Online data supplement

Aclidinium inhibits cigarette smoke-induced lung fibroblast to myofibroblast transition

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Material

Aclidinium bromide 3*R*-(2-hydroxy-2,2-di-thiophen-2-yl-acetoxy)-1-(3-phenoxy-propyl)-1-azonia-bicyclo [2.2.2] octane bromide was synthesized by the Department of Medicinal Chemistry (Almirall S.A., Barcelona, Spain). Unless indicated otherwise, all other reagents used were obtained from Sigma (Chemical Co, Madrid, Spain). Aclidinium bromide was dissolved in a stock solution of 0.2 to 1% (v/v) HCl. PD98059 was dissolved in a dimethyl sulfoxide (DMSO) 0.1% stock solution. The final concentrations of DMSO (0.1%) or HCl (10 µM) in the cell culture did not affect cellular functions. Other chemicals (dibutyryl cAMP [dbcAMP], apocynin, N-acetyl cysteine [NAC], atropine, hemicholinium-3, neostigmine bromide and acetyl cholinesterase [AChE]) were dissolved in medium.

Methods

Isolation and Cultivation of Human Fibroblasts

Human lung tissue was obtained from patients who were undergoing surgery for lung carcinoma and who gave informed consent. Bronchial (~2 mm of internal diameter) healthy areas of surgically resected lung tissue were used to obtain human bronchial fibroblasts. The protocol for obtaining human tissue was approved by the local ethical review board for human studies (General Hospital of Valencia, Spain). Bronchial tissue was: cut into small pieces; treated with pronase (1 mg/ml^{-1}; Calbiochem®, Novabiochem®, San Diego, CA, USA) at 37ºC for 30 minutes; placed in cell culture plates and incubated in Dulbecco's Modified Eagle's Medium (DMEM); and supplemented with 10% fetal calf serum (Sigma, St Louis, MO, USA), 100 U/ml^{-1} penicillin/streptomycin and 2% fungizone (GIBCO, Grand Island, NY, USA). After approximately 2 weeks, fibroblasts had grown from the tissue and were passaged by standard trypsinization. Cells from passages 3 to 10 were used in all experiments described in the present study.

Preparation of Cigarette Smoke Extract and Incubations

The smoke of a research cigarette (2R4F, from Tobacco Health Research, University of Kentucky) was bubbled into a flask containing 25 ml of pre-warmed (37°C) DMEM medium using a respiratory pump model (Harvard Apparatus Rodent Respirator 680, Harvard Apparatus, Hollister, MA, USA), that operates through a puffing mechanism corresponding to the human smoking pattern (3 puffs min^{-1}; 35 ml per each puff of 2-s duration with a volume of 0.5 cm above the filter). The cigarette smoke solution was then drawn into a syringe through a 0.22 µm pore size filter to remove particles and the tar phase and to obtain a sterile solution (Corning, NY, USA). The resulting solution was defined as CSE at 100% and used in the different experiments within 30 minutes of preparation following appropriate dilution as indicated. CSE at 10% reportedly corresponds to an exposure associated with smoking of approximately 1–2 packs/d [1]. Cytotoxicity possibly emanating from CSE was analyzed by exposing bronchial fibroblasts to 10% CSE for up to 3 days (CSE-and culture medium were replaced every 24 h) based on the release of lactate dehydrogenase (LDH) in culture supernatants. No significant differences in LDH activities were observed in the culture supernatants between the CSE and control group (LDH cytotoxicity assay, Cayman, Madrid, Spain, data not shown).

Before stimulation, subconfluent cell monolayers were deprived of serum for 24 h. Human bronchial fibroblasts were stimulated with CSE (0 to 10%) for different periods of time (0–72 h) replacing culture medium and stimulus every 24 h. Aclidinium bromide (Almirall, Barcelona, Spain), human acetylcholinesterase (10 U/ml^{-1}; AChE; Sigma: catalogue no. C1682), dibutyryl adenosine 3´–5´ cyclic monophosphate sodium salt (1 mM; dbcAMP; Sigma: catalogue no. D0260), PD98059 (described as ERK1/2 inhbitor, 10 µM; Sigma: catalogue no. P215), atropine (muscarinic agonist, 10nM-1µM; Sigma: catalogue no. A0132), hemicholinium-3 (choline uptake inhibitor, 50µM; Sigma: catalogue no. H108), neostigmine bromide (inhibitor od acetylcholinesterase, 10µM; Sigma: catalogue no. N2001), N-acetyl-L-cysteine (described as antioxidant, 1 mM; Sigma: catalogue no. A7250), apocynin (described as NADPH oxidase inhibitor, 100 µM; Sigma: catalogue no. w508454) were added 30 minutes before stimulus.

Real Time RT–PCR

Total RNA was isolated from cultured human bronchial fibroblasts by using TriPure® Isolation Reagent (Roche, Indianapolis, IN, USA). Integrity of the extracted RNA was confirmed with Bioanalizer (Agilent, Palo Alto, CA, USA). The reverse transcription was performed in 300 ng of total RNA with the TaqMan reverse transcription reagents kit (Applied Biosystems, Perkin-Elmer Corporation, CA, USA). cDNA was amplified using assays-on-demand specific primers pre-designed by Applied Biosystems for muscarinic acetylcholine receptors (mAChR) M1, M2 and M3, α_1(I)-collagen (Col Type I), alpha-smooth muscle actin (α-SMA), p46phox, p67phox, NOX1 and NOX4 (catalogue no. Hs00912795_m1, Hs00265208_s1, Hs00327458_m1, Hs00164004_m1, Hs00559403_m1, Hs00417167_m1, Hs01084940_m1, Hs00246589_m1 and Hs00418356_m1) in a 7900HT Fast Real-Time PCR System (Applied Biosystems) using Universal Master Mix (Applied Biosystems). Relative quantification of these different transcripts was determined with the $2^{-\Delta\Delta Ct}$ method using glyceraldehyde phosphate dehydrogenase (GAPDH) as endogenous control (Applied Biosystems; 4352339E) and normalized to control group.

Transfection of siRNAs

Small interfering RNA (siRNA), including the scrambled siRNA control, were purchased from Ambion (Huntingdon, Cambridge, UK). M1, M2 and M3 muscarinic receptor gene-targeted siRNAs (identification nos s3024, s3026 and s230642, respectively) were designed by Ambion. The human bronchial fibroblasts were transfected with siRNA (50 nM) in serum and antibiotic-free medium. After a period of 6 h, the medium was aspirated and replaced with medium containing serum for a further 42 h before carbachol stimulation. The transfection reagent used was lipofectamine-2000 (Invitrogen, Paisley, UK) at a final concentration of 2 $\mu l/ml^{-1}$. The mRNA expression for M1, M2 and M3 transcripts was determined by real-time RT-PCR (as described above) after 48 h post-silencing and compared with siRNA control at the respective time to determine silencing efficiency. Furthermore, M1, M2 and M3 protein expression was measured by Western blot after 48 h of silencing, as described in the Western blotting section.

DCF Fluorescence Measurement of Reactive Oxygen Species

2´, 7´-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Molecular Probes, Nottingham UK) is a cell-permeable compound that following intracellular ester hydrolysis is oxidized to fluorescent 2´, 7´-dichlorofluorescein (DCF) by $O_2^{\cdot-}$ and H_2O_2, and can therefore be used to monitor intracellular generation of reactive oxygen species (ROS) [2]. To quantify ROS levels, cells were seeded to black-walled, clear bottom 96-well plates, washed twice with PBS and incubated for 30 minutes with 50 µM DCFDA diluted in Opti-MEM with 10% FCS. At the end of the incubation period, the cells were again washed twice with PBS and stimulated with CSE at different concentrations (2.5 to 10%) and times as indicated. In other experiments different drug modulators were added 30 minutes before CSE 2.5% addition, and remained together for 24 h; 1 h before the end of the stimulation period, DCFDA was added. Then, cells were washed and fluorescence was measured using a microplate spectrophotometer (Victor 1420 Multilabel Counter, PerkinElmer) at excitation and emission wavelengths of 485 and 528 nm, respectively. Results were expressed as DFC fluorescence in relative fluorescence units (RFU) *versus* time.

Western blotting

Western blot analysis was used to detect changes in collagen type I (138 kD), α-SMA, p-ERK1/2 (42–44 kD), M1 (52 kD), M2 (70 kD), M3 (75 kD), p67phox (67 kD), NOX4 (67 kD) and ChAT (65 kD). Cells were scraped from a confluent 25-cm^2 flask and lysed on ice with a lysis buffer consisting of a complete inhibitor cocktail plus 1 mM ethylenediaminetetraacectic acid (Roche Diagnostics Ltd, West Sussex, UK) with 20 mM Tris base, 0.9% NaCl, 0.1% Triton X-100, 1 mM dithiothreitol and 1 $\mu g\ mL^{-1}$ pepstatin A. The Bio-Rad assay (Bio-Rad Laboratories Ltd., Herts, UK) was used (following manufacturer's instructions) to quantify the level of protein in each sample to ensure equal protein loading. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was used to separate the proteins according to their molecular weight. Briefly, 20 µg proteins (denatured) along with a molecular weight protein marker, Bio-Rad Kaleidoscope marker (Bio-Rad Laboratories), were loaded onto an acrylamide gel consisting of a 5% acrylamide stacking gel stacked on top of a 10% acrylamide resolving gel and run through the gel by application of 100 V for 1 h. Proteins were

transferred from the gel to a polyvinylidene difluoride membrane using a wet blotting method. The membrane was blocked with 5% Marvel in PBS containing 0.1% Tween20 (PBS-T) and then probed with a rabbit anti-human col type I (1:1,000) antibody (polyclonal antibody; Affinity Bioreagents, Golden, USA; catalogue no. PA1-26204), mouse anti-human anti-α-SMA (1:1,000) antibody (monoclonal antibody; Sigma; catalogue no. A5228), rabbit anti-human M1, M2 and M3 (1;1,000) antibodies (polyclonal antibodies; Sigma-Aldrich, CA, USA; catalogue nos. HPA014101, M 9558 and M0194 respectively), rabbit anti-human ChAT (1:1,000) antibody (monoclonal antibody; Millipore Bioscience Research Reagents, Temecula, CA, USA; catalogue no.AB143), rabbit anti-human NOX4 antibody (1:1,000) (monoclonal antibody; Novus Biologicals, CA, USA; catalogue no.NB110-58849) and rabbit anti-human p67phox (1:500) antibody (polyclonal antibody; Sigma-Aldrich, CA, USA; catalogue no.HPA006040) which were normalised to mouse anti-human β-actin (1:10,000) antibody (monoclonal antibody; Sigma; catalogue no. A1978). p-ERK1/2 expression was determined with the rabbit anti-human p-ERK1/2 (1:1,000) antibody (monoclonal antibody; Cell Signalling, Boston, Massachusetts, USA; catalogue no. 4376S) and was normalised to total rabbit anti-human ERK1/2 (1:1,000) antibody (monoclonal antibody; Cell Signalling, Boston, Massachusetts, USA; catalogue no. 4695). The enhanced chemiluminescence method of protein detection using enhanced chemiluminescence reagents, ECL plus (Amersham GE Healthcare, Buckinghamshire, UK), was used to detect labelled proteins. Quantification of protein expression was performed by densitometry relative to β-actin or total ERK1/2 expression using the software GeneSnap version 6.08. Results were expressed as ratios of the endogenous controls β-actin or total ERK1/2 as appropriate, and normalised to control group.

RESULTS

The treatment of lung fibroblasts with aclidinium 10^{-7}M or with atropine 10^{-6}M during 48 hours did not show any effect on collagen type I and α-SMA mRNA expression *versus* control levels (Figure E1). In the same manner, the effect of aclidinium 10^{-7}M, atropine 10^{-6}M, hemicholinium-3 50μM, neostigmine 10μM, NAC 1mM, APO 100μM and AChE 10 U/mL^{-1} did not show any effect on collagen type I, α-SMA or pERK1/2 expression *versus* control levels (Figure E2, E3 and E4).

References

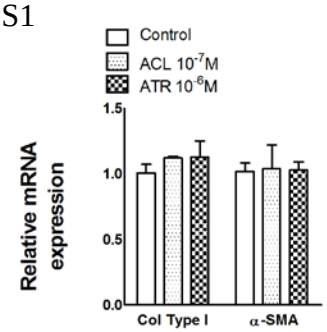
1. Su Y, Han W, Giraldo C, De Li Y, Block ER. Effect of cigarette smoke extract on nitric oxide synthase in pulmonary artery endothelial cells. *Am J Respir Cell Mol Biol* 1998: 19(5): 819-825.

2. Trayner ID, Rayner AP, Freeman GE, Farzaneh F. Quantitative multiwell myeloid differentiation assay using dichlorodihydrofluorescein diacetate (H2DCF-DA) or dihydrorhodamine 123 (H2R123). *J Immunol Methods* 1995: 186(2): 275-284.

Effect of aclidinium and atropine by itself on collagen type I and α-SMA expression in lung fibroblasts.
Human lung fibroblasts were incubated for 48 hours with aclidinium bromide (ACL), atropine (ATR), hemicholinium-3 (HC-3, 50μM), neostigmine 10μM, apocynin (APO: 100 μM), N-acetyl-L-cysteine (NAC: 1 mM) or acetylcholinesterase (AChE: 10 U/mL^{-1}) during 48 hours. E1 graph shows RT-PCR for Col Type I or α-SMA. E2 and E4 graph represent western blot representative images of Col Type I, α-SMA or p-ERK1/2 protein expression relative to β-actin. E3 graph represents western blot representative images of p-ERK1/2 protein expression relative to total ERK1/2. Graph S1 represents the mean ± SEM of three experiments for RNA experiments.

Figures

S1



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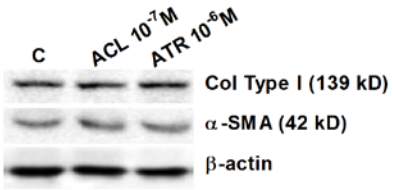

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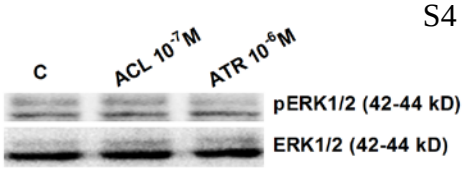

S4