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β_2-adrenoceptors and muscarinic receptors mediate opposing effects on endothelin-1 expression in human lung fibroblasts

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ABSTRACT

Human lung fibroblasts are a potential source of endothelin-1 (ET-1), a pro-fibrotic mediator. The present study explored possible muscarinic and β-adrenergic modulations of ET-1 expression in human lung fibroblasts. MRC-5 human lung fibroblasts were cultured. Expression of prepro-endothelin-1 (ppET-1) mRNA was determined by quantitative real time PCR. [^{3}H]-Proline incorporation was determined as measure of collagen synthesis. The muscarinic agonist oxotremorine induced, in a tiotropium-sensitive manner, a three-fold increase in ppET-1 mRNA. The β_2-adrenoceptor agonist olodaterol caused a reduction of ppET-1 mRNA by 45%. Olodaterol also opposed the stimulatory effect of oxotremorine. The effect of olodaterol was mimicked by the protein kinase A agonist 6-Bnz-cAMP, whereas the Epac (exchange protein activated by cAMP) agonist 8-CPT-2′-*O*-Me-cAMP was less effective. Transforming growth factor-β_1 (TGF-β, 0.3 and 1 ng/ml) induced a three- and eight-fold increase in pp-ET-1 mRNA, respectively. Olodaterol opposed the effect of 0.3, but not that of 1 ng/ml TGF-β. Likewise, 6-Bnz-cAMP opposed the effect of 0.3, but not that of 1 ng/ml TGF-β. TGF-β inhibited β_2-adrenoceptor mRNA expression, maximally by 90%. Muscarinic agonist-induced stimulation of [^{3}H]-proline incorporation was attenuated by the endothelin ET1 receptor antagonist bosentan. In conclusion, ET-1 expression in human lung fibroblasts is regulated by stimulatory muscarinic receptors and inhibitory β_2-adrenoceptors. Since muscarinic up-regulation of ET-1 contributes to pro-fibrotic effects of muscarinic stimuli, inhibition of ET-1 expression could contribute to long-term beneficial effects of long-acting β_2-adrenoceptor agonists and long-acting muscarinic antagonists. However, excessive exposure to TGF-β results in loss of β-adrenoceptor expression and function of its down-stream signaling.

1. Introduction

Endothelins (ET-1, -2 and -3) are a family of small peptides of 21 amino acids. They are synthesized as large precursor proteins (prepro-endothelin) which are processed by a cascade of different proteases resulting in big-endothelin and finally the active endothelin (for review see e.g., Khimji and Rockey, 2010; Polikepahad et al., 2006). ET-1 is a potent constrictor of vascular and airway of smooth muscle and appears to exert in addition various pro-fibrotic effects (for review see Shao et al., 2011). Its role in the pathophysiology of pulmonary hypertension has extensively been studied, but it is also considered to participate in the pathogenesis of pulmonary fibrosis and obstructive airway disease (e.g., Chalmers et al., 1997; Goldie and Henry, 1999; Polikepahad et al., 2006; Ross et al., 2010, Swigris and Brown, 2010). Lung fibroblasts are one important source of pulmonary ET-1 (Ahmedat et al., 2010a, b).

Increased levels of ET-1 were observed in bronchial epithelial cells (Pégorier et al., 2007) and exhaled air (Zietkowski et al., 2008) of asthmatic patients as well as in exhaled air, sputum and plasma of patients with COPD (e.g., Carratu et al., 2008; Roland et al., 2001). Transforming growth factor-β_1 (TGF-β) is a cytokine which is markedly up-regulated in lung and airway tissue of patients with bronchial asthma and chronic obstructive pulmonary disease (COPD), and there is evidence that it plays a key role in driving various remodeling processes including fibrosis (for review see Makinde et al., 2007; Königshoff et al., 2009; Araya and Nishimura, 2010; Halwani et al., 2011). Moreover, there is evidence that ET-1 could be a downstream mediator of pro-fibrotic responses to TGF-β (e.g., Shi-Wen et al., 2007).

Based on their bronchodilatory effects, β_2-adrenergic agonists and anticholinergic drugs constitute essential elements in the treatment of bronchial asthma and/or COPD (e.g., Sin et al., 2003; Barnes, 2004; Walters et al., 2005; Fitzgerald and Fox, 2007).

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There is increasing evidence, that the action of these drugs is not confined to bronchodilation (see Racké et al., 2008; Bateman et al., 2009). Thus, studies from our laboratory demonstrated that human lung fibroblasts express muscarinic receptors (Matthiesen et al., 2006; Haag et al., 2008a) and β_2-adrenoceptors (Lamyel et al., 2011), and showed further that proliferation and collagen synthesis in these cells is stimulated by muscarinic receptors (Matthiesen et al., 2006; Haag et al., 2008a), but inhibited by β_2-adrenoceptors (Lamyel et al., 2011).

The primary aim of the present study was to investigate, whether the expression of ET-1 in human lung fibroblasts is regulated by muscarinic and/or β-adrenergic mechanisms. In addition, possible functional consequences of an altered ET-1 expression were explored.

2. Materials and methods

2.1. Culture of lung fibroblasts

MCR-5 human lung fibroblasts (CCL-171, ATCC, Manassas, USA) were grown in Eagle's MEM supplemented with 10% FCS, 2 mM L-glutamine, Earle's BBS adjusted to contain 2.2 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were grown in a humidified incubator at 37 °C and 5% CO_2, and passaged by trypsinization at nearly confluence.

2.2. Extraction of RNA and real time reverse transcription-polymerase chain reaction

Total RNA was isolated by help of silica-gel-based membranes according to manufacturer's instructions including an additional DNase digestion protocol to beware any contamination by genomic DNA (Qiagen, Hilden, Germany). First strand cDNA was synthesised using Omniscript reverse transcriptase (Qiagen).

Quantitative PCR was performed by monitoring the fluorescence of SYBR Green dye on a Statagene Mx3000P real time PCR system. Applied primer pairs (based on human EMBL sequences) were specific for prepro-ET-1, 5′-TTATCAGCAGTTAGTGAGAGG-3′ and 5′-GAAGGTCTGTCACCAATGTG-3′; the β_2-adrenoceptor 5′'-GATTTCAGGATTGCCTTCCAG-3′ and 5′-GTGATATCCACTCTGCTCCCC-3′ and the housekeeping gene GAPDH, 5′-CTGCACCACCAACTGCTTAGC-3′ and 5′-GGCATGGACTGTGGTCATGAG-3′, which was used for normalization. The cycling conditions were: 10 min polymerase activation at 95 °C and 40 cycles at 95 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s. The threshold was automatically set by the software. The crossing point of the amplification curve with the threshold represents the 'Ct'.

Fluorescence data from each sample were analyzed with the $2^{-[\Delta\Delta Ct]}$ method: fold induction$=2^{-[\Delta\Delta Ct]}$, where $\Delta\Delta Ct=$[Ct GI (unknown sample)—Ct GAPDH (unknown sample)]—[Ct GI (calibrator sample)—Ct GAPDH (calibrator sample)], GI is the gene of interest.

2.3. [^{3}H]-proline incorporation

Collagen synthesis and deposition into the extracellular matrix were assessed by [^{3}H]-proline incorporation assays originally developed by Peterkofsky and Diegelmann (1997) and subsequently established also in our laboratory (Haag et al., 2008a, b). Cells were trypsinized, harvested and seeded into 12-well dishes at a density of 10^5 cells per well. Cells were first cultured for 24 h in presence of 10% FCS, followed by an additional 18–24 h under FCS-free conditions. Thereafter, [^{3}H]-proline (37 kBq) was added alone or in combination with test drugs, and cells were cultured for further 24 h. At the end, culture medium was removed and cells were washed twice with 4 °C cold PBS followed by 1–2 h incubation in 1 ml 20% trichloroacetic acid (TCA) at 4 °C. Denaturated cells were scraped off, transferred into a reaction tube and centrifuged at 13,000 rpm for 10 min. The pellet was washed with 1 ml 10% TCA and centrifuged again at 13,000 rpm for 5 min dissolved in 300 μl 0.2 M NaOH followed by neutralization with 300 μl 0.2 M HCl. 300 μl portions were combined with scintillation cocktail (PerkinElmer, Rodgau-Jügesheim, Germany) and radioactivity was determined by liquid scintillation spectrometry in a Packard 2100 TR liquid scintillation analyzer. External standardisation was used to correct for counting efficiency. [^{3}H]-proline incorporation was expressed as percentage of the mean of the control group of each cell preparation.

In previous experiments it was confirmed by collagenase digestion that total radioactivity incorporated into proteins largely reflects *de novo* synthesis of collagen (Haag et al., 2008a, b).

2.4. Statistical analysis

All values are means with S.E.M. of n experiments. Normal distribution was confirmed by performing the D'Agostino–Pearson omnibus normality test. Statistical significance of differences was evaluated by ANOVA followed by Dunnett or Bonferroni test using GraphPad InStat (GraphPad Software, San Diego, USA). When normal distribution could not be confirmed significance of differences was evaluated by the Kruskal–Wallis test followed by Dunn's test. $P<0.05$ was accepted as significant.

2.5. Drugs and materials

Olodaterol (BI 1744 CL) and tiotropium was from Boehringer Ingelheim (Germany) and bosentan from Actelion (Freiburg, Germany). All other drugs were purchased, oxotremorine sesquifumarate, penicillin–streptomycin solution and transforming growth factor-β_1 (TGF-β) and trypsin were purchased from Sigma (Deisenhofen, Germany); CGP 20712 (1-[2-((3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride) and ICI 118551 ((±)-1-[(2,3-dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl) amino]-2-butanol hydrochloride) from Biozol (Eching, Germany); 6-Bnz-cAMP (N^6-benzyladenosine-3′,5′-phosphate) and 8-pCPT-2′-*O*-Me-cAMP (8-(4-chlorophenylthio)-2′-*O*-methyladenosine-cAMP) from Biolog Life Science Institute (Bremen, Germany); desoxynucleotide mixture from Fermentas (St. Leon-Rot, Germany); Eagle's minimal essential medium (MEM) with Earl's salts and L-glutamine, non-essential amino acids from PAA (Cölbe, Germany); fetal calf serum (FCS) from Biochrom (Berlin, Germany); Taq DNA-polymerase from Invitrogen (Karlsruhe, Germany); Omniscript reverse transcriptase, RNeasy Mini kit, QuantiTect™ SYBR Green PCR kit and RNase-free DNase set from Qiagen (Hilden, Germany). Oligodesoxynucleotides for qPCR were obtained from Eurofins MWG Operon (Ebersberg, Germany).

3. Results

In human lung MCR-5 fibroblasts exposed for 24 h to the long acting β_2-adrenoceptor agonist olodaterol a marked reduction of the expression of prepro-ET-1 mRNA was observed, the maximum effect, an inhibition by 45%, was observed at 10 nM (Fig. 1A). The effect of olodaterol was prevented by the β_2-adrenoceptor selective antagonist ICI 118551 (1 μM), but not affected by the β_1-adrenoceptor selective antagonist CGP 20712 (3 μM) (Fig. 1A). The effect of the β_2-adrenoceptor agonist was fully mimicked by