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Endothelin-1 enhances β_2-adrenoceptor gene transcription in human lung fibroblasts

Kurt Racké *, Lisa J. Juergens, Ina Schütz, Nora Kämpfer, Margarita Fuhrmann, Mareille Warnken

Institute of Pharmacology & Toxicology University of Bonn, Germany

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ABSTRACT

Aims: The present study aimed to explore possible effects of endothelin-1 (ET-1) on ß$_2$-adrenoceptor gene transcription in human lung fibroblasts.
Main methods: MRC-5 human lung fibroblasts were cultured in absence or presence of test substances, followed by ß$_2$-adrenoceptor mRNA determination by quantitative real time PCR.
Key findings: ET-1 caused a marked and rapid in onset (1 hr) increase in β_2-adrenoceptor mRNA, an effect additive to that of short time (1 hr) exposure to the β_2-adrenoceptor agonist olodaterol. The stimulatory effect of ET-1 on β_2-adrenoceptor mRNA was prevented by the non-selective ET-A/ET-B receptor antagonist bosentan, indicating that it was mediated via specific ET receptors. In the presence of actinomycin D the effect of ET-1 was prevented indicating that ET-1 acts via increased transcription of the β_2-adrenoceptor gene. ET-1-induced up-regulation of β_2-adrenoceptor mRNA was also seen in the presence of cycloheximide excluding indirect effects via up-regulation of other regulatory proteins.
Conclusions: ET-1 can up-regulate β-adrenoceptor gene transcription in human lung fibroblasts.

Introduction

Endothelin-1 (ET-1) is a potent constrictor of vascular and airway of smooth muscle and appears to exert in addition various pro-fibrotic and pro-inflammatory effects. Its role in the pathophysiology of pulmonary hypertension is well documented (for review see Shao et al., 2011), but in addition there is increasing evidence that ET-1 may also 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). Human lung fibroblasts express ET-1 as well as its receptors, and ET-1 has been shown to stimulate various pro-fibrotic processes, such fibroblast proliferation, collagen synthesis and myo-fibroblast differentiation (Ahmedat et al., 2010a,b; Gallelli et al., 2005).

Based on their bronchodilatory effects, β_2-adrenergic agonists constitute an essential element in the treatment of bronchial asthma and COPD (e.g. Barnes, 2004; Fitzgerald and Fox, 2007; Sin et al., 2003; Walters et al., 2005). However, there is substantial evidence that they may exert a number of additional effects of potential therapeutic value. Thus, we recently showed that human lung fibroblasts express β_2-adrenoceptors which mediate inhibitory effects on various pro-fibrotic features (Lamyel et al., 2011) and ET-1 expression (Racké et al., 2011b). The expression of β_2-adrenoceptors in human lung fibroblasts appears to be highly regulated at the level of mRNA; for example, corticosteroids cause a marked up- and TGF-β a marked down-regulation and β_2-adrenergic agonists exert in a time-dependent manner opposing effects (Racké et al., 2011a; Warnken-Uhlich et al., 2011).

The present study aimed to explore potential effects of ET-1 on β_2-adrenoceptor mRNA expression in human lung fibroblasts.

Materials and methods

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.

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).

* Corresponding author at: Institute of Pharmacology & Toxicology, University of Bonn, Biomedical Center, Sigmund-Freud-Str. 25, D-53105 Bonn, Germany. Tel.: +49 228 28751390; fax: +49 228 28751392.
E-mail address: racke.kurt@uni-bonn.de (K. Racké).

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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 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 sec, 59 °C for 30 sec and 72 °C for 30 sec. 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.

Statistical analysis

All values are means with S.E.M. of *n* experiments. Normal distribution was confirmed by performing the Kolmogorov–Simirnov test and in series with $n > 7$ additionally by the D'Agostino–Pearson omnibus normality test using Prim5 (GraphPad Software, San Diego, USA). Statistical significance of differences was evaluated by ANOVA or (when applicable) repeated measures ANOVA followed by Bonferroni test, again using Prim5.

Drugs and materials

Olodaterol was a gift from Boehringer Ingelheim (Biberach, Germany) and bosentan from Actelion (Freiburg, Germany). All other drugs were purchased, actinomycin D, cycloheximide, human endothelin-1, penicillin-streptomycin solution and trypsin were purchased from Sigma (Deisenhofen, 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).

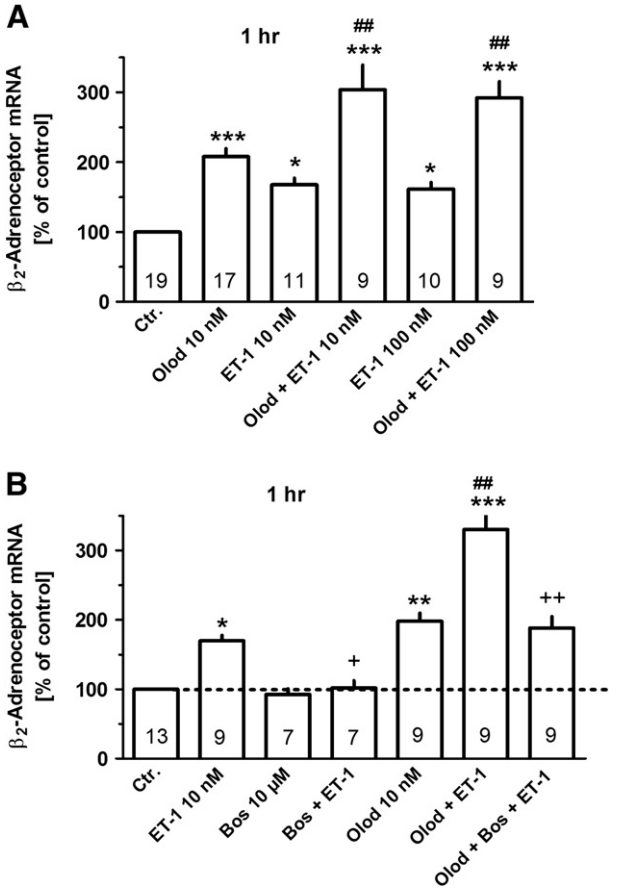

Fig. 1. Effects of short time (1 hr) exposure to ET-1 and/or olodaterol (Olod) in absence or presence of bosentan (Bos) on β_2-adrenoceptor mRNA expression in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hr in presence of 10% FCS followed by 1 hr in FCS-free medium in absence or presence of test drugs at the concentration indicated. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* β_2-adrenoceptor mRNA ($-2^{\Delta\Delta Ct} \times 100$) is expressed as % of the respective control of the individual cell preparation, given are means with S.E.M. of the number of experiments given in the columns. Significance of differences: $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ vs respective control; $\#\#P<0.01$ vs respective value in presence of ET-1 or Olod alone; $+P<0.05$; $++P<0.01$ vs respective value in absence of Bos.

Results

In confirmation of previous observations (Racké et al., 2011a; Warnken-Uhlich et al., 2011; Kämpfer et al., 2011) the long acting β_2-adrenoceptor agonist olodaterol (Bouyssou et al., 2010) (10 nM, a maximally effective concentration as previously observed) induced a rapid (1 hr), but transient increase in β_2-adrenoceptor mRNA expression by about 100% followed by a reduction by about 55% after 4 hr of agonist exposure. ET-1 (10 nM) induced also a rapid (1 hr) increase in β_2-adrenoceptor mRNA expression by about 70% and the effect of ET-1 was at least additive to that of olodaterol, resulting in an increase by about 210–230% in presence of ET-1 in combination with olodaterol (Fig. 1A and B). At a higher concentration (100 nM), ET-1 did not show greater effects neither alone nor in combination with olodaterol (Fig. 1A). The effect of ET-1, both in absence and presence of olodaterol, was blocked by bosentan, which alone had no effect (Fig. 1B).

In contrast to the effect of the β-adrenoceptor agonist, that of ET-1 was preserved after 4 hr of exposure, although somewhat smaller in magnitude, and ET-1 significantly opposed the down-regulation of β_2-adrenoceptor mRNA caused by the β-adrenoceptor agonist (Fig. 2A). As already reported previously (Racké et al., 2011a; Warnken-Uhlich et al., 2011; Kämpfer et al., 2011), inhibition of de-novo protein synthesis by cycloheximide caused an about 6-fold increase in β_2-adrenoceptor mRNA (Fig. 2B). Moreover, the down-regulation caused by 4 hr exposure to olodaterol was prevented by cycloheximide and converted into a marked additional up-regulation, resulting in an about 12-fold increase in β_2-adrenoceptor mRNA in the presence of olodaterol and cycloheximide (Fig. 2B). The effect of ET-1 was also seen in the presence of cycloheximide, resulting also in about a 9-fold increase in β_2-adrenoceptor mRNA in the presence of ET-1 and cycloheximide (Fig. 2B). The strong stimulatory effects of the β-adrenoceptor agonist and of ET-1 in presence of cycloheximide were still additive (Fig. 2B). Like the stimulatory effect of ß-agonists (Racké et al., 2011a; Warnken-Uhlich et al., 2011; Kämpfer et al., 2011) that of ET-1 was prevented by inhibition of RNA synthesis by actinomycin D. After 100 min incubation with actinomycin (30 µM) β_2-adrenoceptor mRNA was reduced to $34.6 \pm 5.7\%$ of controls ($n = 7$, $P<0.001$ vs controls) and by $33.4 \pm 4.8\%$ in experiments in which ET-1 (10 nM) was additionally present for the last 60 min ($n = 7$; not significantly different vs actinomycin alone).

Discussion

In previous studies we observed that MRC-5 and primary human lung cells are very similar with regard to the expression pattern of

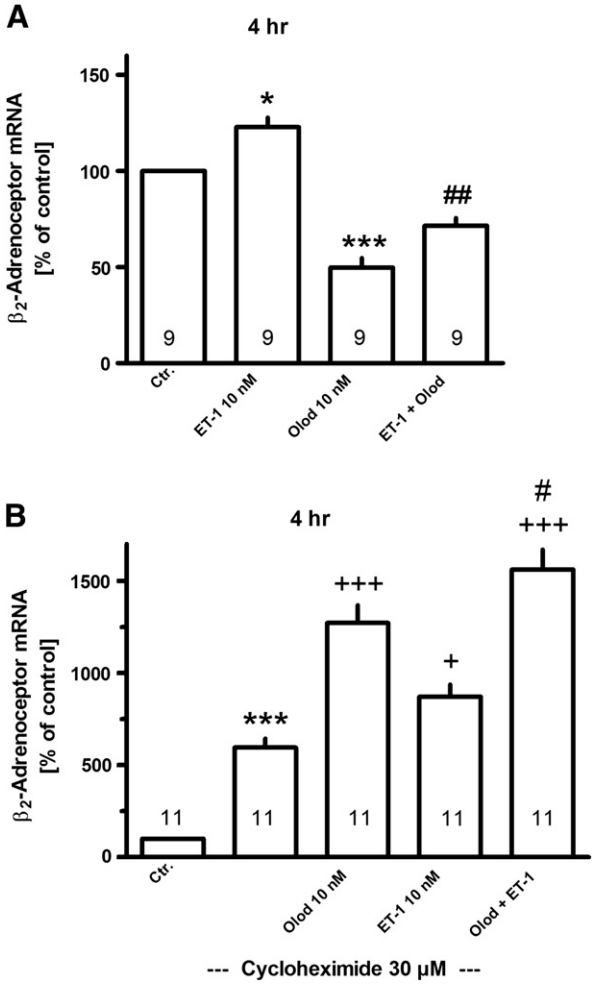

Fig. 2. Effects of 4-hr exposure to ET-1 and/or olodaterol (Olod) on β_2-adrenoceptor mRNA expression in MRC-5 human lung fibroblasts. After dissemination, cells were cultured for 24 hr in the presence of 10% FCS followed by 4 hr in FCS-free medium in absence or presence of test drugs at the concentration indicated, cycloheximide being present already 30 min prior. Thereafter, total RNA was isolated, treated with DNase and used for quantitative real time PCR. *Height of columns:* β_2-adrenoceptor mRNA ($-2^{\Delta\Delta Ct}\times 100$) is expressed as % of the respective control of the individual cell preparation, given are means with S.E.M. of the number of experiments given in the columns. Significance of differences: $^{*}P<0.05$; $^{***}P<0.001$ vs respective control; $+<0.05$; $+++<0.01$ vs respective value in presence of cycloheximide alone; $\#<0.05$; $\#<0.01$ vs respective Olod value alone.

several G-protein coupled receptors and their functional responses (Haag et al., 2008a,b; Matthiesen et al., 2006) and so far studied cAMP-mediated signal transduction mechanisms (Haag et al., 2008b). Furthermore, and of particular importance for the present study, MRC-5 and primary human lung cells showed the same expression pattern of β-adrenoceptor subtypes, solely the β_2-adrenoceptor, and the same functional response upon β-adrenoceptor activation (Lamyel et al., 2011). Therefore, MRC-5 cells were used in the present study as a cell line which has been proven to allow to study physiologically relevant β-adrenergic regulatory mechanisms in human lung fibroblasts.

The expression of β_2-adrenoceptors in human lung fibroblasts appears to be highly regulated at the transcriptional level. Thus, the half life of β_2-adrenoceptor mRNA was about 25 min (Racké et al., 2011a; Warnken-Uhlich et al., 2011) indicating a relatively high turnover rate resulting in close relation between mRNA levels and β_2-adrenoceptor gene transcription, and in line with this conclusion, marked changes in β_2-adrenoceptor mRNA levels were seen for example after short time exposure to corticosteroids, TGF-β or β-adrenoceptor agonists. The present experiments show that ET-1 is a further mediator involved in the regulation of β_2-adrenoceptor expression in human lung fibroblasts. ET-1 caused a rapid increase in β_2-adrenoceptor mRNA which was prevented by the non-selective ET-A/ET-B receptor antagonist bosentan, indicating a specific ET receptor mediated effect. Interestingly, the stimulatory effect of ET-1 was additive to that of short time exposure to a β-adrenoceptor agonist. In general, β-adrenoceptors couple via Gs to adenylyl cyclase and direct activation of adenylyl cyclase by forskolin mimicked the stimulatory effect of β-adrenoceptor activation (Racké et al., 2011a; Warnken-Uhlich et al., 2011; Kämpfer et al., 2011). On the other hand, ET receptors are able to couple in addition to various other G proteins including $G_{q/11}$ and $G_{i/o}$ (Henry and Goldie, 2001). Since the effect of forskolin and β-adrenoceptor agonists was not additive (Kämpfer et al., 2011), the additivity of the effect of ET-1 and the β-adrenoceptor agonist suggests that ET-1 may cause the up-regulation of the β_2-adrenoceptor expression via a signaling pathway different from the G_s–adenylyl cyclase pathway. The fact that actinomycin D prevented the up-regulation by ET-1 indicates that an increased transcription of the β-adrenoceptor gene might be responsible for the enhanced mRNA levels. Furthermore, the rapid onset of the ET-1 effect and the observation that ET-1 caused an up-regulation also in the presence of cycloheximide support the conclusion that the up-regulation of β-adrenoceptor mRNA expression by ET-1 is the result of a direct stimulation of β-adrenoceptor gene transcription and does not involve the up-regulation of other regulatory proteins. Whereas the direct stimulatory effect of β-adrenoceptor–cAMP pathway on β-adrenoceptor mRNA was transient because of delayed up-regulated inhibitory proteins, the stimulatory effect of ET-1 was more prolonged and ET-1 was able to oppose the delayed down-regulation of β-adrenoceptor mRNA following β-adrenoceptor agonist exposure.

Receptor desensitization following prolonged agonist exposure is a generally occurring phenomenon that is also seen for β_2-adrenoceptors (for review see Shore and Moore, 2003; Johnson, 2006). However, several mechanisms appear to exist by which an agonist-induced down-regulation and loss of function of β_2-adrenoceptors may be limited. Thus, corticosteroids have been shown to stimulate β_2-adrenoceptor expression (Mak et al., 1995; Racké et al., 2011a) and to reverse agonist induced β_2-adrenoceptor tolerance (e.g. Cooper and Panettieri, 2008). The present observations suggest that endothelinergic stimulation of β_2-adrenoceptor expression is a further mechanism that could contribute to maintenance of β_2-adrenoceptor function during prolonged agonist exposure.

Conclusion

In human lung fibroblasts ET-1 mediates up-regulation of β-adrenoceptor gene transcription. Whether ET-1 exerts similar effects in other cells such as airway smooth muscle and whether ET-1 might be able to enhance β-adrenoceptor function and/or to oppose agonist-induced loss of β-adrenoceptor function, are important questions, which have to be addressed in future studies.

Conflict of interest statement

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