administration. Phospho-ERK 1/2 MFI increased from 10.9 ± 3 of the unstimulated cells to 32.5 ± 5.6 at 7.5 minutes ($P < .05$). Western blot analysis confirmed the flow-cytometry results. Indeed, bradykinin induced a peak of phosphorylation of ERK 1/2 at 7.5 minutes as shown in Fig 6, B. To examine whether the activation of ERK 1/2 was essential for the bradykinin-induced α -SMA expression, lung fibroblasts were incubated with PD98059, a specific ERK 1/2 inhibitor.

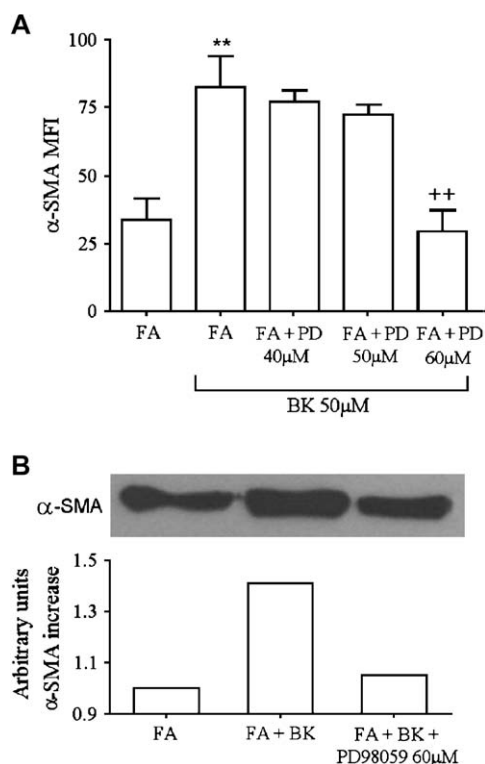


FIG 7. A, α-SMA expression in fibroblasts cultured for 72 hours with bradykinin (BK; 50 µmol/L) in the presence or absence of PD98059, N = 3. ** $P < .01$ vs unstimulated fibroblasts; ++ $P < .01$ vs BK-stimulated fibroblasts without PD98059. **B**, α-SMA protein content of stimulated and unstimulated cells in the presence or absence of PD98059. FA, Fibroblasts alone.

Fig 7, A, shows that PD98059 at the dose of 60 µmol/L completely blocked the expression of α-SMA in cultures of lung fibroblasts stimulated with bradykinin. Western blot analysis further confirmed the inhibitory effect of PD98059 on α-SMA expression after incubation with bradykinin (Fig 7, B).

Role of TGF-β in bradykinin-induced α-SMA expression

Finally, the possibility that a fibrogenic cytokine like TGF-β could mediate, in an autocrine way, the transition of fibroblasts into myofibroblasts induced by bradykinin was explored. Some experiments to evaluate, by means of flow cytometry, the number of fibroblasts expressing TGF-β at the intracellular level were performed. Bradykinin increased the number of TGF-β-positive cells from $25.1\% \pm 7.1\%$ of the unstimulated cells to $35\% \pm 11\%$ (Fig 8, A). Western blot analysis confirmed the increase of TGF-β protein expression (119% over control) in bradykinin-stimulated fibroblasts (Fig 8, B). Although the increased number of TGF-β-positive cells was not statistically significant, it was attempted to understand whether this increase could have a functional meaning. Blocking the activity of the neosynthesized cytokine with a neutralizing antibody (20 µg/mL), only a slight reduction of the expression of α-SMA on bradykinin-

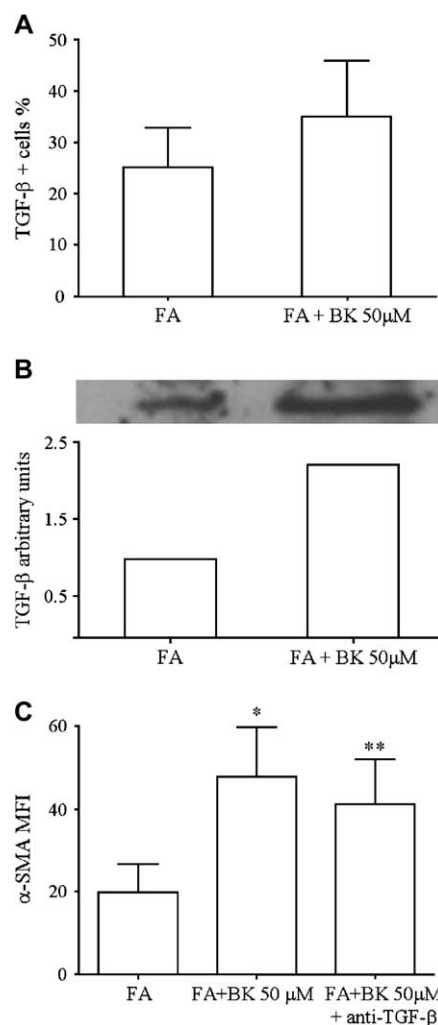


FIG 8. A, TGF-β-positive cells in fibroblast cultures incubated with or without bradykinin (BK); N = 3; $P = NS$. **B**, TGF-β protein expression. **C**, α-SMA expression in fibroblasts cultured with BK in the presence or absence of a blocking anti-TGF-β antibody. N = 3, * $P < .05$ vs unstimulated fibroblasts, ** $P = NS$ vs BK-stimulated fibroblasts. FA, Fibroblasts alone.

stimulated fibroblasts ($-14.3\% \pm 4.8\%$ over control) was found that did not reach statistical significance (Fig 8, C).

DISCUSSION

In this study, the effect of bradykinin on the phenotypic change of normal human lung fibroblasts into myofibroblasts was analyzed. Bradykinin is a mediator generated during inflammation and tissue injury and exerts its activity through interaction with 2 specific cell surface receptors, named B1 and B2.^{8,16} Bradykinin is considered a potent bronchoconstrictor in patients with asthma.^{17,18} It participates in the pathophysiology of airway response to gastroesophageal reflux,¹⁹ in virus-induced airway hyperresponsiveness,²⁰ and in the pathophysiology of cough

and neurogenic inflammation.^{21,22} Furthermore, the administration of bradykinin receptor antagonists is reported to inhibit airway hyperresponsiveness and the increase of several inflammatory mediators after allergen challenge.^{23,24} It has also been shown that bradykinin is able to stimulate lung fibroblasts to produce a wide array of cytokines, including IL-1, IL-8, granulocyte colony stimulating factor (G-CSF), GM-CSF, macrophage chemoattractant protein-1 (MCP-1), and eotaxin.^{10,11,25,26} Moreover, bradykinin affects fibroblast contractility *in vitro* in a manner similar to that exerted by a well known fibrogenic mediator such as TGF- β .¹³

On the basis of these findings, bradykinin may be involved not only in the development of airway inflammation but also in the control of fibroblast activation. One interesting aspect of fibroblasts activation is the transformation of these cells into myofibroblasts. This event is in fact considered crucial both for airway remodeling and lung fibrosis.

In the current study, it has been shown for the first time that bradykinin treatment induces the expression of α -SMA in lung and bronchial fibroblasts. The increased expression of α -SMA is associated with an enhancement of fibroblast proliferation, collagen production, and TGF- β expression, all features that distinguish myofibroblasts.

According to the literature, most of the effects exerted by bradykinin on fibroblasts, including cytokine gene expression, are mediated through the activation of B2 receptors.^{27,28} Similarly, it has been shown here that the stimulatory effect of bradykinin on α -SMA expression is a result of the activation of the B2 receptor, because this effect can be blocked by a specific B2 receptor antagonist. Moreover, it has been shown that the stimulation of the B2 receptor by bradykinin leads to MAPK activation. Although we did not test earlier times, stimulation of lung fibroblasts with bradykinin induces the phosphorylation of ERK 1/2 after 7.5 minutes. In addition, the specific inhibitor of ERK 1/2, PD98059, suppresses α -SMA expression induced by bradykinin. Although these data do suggest that ERK 1/2 is involved in bradykinin-induced α -SMA production in human lung fibroblasts, a contemporary or partial involvement of the other MAPK signaling pathways cannot be excluded.

As mentioned, bradykinin is able to stimulate lung fibroblasts to produce a wide array of cytokines. On this basis, it is hypothesized that the effect exerted by bradykinin on α -SMA expression could be indirectly mediated by the release of a fibrogenic cytokine in an autocrine way. Indeed, it was found that bradykinin enhances the release of TGF- β by lung fibroblasts. Although this increase was not statistically significant, we attempted to determine whether TGF- β so induced was responsible for the expression of α -SMA by fibroblasts. To this end, a neutralizing anti-TGF- β antibody was used with the intent to reduce or block the expression of α -SMA. At the amount of antibody we used, only a slight decrease of the expression of α -SMA was observed, suggesting a direct enhancing effect of bradykinin on α -SMA expression. However, we cannot exclude that higher amounts of

antibody could be more effective. Further studies are required to elucidate the role of TGF- β and/or other cytokines induced by bradykinin in the transformation of fibroblasts into myofibroblasts.

In conclusion, the data support the hypothesis that bradykinin may be involved in bronchial remodeling and lung fibrosis beyond its well recognized proinflammatory activity. Indeed, airway inflammation in asthma, tissue injury and repair in lung fibrosis, and lung inflammation in acute respiratory distress syndrome are all conditions characterized by increased levels of bradykinin. According to our findings, the chronic presence of bradykinin generated by ongoing inflammation in airway or lung parenchyma may lead to fibroblast activation and to the appearance of myofibroblasts.

This study also suggests a potentially new therapeutic strategy for controlling the phenotypic transformation of normal lung fibroblasts into myofibroblasts.

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