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β-adrenergic receptor-mediated growth of human airway epithelial cell lines

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β-adrenergic receptor-mediated growth of human airway epithelial cell lines. K. Nishimura, J. Tamaoki, K. Isono, K. Aoshiba, A. Nagai. ©ERS Journals Ltd 2002.
ABSTRACT: Abnormal growth of airway epithelium and the resultant thickening of airway walls may produce narrowing of airway calibre, thereby contributing to deterioration of bronchoconstriction in chronic obstructive pulmonary disease (COPD). β_2-adrenergic agonists have been widely used for the treatment of COPD, but their effects on the growth of airway epithelial cells is unknown.

Growth of three human airway epithelial cell lines was studied *in vitro*. Exposure to salbutamol in serum-free medium increased 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide reduction and intracellular deoxyribonucleic acid (DNA) contents in 16-human bronchial epithelium (16-HBE) cells and NCI-H292 cells, but not in A549 cells. The growth-promoting effect of salbutamol in 16-HBE cells was equipotent to 10% foetal bovine serum and was inhibited by propranolol and a cyclic adenosine monophosphate (cAMP) antagonist, Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS). Likewise, forskolin and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) caused cell growth and DNA synthesis. Western blot analysis showed that salbutamol, forskolin, and 8-Br-cAMP each induced expression of the phosphorylated form of mitogen-activated protein (MAP) kinase, and that the salbutamol-induced phosphorylation was inhibited by propranolol, Rp-cAMPS, and the MAP kinase-kinase inhibitor PD98059.

These results suggest that in certain airway epithelial cell lines stimulation of β_2-adrenergic receptors and the consequent production of cyclic adenosine monophosphate may upregulate cell growth, probably through activation of the mitogen-activated protein kinase cascade.
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Mitogen-activated protein (MAP) is a family of 42 and 44 kDa cytosolic serine-threonine kinases [1, 2], and has been identified as an important regulatory component in the growth and proliferative responses of many cell types [3]. There is increasing evidence that MAP kinase can be activated by a variety of signals, including growth factors [4], phorbol esters [5] and Ca^{2+} [6]. In addition, stimulation of cell surface receptors, linked to Gi and Gq proteins, has been shown to potently activate MAP kinase [7, 8], but the effect of Gs protein-coupled receptors on the enzyme activity is poorly understood.

β-adrenergic receptors are Gs protein-coupled receptors and stimulate the synthesis of intracellular cyclic adenosine monophosphate (cAMP). Inhaled β_2-adrenoceptor agonists have been widely used in the treatment of chronic obstructive pulmonary disease and asthma, and it is likely that airway epithelial cells are frequently exposed to these drugs in high concentrations. It has recently been demonstrated that cAMP stimulates proliferation of auditory receptor epithelial cells [9]. However, the role of the cAMP-dependent signalling pathway in the proliferative responses of airway epithelial cells is unknown. Therefore, the current authors studied three human airway epithelial cell lines *in vitro*, in order to determine whether β_2-adrenergic agonists affect the growth of airway epithelial cells and to assess a possible involvement of cAMP-mediated stimulation of the MAP kinase cascade.

Materials and methods

Cell growth

A human bronchial epithelium (HBE) cell line, 16-HBE, was obtained from the Cardiovascular Research Institute (University of California, San Francisco, CA, USA). A human airway mucoepidermoid carcinoma cell line, NCI-H292, and an epithelial cell line derived from lung carcinoma, A549, were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were incubated in Ham′s F12 medium, supplemented with 10% foetal bovine serum (FBS), 100 $U \cdot mL^{-1}$ penicillin, 100 $\mu g \cdot mL^{-1}$ streptomycin and 100 $\mu g \cdot mL^{-1}$ amphotericin B in a carbon dioxide (CO_2) incubator (5% CO_2:95% air) at 37°C. After growth to confluence, the cells were detached with a 0.25% trypsin/0.02% ethylenediamine

tetraacetic acid (EDTA) solution for passage. In the preliminary experiment, the cells prepared in this manner maintained the same morphology for $\geqslant 15$ passages.

The cells were plated in 100 μL of Ham′s F12 medium containing 10% FBS at a density of 1×10^4 cells per flat-bottomed well in 96-well microtitre plates, and grown to subconfluence. After washing the cells three times with phosphate-buffered saline (PBS), the cell growth was arrested by incubation in Ham′s F12 medium without FBS for 72 h. The medium was then replaced with serum-free Ham′s F12 medium to which salbutamol (1×10^{-7} M) (Sigma Chemical Co., St Louis, MO, USA) was added, and the cells were further cultured. In the control experiment, cell culture was performed in the serum-free medium alone. Growth of epithelial cells was assessed at indicated times after exposure to salbutamol, using the MTT dye technique, which relies on the specific metabolic reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) (Sigma Chemical Co.) by living cells [10]. MTT (100 μL of 10 $\mu g\cdot mL^{-1}$ in PBS) was added to each well, and the cells were incubated for 4 h at 37°C. The medium and MTT were then removed from the wells, and 25 μL of Sorenson′s glycine buffer (0.1 M glycine and 0.1 M NaCl equilibrated to pH of 10.5 with 0.1 N NaOH) and 200 μL dimethylsulfoxide were consecutively added. The optical density was determined using an immunosorbent assay plate reader (Model 450; Bio-Rad Laboratories Ltd, Watford, UK) against a reagent blank (*i.e.* no cells) at a test wavelength of 570 nm and a reference of 630 nm.

To confirm that the effect of salbutamol on cell proliferation is mediated by β-adrenergic receptors and the consequent synthesis of cAMP, 16-HBE cells were cultured in serum-free medium containing both salbutamol (1×10^{-7} M) and the β-adrenergic receptor antagonist propranolol (1×10^{-5} M) (Sigma Chemical Co.) or the cAMP antagonist Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS) (1×10^{-4} M), and the MTT assay was performed (BIOLOG Life Science Institute, Bremen, Germany). To assess a contribution of the cAMP-dependent signal transduction pathway, the effects on cell growth of forskolin (1×10^{-7} M) (Sigma Chemical Co.), a stimulator of adenylyl cyclase and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) (Sigma Chemical Co.) (1×10^{-7} M), a stable analogue of cAMP, were studied in a similar manner. In evaluating the potency of these drugs, the response was compared to that induced by 10% FBS at 48 h after initiation of culture.

Deoxyribonucleic acid synthesis

The deoxyribonucleic acid (DNA) contents in cultured epithelial cells were assayed by the method of RAGO *et al.* [11]. Briefly, the cells were cultured in serum-free Ham′s F12 medium containing salbutamol (1×10^{-7} M). Then, 100 μL distilled water was added to each well, and the cells were incubated for 1 h. To destruct cell membranes, the cells were frozen at -80°C and thawed at room temperature. A stock solution of Hoechst 33258 (200 $\mu g\cdot mL^{-1}$ in distilled water) (Sigma Chemical Co.) was diluted 10-fold before use with TNE buffer (10 mM Tris, 2 M NaCl, pH 7.4, 1 mM EDTA), and 100 μL of diluted Hoechst 33258 was added to each well. Fluorescence was measured by a spectrofluorimeter (Ultrospec Plus; Phamacia LKB Biochrom Ltd, Cambridge, UK) at excitation and emission lengths of 350 nm and 460 nm, respectively. In addition, the DNA contents were likewise determined after exposure to forskolin (1×10^{-7} M), 8-Br-cAMP (1×10^{-7} M) and salbutamol (1×10^{-7} M) plus propranolol (1×10^{-5} M).

To determine the role of MAP kinase cascade and cAMP in the salbutamol-induced DNA synthesis, 16-HBE cells were cultured in serum-free medium containing salbutamol (1×10^{-7} M) alone or in combination with various concentrations of PD98059 (1, 30, and 100 μM) (New England Biolabs, Inc., Beverly, MA, USA), a specific inhibitor of MAP kinase-kinase [12], or with Rp-cAMPS (1×10^{-4} M), and the DNA contents were measured 48 h later. In this experiment, to block MAP kinase cascade, a MAP kinase-kinase inhibitor was used because a specific MAP kinase inhibitor was not available.

Western blotting

Cell lysates were solubilised in radioimmunoprecipitation (RIPA) buffer (0.15 M NaCl, 50 mM Tris-Cl pH 7.4, 0.5% Nonidet P40 (NP40), and 0.1% sodium dodecylsulphate (SDS)) containing 10 $\mu g\cdot mL^{-1}$ leupeptin, 1 mM phenylmethylsulfonylfluoride, 10 $\mu g\cdot mL^{-1}$ aprotinin, and 1 mM sodium vanadate, and then fractionated by SDS-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and probed with antibodies (1:1,000) against MAP kinase and phosphorylated MAP kinase (New England Biolabs, Inc.). Primary antibody (1:2,500) was visualised using enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL, USA).

Statistical analysis

All values are expressed as mean±SEM. One-way analysis of variance and Newman Keuls multiple comparison test were used to determine statistically significant differences between groups, and a $p<0.05$ was considered significant.

Results

Incubation with salbutamol (1×10^{-7} M) for 48 h in serum-free medium caused increases in both MTT reduction and intracellular DNA content in 16-HBE cells and NCI-H292 cells, but not in A549 cells (fig. 1). Therefore, the growth-promoting effect of salbutamol is different among epithelial cell lines, and the subsequent experiments were conducted using 16-HBE cells.

As demonstrated in figure 2, the 16-HBE cells that

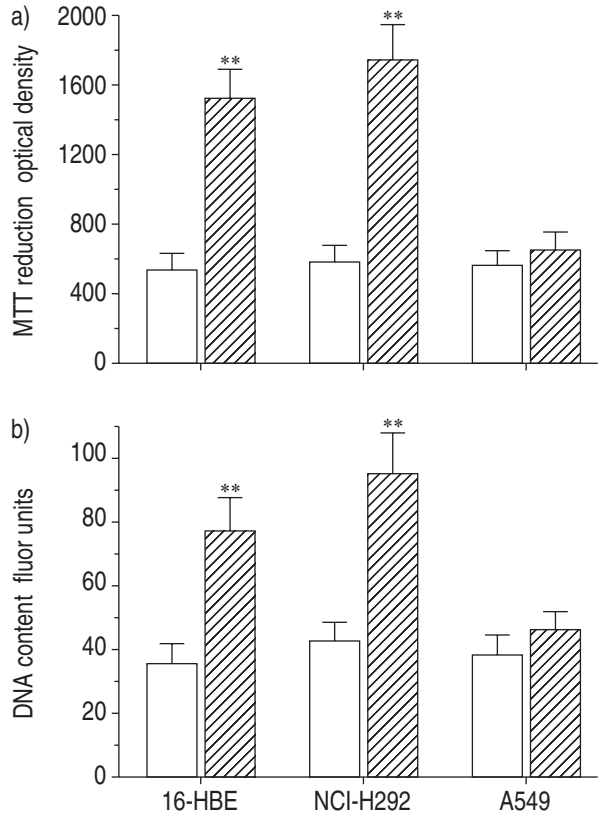

Fig. 1. – a) Effect of salbutamol on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) reduction and b) intracellular deoxyribonucleic acid (DNA) contents in 16-human bronchial epithelium (16-HBE) cells, NCI-H292 cells and A549 cells. Data are expressed as optical density determined by MTT assay and fluor units using Hoechst 33258, respectively. After a 72-h incubation in serum-free medium to arrest cell growth, salbutamol (1×10^{-7} M, ▨) was added and the cells were cultured for 48 h. In the control experiment, no drug was added (□). Data are shown as mean±SEM; n=5 for 16-HBE cells and n=6 for others. **: $p<0.01$, significantly different from control values.

had been incubated for 72 h in Ham′s F12 medium, without FBS, did not grow during the next 48 h in the serum-free medium. However, addition of salbutamol (1×10^{-7} M) to the serum-free medium stimulated the growth of epithelial cells, the values of MTT reduction compared with controls (no salbutamol) being 197±20% ($p<0.01$, n=10) at 24 h and 233±27% ($p<0.01$, n=10) at 48 h after initiation of the culture. The potency of the effect of salbutamol on cell growth was similar to that of 10% FBS. When the cells were cultured in the serum-free medium containing salbutamol (1×10^{-7} M) plus propranolol (1×10^{-5} M), the response of growth was completely abolished. To determine the role of cAMP in epithelial cell proliferation, the effects of cAMP-generating agents were determined.

As shown in figure 3, incubation for 48 h with forskolin (1×10^{-7} M) or 8-Br-cAMP (1×10^{-7} M) in the serum-free medium increased MTT reduction to the same degree as with salbutamol (1×10^{-7} M). The salbutamol-induced increase in MTT reduction was reduced by 86±10% ($p<0.01$, n=8) by co-incubation with Rp-cAMPS (1×10^{-4} M).

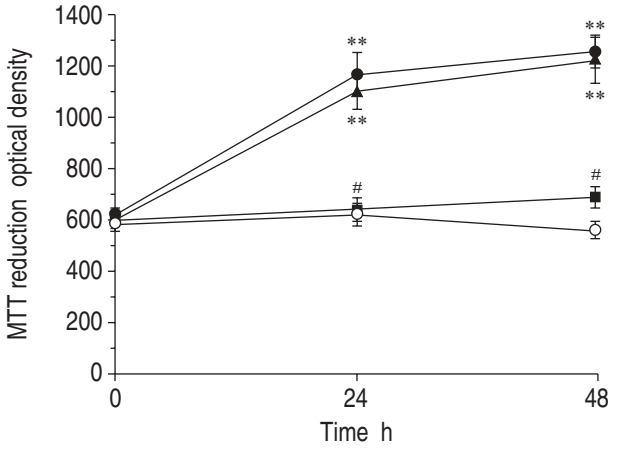

Fig. 2. – Time course of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) reduction of 16-human bronchial epithelium (16-HBE) cells in response to salbutamol (1×10^{-7} M, ●), salbutamol (1×10^{-7} M) plus propranolol (1×10^{-5} M, ■), and 10% foetal bovine serum (▲). After a 72-h incubation in serum-free medium to arrest cell growth, each drug was added and the cells were cultured for up to 48 h. In the control experiment, no drug was added (○). Data are shown as mean±SEM; n=10 for each point. **: $p<0.01$, significantly different from control values; #: $p<0.01$, significantly different from corresponding values for salbutamol alone.

The DNA content of the 16-HBE cells was not changed in the serum-free medium during the 48-h culture period, but increased in a time-dependent manner after the addition of salbutamol (1×10^{-7} M) to the medium (fig. 4). As with the finding in the MTT assay, the effect of salbutamol on DNA synthesis was

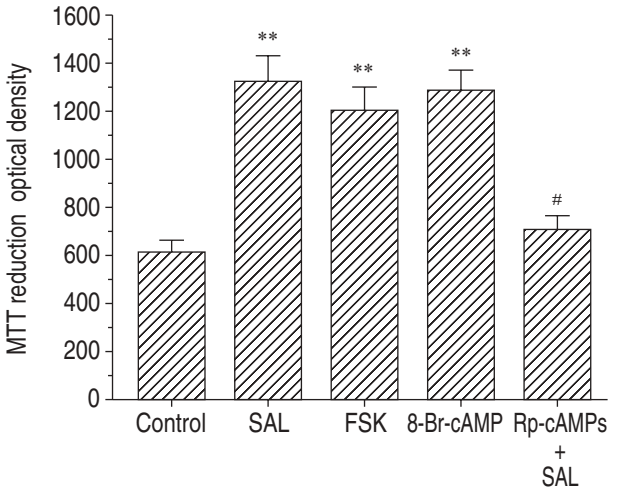

Fig. 3. – Effects of salbutamol (SAL), forskolin (FSK), and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) reduction of 16-human bronchial epithelium (16-HBE) cells, and the effect of Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS) (1×10^{-4} M) on salbutamol-induced MTT reduction. The response was assessed 48 h after incubation in serum-free medium alone (control) or containing each agonist at 1×10^{-7} M. Data are shown as mean±SEM; n=8 for each column. **: $p<0.01$, significantly different from control values; #: $p<0.01$, significantly different from values for salbutamol alone.

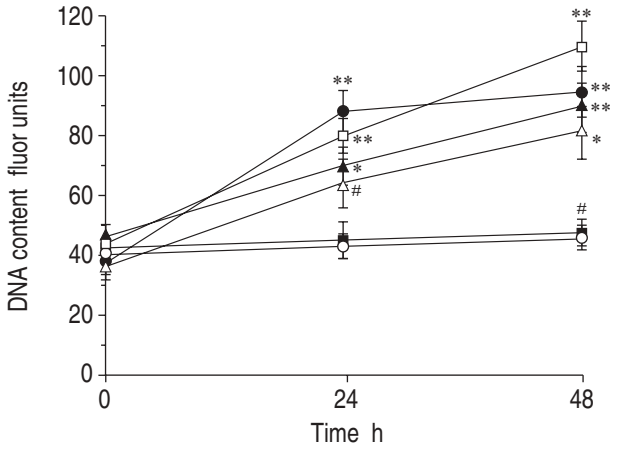

Fig. 4. – Time course of deoxyribonucleic acid (DNA) content in 16-human bronchial epithelium (16-HBE) cells in response to salbutamol (1×10^{-7} M, ●), salbutamol (1×10^{-7} M) plus propranolol (1×10^{-5} M, ■), forskolin (1×10^{-7} M, □), 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) (1×10^{-7} M, △), and 10% foetal bovine serum (▲). After a 72-h incubation in serum-free medium to arrest cell growth, each drug was added and the cells were cultured for up to 48 h. In the control experiment, no drug was added (○). Data are shown as mean±SEM; n=10 for each point. *: $p<0.05$, **: $p<0.01$, significantly different from control values; #: $p<0.01$, significantly different from corresponding values for salbutamol alone.

as potent as forskolin, 8-Br-cAMP and 10% FBS, and was abolished in the presence of propranolol.

To assess the involvement of MAP kinase cascade and cAMP in salbutamol-induced DNA synthesis, the effects of the MAP kinase-kinase inhibitor PD98059 and Rp-cAMPS were examined. As shown in figure 5,

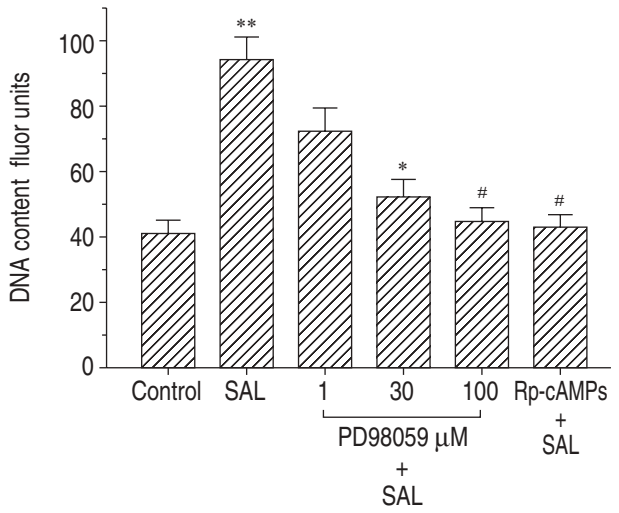

Fig. 5. – Effects of PD98059 and Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS) on salbutamol-induced deoxyribonucleic acid (DNA) synthesis in 16-human bronchial epithelium (16-HBE) cells. DNA content was determined 48 h after incubation in serum-free medium alone (control) or that containing salbutamol (SAL, 1×10^{-7} M) in the absence and presence of various concentrations of PD98059 or 1×10^{-4} M Rp-cAMPS. Data are shown as mean±SEM; n=9 for each point. *: $p<0.05$, #: $p<0.01$, significantly different from values for salbutamol alone; **: $p<0.01$, significantly different from control values.

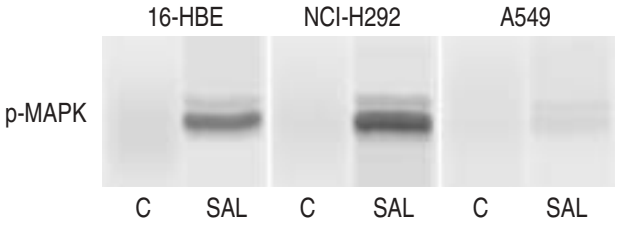

Fig. 6. – Western blotting of phosphorylated mitogen-activated protein (MAP) kinase (p-MAPK) in 16-human bronchial epithelium (16-HBE) cells, NCI-H292 cells, and A549 cells after a 5-min exposure to serum-free medium alone (C) or that containing salbutamol (SAL, 1×10^{-7} M).

the increase in DNA content induced by salbutamol was inhibited by PD98059 in a concentration-dependent manner, and by Rp-cAMPS.

Western blot analysis showed that incubation with salbutamol (1×10^{-7} M) caused phosphorylation of MAP kinase in 16-HBE cells and NCI-H292 cells, but it had little effect in A549 cells (fig. 6).

In 16-HBE cells, MAP kinase activation was induced by salbutamol, forskolin, and 8-Br-cAMP, the salbutamol-induced expression of phosphorylated MAP kinase was greatly inhibited by propranolol (fig. 7). Moreover, the phosphorylation of MAP kinase produced by salbutamol was inhibited by PD98059 and Rp-cAMPS.

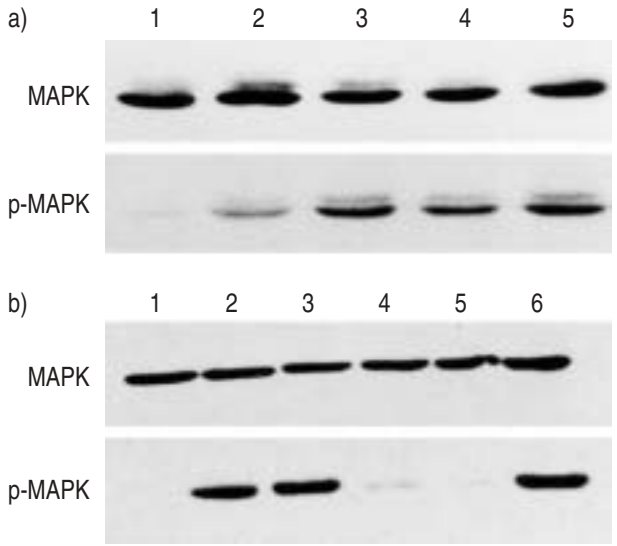

Fig. 7. – Western blotting of mitogen-activated protein (MAP) kinase (MAPK) and phosphorylated MAP kinase (p-MAPK) in 16-human bronchial epithelium (16-HBE) cells after a 5-min exposure to serum-free medium alone (control) or containing various drugs. a) Effects of cyclic adenosine monophosphate (cAMP)-generating agents and the effect of β-adrenoceptor antagonist on salbutamol-induced MAPK phosphorylation. Lane 1: control; lane 2: salbutamol plus propranolol; lane 3: salbutamol; lane 4: forskolin; lane 5: 10% foetal bovine serum (FBS). b) Effects of MAP kinase-kinase inhibitor and cAMP antagonist on salbutamol-induced MAPK phosphorylation. Lane 1: control; lane 2: salbutamol; lane 3: 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP); lane 4: salbutamol plus PD98059; lane 5: salbutamol plus Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS); lane 6: 10% FBS.

Discussion

The present study shows that salbutamol, a β_2-adrenergic receptor agonist, stimulates the proliferation of human airway epithelial cell lines. The current authors found that, in 16-HBE and NCI-H292 cells (but not A549 cells), exposure to salbutamol caused remarkable increases in MTT reduction and intracellular DNA content in a time-dependent manner, even in the culture medium from which FBS was substituted in order to arrest cell growth. This effect in 16-HBE cells is mediated by stimulation of β_2-adrenergic receptors, because co-incubation with propranolol abolished the salbutamol-induced cell growth and DNA synthesis. Furthermore, cAMP-generating agents, including the adenylyl cyclase stimulator, forskolin, and the stable cAMP analogue, 8-Br-cAMP, also showed a growth-promoting effect. In this experiment, time course of DNA synthesis was different between salbutamol and other drugs; the response to salbutamol reached a plateau during the first 24 h, whereas forskolin and 8-Br-cAMP seemed to induce a continued increase in DNA contents over the full 48 h. The reason for this difference is unknown, but could be due to desensitisation of β_2-adrenergic receptors by salbutamol. In addition, the present authors found that the salbutamol-induced MTT reduction and DNA synthesis were inhibited by the cAMP antagonist, Rp-cAMPS. Taken together, cAMP formation may represent a mitogenic signal in certain airway epithelial cell lines.

The role of cAMP in the regulation of cell growth is controversial, probably because of the differences in cell types and experimental conditions. Previous studies have shown that the elevation of intracellular cAMP content may link to the inhibition of growth of human and guinea pig airway smooth muscle cells [13, 14]. Conversely, in rat parotid cells [15], dog thyroid cells [16], and rat cardiac myocytes [17], the increases in intracellular content of cAMP have been shown to stimulate cell growth. In addition, according to the recent study on cochlear sensory epithelium, forskolin and 8-Br-cAMP stimulate cell growth, an effect that can be blocked by the inhibitors of protein kinase A [9]. These findings are in accordance with the present results and suggest a stimulatory role for the cAMP-dependent signalling pathway in the proliferation of epithelial cells. However, this experiment was conducted using only airway epithelial cell lines, and the effect of salbutamol was different between the cell lines. Thus, it remains to be determined whether such a signalling pathway is also present in primary culture of human airway epithelium.

The MAP kinases actively participate in converting extracellular stimuli detected by cell surface receptors to intracellular signals, leading to the expression of genes necessary for cell growth and differentiation [18]. For example, there is ample evidence that stimulation of Gi and Gq protein-coupled receptors potently activates MAP kinase [7, 8]. Regarding the effect of Gs protein-coupled receptor stimulation, isoproterenol has been shown to activate and inhibit the enzymatic activity in cardiac myocytes [17] and COS-7 cells [19], respectively. In the present study, the induction of phosphorylated MAP kinase was observed in 16-HBE and NCI-H292 cells, but not A549 cells after incubation with salbutamol. In 16-HBE cells, other cAMP-generating agents also caused activation of MAP kinase, and the salbutamol-induced MAP kinase phosphorylation was abolished by Rp-cAMPS. These results indicate that there may be a Gs-induced stimulatory signal mediated by cAMP for MAP kinase activity in certain human airway epithelial cells.

The major pathway involved in mitogen-activated protein kinase activation generally requires the sequential activation of Ras, Raf-1, and mitogen-activated protein kinase-kinase [20, 21]. The present authors found that a specific inhibitor of mitogen-activated protein kinase-kinase, PD98059 [12], inhibited the mitogen-activated protein kinase phosphorylation induced by salbutamol, and the salbutamol-induced deoxyribonucleic acid synthesis in a concentration-dependent fashion. Thus, activation of mitogen-activated protein kinase-kinase is likely to be involved in the observed growth of 16-human bronchial epithelium cells. However, the link between the cyclic adenosine monophosphate-dependent pathway and the initially activated molecule that localises proximal to mitogen-activated protein kinase-kinase in the mitogen-activated protein kinase cascade is still unknown and warrants further investigation.

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References

1. Ray LB, Sturgill TW. Characterization of insulin-stimulated microtubule-associated protein kinase: rapid isolation and stabilization of a novel serine/threonine kinase from 3T3-L1 cells. *J Biol Chem* 1988; 263: 12721–12727.
2. Ahn NG, Krebs EG. Evidence for an epidermal growth factor-stimulated protein kinase cascade in Swiss 3T3 cells: activation of serine peptide kinase activity by myelin basic protein kinases *in vitro*. *J Biol Chem* 1990; 265: 11495–11501.
3. Anderson NG, Maller JL, Tonks NK, Sturgill TW. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature* 1990; 343: 651–653.
4. Ahn NG, Seger R, Bratlien RL, Diltz CD, Tonks NK, Krebs EG. Multiple components in an epidermal growth factor-stimulated protein kinase cascade: *in vitro* activation of a myelin basic protein/microtubule-associated protein 2 kinase. *J Biol Chem* 1991; 266: 4220–4227.
5. Chao TS, Foster DA, Rapp UR, Rosner MR. Differential Raf requirement for activation of mitogen-activated protein kinase by growth factors, phorbol esters, and calcium. *J Biol Chem* 1994; 269: 7337–7341.
6. Chao TS, Byron KL, Lee KM, Villereal M, Rosner MR. Activation of MAP kinases by calcium-dependent and calcium-independent pathways: stimulation by

thapsigargin and epidermal growth factor. *J Biol Chem* 1992; 267: 19876–19883.
7. Crespo P, Xu N, Simonds WF, Gutkind JS. Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature* 1994; 369: 418–420.
8. Faure M, Voyno-Yasenetskaya TA, Bourne HR. cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J Biol Chem* 1994; 269: 7851–7854.
9. Navaratnam DS, Su HS, Scott SP, Oberholtzer JC. Proliferation in the auditory receptor epithelium mediated by a cyclic AMP-dependent signaling pathway. *Nat Med* 1996; 2: 1136–1139.
10. Plumb JA, Milroy R, Kaye SB. Effects of the pH dependence of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-formazan absorption on chemosensitivity determined by a novel tetrazolium-based assay. *Cancer Res* 1989; 49: 4435–4440.
11. Rago R, Mitchen J, Wilding G. DNA fluorometric assay in 96-well tissue culture plates using Hoechst 33258 after cell lysis by freezing in distilled water. *Anal Biochem* 1990; 191: 31–34.
12. Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc Natl Acad Sci USA* 1995; 92: 7686–7689.
13. Tomlinson PR, Wilson JW, Stewart AG. Inhibition by salbutamol of the proliferation of human airway smooth muscle cells grown in culture. *Br J Pharmacol* 1994; 111: 641–647.
14. Pyne NJ, Moughal N, Stevens PA, Tolan D, Pyne S. Protein kinase C-dependent cyclic AMP formation in airway smooth muscle: the role of type II adenylate cyclase and the blockade of extracellular-signal-regulated kinase-2 (ERK-2) activation. *Biochem J* 1994; 304: 611–616.
15. Tsang BK, Rixon RH, Whitfield JF. A possible role for cyclic AMP in the initiation of DNA synthesis by isoproterenol-activated parotid gland cells. *J Cell Physiol* 1980; 102: 19–26.
16. Roger PP, Servais P, Dumont JE. Stimulation by thyrotropin and cyclic AMP of the proliferation of quiescent canine thyroid cells cultured in a defined medium containing insulin. *FEBS Lett* 1983; 157: 323–329.
17. Lazou A, Bogoyevitch MA, Clerk A, Fuller S, Marshall JC, Sugden PH. Regulation of mitogen-activated protein kinase cascade in adult rat heart preparations *in vitro*. *Circ Res* 1994; 75: 932–941.
18. Cobb MH, Boulton TG, Robbins DJ. Extracellular signal-regulated kinases: ERKs in progress. *Cell Regul* 1991; 2: 965–978.
19. Crespo P, Cachero TG, Xu N, Gutkind JS. Dual effect of beta-adrenergic receptors on mitogen-activated protein kinase: evidence for a beta gamma-dependent activation and a G alpha s-cAMP-mediated inhibition. *J Biol Chem* 1995; 270: 25259–25265.
20. Kyriakis JM, App H, Zhang XF, *et al.* Raf-1 activates MAP kinase-kinase. *Nature* 1992; 358: 417–421.
21. Macdonald SG, Crews CM, Wu L, *et al.* Reconstitution of the Raf-1-MEK-ERK signal transduction pathway *in vitro*. *Mol Cell Biol* 1993; 13: 6615–6620.