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EFFECT OF PHOSPHODIESTERASE INHIBITORS ON ERK1/2 MAP KINASE SIGNALING PATHWAY

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Summary. Phosphodiesterase (PDE) enzymes catalyze the breakdown of cAMP and thus may have potent immunomodulatory activity. We were interested to investigate whether PDE4 inhibitor rolipram and a non-specific PDE inhibitor theophylline modulate ERK1/2 signaling pathway in freshly isolated human PBMCs. To determine the effect of rolipram and theophylline on the phosphorylation and activation of ERK1/2 signaling pathway, human PBMCs (5 x 10^6) ml were serum-starved and treated with 1 μg/ml Der f. After 15 min incubation cells were harvested and lysed in cell lytic buffer. Equal amounts of lysates (40 μg) were subjected to SDS-PAGE and Western blot analysis using an antibody phospho-p44/42 MAP kinase (Thr 202/Tyr 204), specific for the activated form of ERK1/2. This antibody recognizes ERK when phosphorylated on both threonine and tyrosine residues. Cells treated with Der f exhibited detectable phosphorylation of ERK1/2 at 15 min. Our present study demonstrates that PDE4 inhibitor rolipram (10^{-5} M) and nonspecific phosphodiesterase inhibitor theophylline (10^{-5} M) decrease ERK phosphorylation induced by Der f. MEK/ERK inhibitor U0126 (10 μM) alone or in combination with rolipram (10^{-5} M) downregulated the gene expression of IL-5 mRNA of human PBMCs stimulated with Der f.

Key words: human PBMCs, PDE inhibitors, rolipram, theophylline, Der f antigen, ERK MAP kinase

ЕФЕКТ НА ФОСФОДИЕСТЕРАЗНИТЕ ИНХИБИТОРИ ВЪРХУ ERK1/2 MAP КИНАЗНИЯ СИГНАЛЕН ПЪТ

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Резюме. Фосфодиестеразата (PDE) катализира разграждането на cAMP и в резултат притежава имуномодулираща активност. Ние изследвахме ефекта на PDE4 инхибитора ролипрам и на неселективния PDE инхибитор теофилин върху активирането на ERK1/2 сигнален път при прясно изолирани човешки периферни мононуклеарни клетки (PBMCs). PBMCs (5 x 10^6) бяха третирани с 1 μg/ml Der f антиген. След 15-минутна инкубация клетките бяха лизирани с лизиращ буфер и с участието на phospho-p44/42 MAP kinase (Thr 202/Tyr 204) антитяло, специфично за активираната форма на ERK1/2, се проведе Western blot анализ. Третираните с 1 μg/ml Der f клетки показаха значително фосфорилиране на ERK1/2 на 15 min. Нашите резултати демонстрират, че PDE4 инхибиторът ролипрам (10^{-5} M) и неселективният PDE инхибитор теофилин (10^{-5} M) потискат Der f индуцираното фосфорилиране на ERK1/2 сигнален път. MEK/ERK инхибиторът U0126 (10 μM), приложен самостоятелно или в комбинация с ролипрам (10 μM), потиска генната експресия на IL-5 mRNA при човешки PBMCs, стимулирани с Der f антиген.

Ключови думи: човешки периферни мононуклеарни клетки, фосфодиестеразни инхибитори, ролипрам, теофилин, Der f антиген, ERK MAP киназа

Although the precise mechanisms underlying cell activation are not clearly understood, adenosine 3'5'-cyclic monophoshate (cAMP) is widely accepted to exert a broad suppressive effect on the activation of most inflammatory cells [1]. Agents which elevate cAMP levels thus have the potential to inhibit

cellular activation. The phosphodiesterase (PDE) enzymes catalyze the breakdown of cAMP and guanosine 3'5'-cyclic monophosphate (cGMP). To date, at least eleven heterologous families of PDEs were reported, based on their substrate selectivity, enzyme kinetics and inhibitor potency [12]. In particular, the cAMP-specific PDE4 is preferentially expressed in immune and inflammatory cells [10].

Rolipram ((±) 4-(3'-cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone), is a specific PDE4 inhibitor, whose therapeutic utility has been investigated in treatment of depression [13]. Furthermore and even more importantly, rolipram and other specific PDE4 inhibitors have the capacity to suppress inflammatory processes. A large body of evidence suggests that PDE4 inhibitors might possess anti-inflammatory effects and thus be a newer strategy to control allergic diseases [3, 5, 6, 7, 11]. Theophylline has been in clinical use for the treatment of bronchial asthma and other respiratory diseases for over 50 years. Over this time, a considerable body of evidence has accumulated to show that this drug has wide range of pharmacological actions, in addition to the well-recognised action on airway smooth muscle function. Current evidence suggests that part of the therapeutic value of theophylline in the treatment of asthma is virtue of an anti-inflammatory or immunomodulatory effect, although the actual mechanism of action remains unclear. It has been proposed that the observed anti-inflammatory effects of theophylline could be attributed to phosphodiesterase inhibition.

Currently, much research is being undertaken to develop novel PDE4 inhibitors for the treatment of allergic and inflammatory diseases including asthma and greater understanding of their mechanism of action may encourage further research and would provide new therapeutic approach for the treatment of such conditions.

Based on the above mentioned concerns, we were interested to investigate whether PDE4 inhibitor rolipram and a non-specific phosphodiesterase inhibitor theophylline modulate Der f-initiated ERK1/2 signaling in freshly isolated human PBMCs.

Reagents

The following reagents were obtained from manufactures described as follows: Ficoll-Paque™ from Amersham Pharmacia Biotech AB, Uppsala, Sweden. Rolipram and theophylline (Wako Pure chemical industries), RP-8-Br-cAMP and MEK/ERK inhibitor U0126 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Cell lysis extraction reagent and phosphatase inhibitor cocktail, casein blocking buffer were all from Sigma, St. Louis, Missouri, USA. Protease inhibitor cocktail was from Boehringer Mannheim, Mannheim, Germany. Dermatophagoides Farinae (Der f) antigen from DANI, Japan. Phospho-p44/42 MAP kinase (Thr 202/Tyr 204) and p44/42 MAP kinase antibodies were obtained from Cell Signaling Technology, Beverly, MA, USA, RNeasy kit (Qiagen), Reverse transcriptase system (Promega), Taq PCR Core kit (Qiagen), IL-5 primers were obtained from TaKaRa Bio Inc, Shiga, Japan.

Methods

Preparation of freshly isolated peripheral blood mononuclear cells. Human peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood of healthy volunteers through a density gradient centrifugation method (Ficoll-Paque®). PBMCs were collected from the interface and washed twice with PBS. Rolipram was dissolved in DMSO at a concentration of 10^{-2} M and prepared immediately before use. The final concentration of DMSO in each well was no greater than 0.1%. All other compounds were solubilized in medium.

PBMCs stimulation. PBMCs were cultured in RPMI1640 medium supplemented with 1% L-glutamine and 1% antibiotics/antimycotics, at a density of 5 x 10^6 cells/ml for all experiments. Cells were pre-incubated for 30 min with rolipram (10^{-7}, 10^{-6} or 10^{-5} M), theophylline (10^{-7}, 10^{-6} or 10^{-5} M) or MEK/ERK inhibitor U0126 (10 μM) before stimulation with 1 μg/ml Der f antigen. Cells were harvested at 18 h after stimulation with Der f antigen.

RT-PCR. Cells were first harvested, and RNA samples prepared by use of RNeasy kit (Qiagen). All RNA samples were subjected to reverse-transcription of cDNA using a reverse transcriptase system (Promega) according to the manufacturer's instructions. cDNA products were amplified with the designated primer pairs using Taq PCR Core kit (Qiagen). To perform following PCR amplification in a semi-quantitative way, serially diluted samples were amplified with β-actin primers with different cycle numbers, and PCR conditions yielding exponential increase in PCR signals were used for further study. cDNA samples then were amplified using specific primers for IL-5. Cycling parameters were as follows:

Denaturation at 95°C for 30 seconds; annealing at 55°C for 45 seconds and extention at 72°C for 1 minute for the appropriate number of cycles giving a visible signal on agarose gels without reaching saturation (30 cycles for β-actin and 40 cycles for IL-5), followed by a 10 min extention at 72°C.

PCR products were subsequently size-fractionated on 2% agarose gels, stained with ethidium bromide and visualized under UV light.

Western Blot analysis. PBMCs (5 x 10^6) were seeded in serum free medium, treated with drugs and stimulated with 1 μg/ml Der f antigen. Cells were harvested in 15 min, lysed with cell lysis extraction reagent (Sigma) containing phosphatase inhibitor cocktail (Sigma) and protease inhibitor cocktail (Boehringer Mannhein) and sonicated for 10 s. Cell debris was removed by centrifugation at 15 000 r.p.m. for 15 min at 4°C. Protein concentrations of the supernatants were determined by Bio-Rad protein assay reagent and 40 μg of protein diluted with equal volumes of 2 x sodium dodecyl sulfate (SDS) sample buffer was denaturated by heating at 95°C for 5 min and resolved in 10% SDS-polyacrylamide gels. Proteins were further transferred in a buffer contaning 25 mM Tris-HCI (Ph 8.3), 192 mM glycine, 0.1% SDS and 10% methanol onto nitrocellulose membranes (Bio-Rad). Blots were blocked in casein blocking buffer (Sigma) for 12 h at 4°C. The incubation with primary antibody diluted 1:1000 in casein blocking buffer was performed overnight at 4°C. After three times washing in PBS – 0.1% Tween 20, blots were incubated with secondary anti-rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (Santa Cruz Biotehnology) diluted 1:10 000 in casein blocking buffer at room temperature for 1h. After three washes in PBS – 0.1% Tween 20, primary antibody binding was visualized by using the ECL plus Western blotting Detection System (Amersham). Phosphorylated ERK1/2 were detected by using phospho-p44/42 MAP Kinase (Thr 202/Tyr 204) antibody obtained from Cell Signaling Technology.

Results

Effect of pharmacological blockade of ERK1/2 signaling pathway on Der f-mediated expression of IL-5 mRNA. Human PBMCs were preincubated with a highly specific MEK/ERK inhibitor U0126 (10 μM) for 30 min before stimulation with Der f (1 μg/ml). After 18 h incubation cells were harvested for mRNA expression analysis. As shown on fig. 1a and 1b, the MEK/ERK inhibitor U0126 (10 μM) alone or in combination with rolipram (10 μM) downregulated the expression of IL-5 mRNA of human PBMCs stimulated with Der f.

Effect of rolipram, theophylline and 8-Br-cAMP on phosphorylation and activation of ERK1/2 MAP kinase signaling pathway. To determine the effect of rolipram, theophylline and 8-Br-cAMP on the phosphorylation and activation of ERK1/2 signaling pathway in human PBMCs, cells (5 x 10^6) ml were serum-starved and treated with 1 μg/ml Der f. After 15 min incubation cells were harvested and lysed in cell lytic buffer. Equal amounts of lysates (40 μg) were subjected to SDS-PAGE and Western blot analysis using an antibody phospho-p44/42 MAP kinase (Thr 202/Tyr 204), specific for the activated form of ERK1/2. This antibody recognizes ERK when phosphorylated on both threonine and tyrosine residues. As presented in fig. 2 for PBMCs, two bands were detected at the expected sizes: 42 and 44 kDa when cells were at their resting state. Cells treated with Der f exhibited detectable phosphorylation of ERK1/2 at 15 min. As compared to the positive control (cells activated with Der f), PDE4 inhibitor rolipram, nonspecific phosphodiesterase inhibitor theophylline and cAMP analogue 8-Br-cAMP decreased ERK phosphorylation induced by Der f (fig. 2).

Discussion

In this study, we were trying to elucidate the effects of phosphodiesterase inhibitors on the Der f – stimulated ERK1/2 signaling pathway. Throughout this paper, we stimulated human PBMCs with Der f in our experimental setting. To date there is evidence that rolipram may modulate activation of immunocompetent cells. It was reported that this compound inhibited secretion of IL-5, IL-10, IL-2 and TNF-α, but poorly did so for IFN-γ from human T cells, which were stimulated with immobilized anti-CD3 [2]. Other reports showed as rolipram and other cAMP-elevating agents inhibited release of IL-5 from human PBMC stimulated with anti-CD3 and anti-CD28 [8]. By employing this strict experimental conditions, we showed herein that rolipram (10^{-5}M) and highly specific MEK/ERK inhibitor U0126 (10 μM) clearly downregulated Der f-upregulated expression of IL-5 mRNA of PBMCs from healthy volunteers (fig. 1a and b).

Our previous data showed that Ropilram down-regulated IL-4-induced IL-5 mRNA of human T cells

[4]. In this paper, we presented the inhibitory effect of MEK/ERK inhibitor U 0126 (10 μM) on the Der f-stimulated expression of IL-5 in human PBMCs (fig. 1a and 1b).

Tamir et al., 1996 [9], reported that cAMP-inhibited activation of T cells was associated with downregulation of MAPK pathways, ERK kinases and c-Jun N-terminal kinase (c-JNK). Our western blotting data showed that Der f antigen increases phosphorylation and activation of ERK1/2 MAP kinases in human PBMCs. Rolipram, theophylline and 8-Br-cAMP inhibited this process (fig. 2). ERK MAP kinase signalling pathway is connected with expression of inflammatory genes and control of this process could have therapeutic potential.

This study shows that phosphodiesterase inhibitors, like rolipram and theophylline via suppresson of the reactivity of human PBMCs may be of therapeutic benefit for allergic diseases. Further investigation is warranted to elucidate more precise mode of actions of phosphodiesterase inhibitors at a molecular basis. This may contribute to welfare and health of patients suffering from long-lasting and so far therapy-resistant disorders, such as allergies.

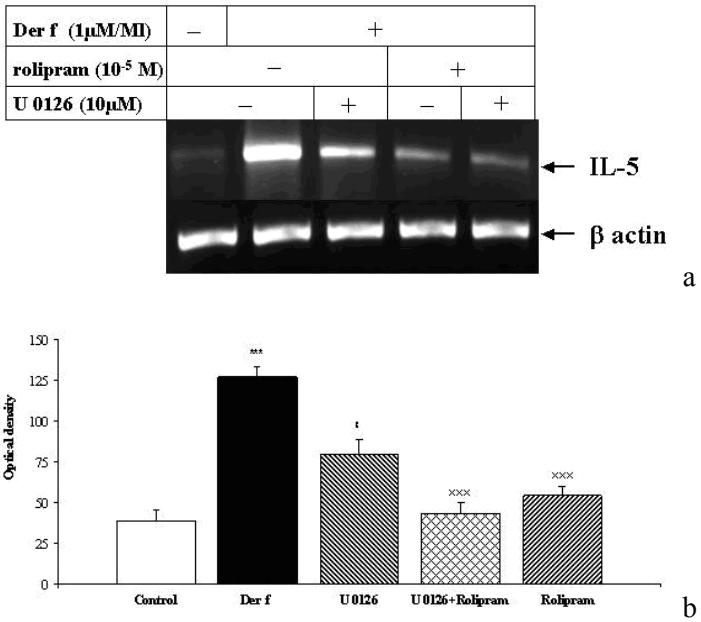

Fig. 1. *Semiquantitative RT-PCR. The effect of pharmacological blockade of ERK1/2 signaling pathway on Der f-mediated expression of IL-5 mRNA. (a) Human PBMCs (5 x 10^6 cells/ml) were cultured in the presence of Der f antigen (1 μg/ml) for 18 h with or without MEK/ERK inhibitor U0126 (10 μM) alone or in combination with rolipram (10^{-5} M). Data from four independent experiments are shown.(b) Densitometry data are depicted for IL-5 cytokine for all subjects. Data were normalized to Der f-treated cells; nonstimulated drug-free controls and with background values subtracted. ***$P < 0.001$ vs. nonstimulated group; $^{x}P < 0.05$, $^{xxx}P < 0.001$, vs. Der f-treated controls. The data presented are one of representative of four independent experiments*

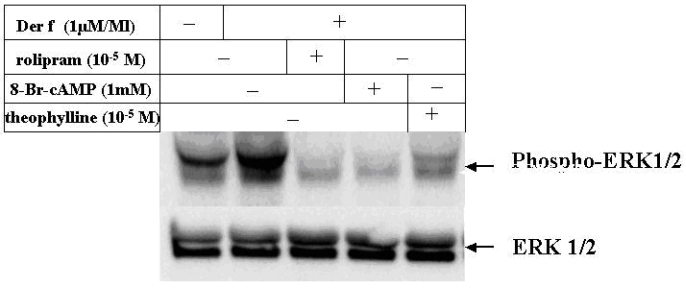

Fig. 2. *Western Blot analysis. The effect of rolipram and theophylline on the phosphorylation and activation of ERK1/2 MAP kinase induced by Der f antigen in human PBMCs. Human PBMCs (5 x 10^6 /ml) were preincubated for 30 min at 37°C in media alone (lane 1) or in the presence of rolipram (10^{-5} M). (lane 3), 8-Br-cAMP (1mM/ml) (lane 4) and theophylline (10^{-5} M). (lane 5). The cells then were stimulated with Der f (1μg/ml) (lane 2). Cells were harvested in 15 min therafter and lysed. The lysates were electrophoresed in 10% tris-glycine polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with an antiphosphorylated ERK1/2 antibody (first pannel) or a non-phosphorylation-dependent anti-ERK1/2 antibody (panel 2). Reactions were detected by chemiluminescence. Data from three independent experiments are shown*

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