

Interleukin-1 β attenuates endothelin B receptor-mediated airway contractions in a murine *in vitro* model of asthma: roles of endothelin converting enzyme and mitogen-activated protein kinase pathways

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Summary

Background Asthma is a chronic airway disease, known to involve several inflammatory mediators. Little is known about how these mediators interact in order to produce or attenuate even basic features of the disease, like airway hyper-reactivity and remodelling. Endothelin-1 (ET-1) and IL-1 β are two mediators suggested to play important roles in the induction of airway inflammation.

Objective To investigate the interactions between ET-1 and IL-1 β , using a novel *in vitro* model of asthma, focusing on airway smooth muscle contractility.

Methods Isolated murine tracheal segments were cultured from 1 to 8 days in the absence and presence of IL-1 β . The subsequent contractile responses to sarafotoxin 6c (S6c) (selective agonist for ET_B receptor) and sarafotoxin 6b (S6b) (ET_A and ET_B receptor agonist) were recorded by a myographs system. In all experiments, ET_B receptors were desensitized before the contractile response to S6b was recorded. Thus, the response to S6b is only mediated by ET_A receptors in the present study. The mRNA expressions for ET-1 and endothelin (ET) receptors were quantified by real-time PCR.

Results Organ culture in the presence of IL-1 β attenuated the maximal contraction induced by S6c, but not S6b. This reduction was concentration-dependent and was significant after 2, 4 and 8 days of culture. To investigate the mechanisms behind this, inhibitors for endothelin converting enzyme (ECE) phosphoramidon, c-JUN N-terminal kinase (JNK) SP600125, extracellular-signal-regulated kinase 1/2 (ERK 1/2) PD98059 and p38 pathway SB203580 were used. Individually, SP600125 and PD98059, but not SB203580, could partly reverse the reduction induced by IL-1 β . An additional effect was obtained when SP600125 and PD98059 were combined. The mRNA expressions for ET-1 and ET_B receptor were up- and down-regulated, respectively, by IL-1 β .

Conclusion Presence of IL-1 β in the airways attenuate the contractile response mediated via ET_B receptors, an effect dependent on ECE, JNK and ERK 1/2 pathways.

Keywords asthma, ECE, endothelin receptor, IL-1 β , MAPK pathways

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Introduction

Asthma is characterized by airway hyper-reactivity and recurrent airway obstruction. The pathological process is linked to chronic inflammation, and its role in the induction of both hyper-responsiveness and the remodelling of the airways has recently been emphasized [1, 2]. Following the infiltration and accumulation of different inflammatory cells (eosinophils, lymphocytes, activated macrophages, mast cells and neutrophils), a cascade of mediators is released [3, 4]. Among them, cytokines have been demonstrated to affect airway smooth muscle functional properties such as regulation of muscle tone, proliferation and matrix secretion. Thus,

alteration of airway smooth muscle plasticity by cytokines is one of the key events in the pathogenesis of asthma [5]. In order to study interaction between different mediators with potential relevance for asthma, we have developed a murine *in vitro* model of asthma [6]. In the present study, we are focusing on the strong and potent airway constrictor endothelin-1 (ET-1) and the pro-inflammatory cytokine IL-1 β , both suggested playing important roles in the asthmatic inflammation [7, 8].

ET-1, a 21 amino acid peptide in airway produced by airway epithelial and smooth muscle cells acts as a potent bronchoconstrictor *in vitro* and *in vivo* [9–12]. Elevated levels of IL-1 β and ET-1 were found in the bronchoalveolar lavage fluids from asthmatic patients [13–15]. Thus, IL-1 β is suggested to have links to ET-1 and ET receptors in airway smooth muscle during the development of asthmatic inflammation. However, mechanisms that link the action between IL-1 β and ET receptors in airway smooth muscle are not

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clear. ET-1 is converted by endothelin converting enzyme (ECE) from big ET (38 amino acid peptide) and acts via two receptors, ET_A and ET_B [16–20]. The two receptor subtypes are targeted to different intracellular fates, i.e. ET_A receptor is directed to the pericentriolar recycling compartment and subsequently reappears at the plasma membrane, the ET_B receptor is directed to lysosomes for degradation [21].

The present study is designed to investigate how the effects of IL-1 β interact with the response mediated by ET receptors using a novel *in vitro* model of asthma focusing on airway smooth muscle contractility [6]. For this purpose two different ET receptor agonists, sarafotoxin 6c (S6c, a highly selective ET_B receptor agonist) and sarafotoxin 6b (S6b, non-selective ET_A/ET_B receptor agonist) were used [22]. Special emphasis was to evaluate the involvement of ECE and intracellular mitogen-activated protein kinases (MAPK) pathways.

Materials and methods

Tissue preparation and organ culture

Male BALB/c J strain mice (10 weeks old) were killed by cervical dislocation. Whole tracheae were placed into Dulbecco's modified Eagle's medium (DMEM; 4500 mg/L D-glucose, 110 mg/L sodium pyruvate, 584 mg/L L-glutamine) supplemented with penicillin (100 U/mL) and streptomycin (100 μ g/mL). Under sterile conditions, the tracheae were cut into segments with three cartilage rings each for organ culture or directly used for myographs studies. Segments were placed individually into wells of a 96-well plate (Ultra-low attachment; Sigma, St Louis, MO, USA) with 300 μ L DMEM for organ culture and incubated at 37 °C in humidified 5% CO₂ in air for 1, 2, 4 and 8 days in the absence or presence of recombinant murine IL-1 β . Segments were moved into a new well containing fresh media every day.

In-vitro pharmacology

Tracheal smooth muscle reactivity was recorded in temperature-controlled (37 °C) myographs (Organ Bath Model 700MO, J.P. Trading, Aarhus, Denmark) containing 5 mL Krebs–Henseleit buffer with 10 mM D-glucose. The solution was continuously equilibrated with 5% CO₂ in O₂ resulting in a stable pH of 7.4. The tracheal segments were mounted for continuous recording of isometric tension by the Chart software (AD Instruments Ltd, Hastings, UK). Briefly, 1-h equilibration was performed to reach a stable basal force of 0.8 mN and the viability of tracheal segments was tested by 60 mM KCl. To inhibit epithelial prostaglandin release, the segments were incubated with 3 μ M indomethacin 30 min before cumulative concentrations of S6c and S6b were administered. In addition, the involvement of nitric oxide (NO) was excluded by using nitric oxide synthase (NOS) inhibitor *N*-monomethyl-L-arginine (L-NMMA 100 μ M). At the end of the experiment a single contractile response of 1 mM carbachol was induced. To study ET_A receptor-mediated contraction, S6c concentration–effect curve was performed first, and the segments remained in contact with the highest concentration of S6c (100 nM) for a further 30 min

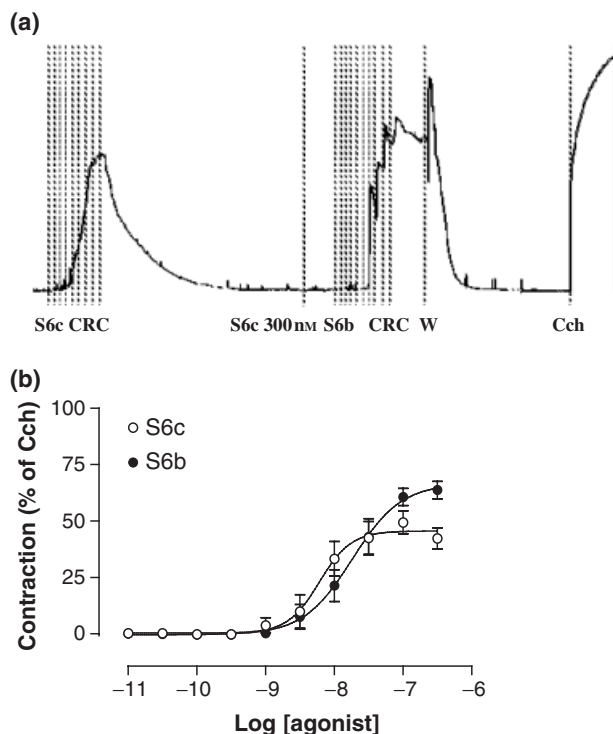


Fig. 1. (a) A typical experiment procedure shows sarafotoxin 6b (S6b) concentration–effect curves after sarafotoxin 6c (S6c) administration. The contractile response to S6c declined after the highest concentration (100 nM) was administered and basal level was reached after ~ 30 min. The desensitization of ET_B receptors has verified by no response to further increase of S6c concentration to 300 nM. Thereafter S6b was administered and after a wash, a high concentration of carbachol (1 mM) completed the experiments. W, wash; Cch, carbachol; CRC, concentration–response curve. S6c CRC concentration ranging from 0.01 to 100 nM; S6b CRC concentration ranging from 0.01 to 300 nM. (b) Cumulative concentration–effect curves induced by S6c and S6b on fresh mouse tracheal segments. Each point represents the mean of all segments $\pm$ SEM ($n = 6$).

to desensitize ET_B receptors [23, 24]. During the 30 min period, the contractile response to S6c faded to the baseline levels even though the S6c still remained in contact with the segments. The desensitization of ET_B receptors was verified by no response to further increase of S6c concentration to 300 nM (Fig. 1a). After ET_B receptors have been desensitized, S6b concentration–effect curve was performed. Thus, the contractile response to S6b was only mediated by ET_A receptors in the present study [25].

Real-time quantitative PCR

Total RNA isolation Whole tracheal segments with intact epithelium for ET-1 mRNA and smooth muscle strips without epithelium for ET_A and ET_B receptor mRNA were stored in RNAlater™ at –80 °C for extraction of total RNA. The tissues were mechanically homogenized in the TRIzol reagent and the total RNA was extracted by using the RNeasy Mini following the kit instructions (QIAGEN GmbH, Hilden, Germany). To remove the contamination of genomic DNA, the samples were further treated with DNA-free™ kits (Ambio Inc., Austin, TX, USA) with DNase I at 37 °C for 1 h. The quality of total RNA was checked by a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.6 and 2.0 in all preparations.

Reverse transcription Reverse transcription of total RNA to cDNA was carried out using Omniscript™ reverse transcriptase kit (QIAGEN GmbH) in 20 µL volume reaction by using Mastercycler personal PCR machine (Eppendorf AG, Hamburg, Germany) at 37 °C for 1 h.

Primer design All the PCR primers used in the present study were designed with primer spans on exons by using Prime Express® 2.0 software (Applied Biosystem, Foster City, CA, USA) and synthesized by DNA Technology A/S (Aarhus, Denmark). The sequences are given below:

GAPDH

Forward: 5'-CATGGCCTTCCGTGTTCTTA-3'

Reverse: 5'-TGCTTACCACCTTCTTGATG-3'

ET_A receptor

Forward: 5'-GATGGATAAGAACCGGTGTGAAC-3'

Reverse: 5'-GAGCTATTGGGTTTATGCAAGAATTC-3'

ET_B receptor

Forward: 5'-ACCAGAGCAATCCACACAGG-3'

Reverse: 5'-AGAGCGATTGGATTGATGCAG-3'

ET-1

Forward: 5'-CCACAGACCAGGCAGTTAGATG-3'

Reverse: 5'-CATGGTACTTTGGGCCCTGA-3'

The real-time quantitative PCR The real-time quantitative PCR was performed with the LightCycle-FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH, Mannheim, Germany) in the LightCycler detection system (Roche Applied Science, Mannheim, Germany). The system automatically monitors the binding of a fluorescent dye SYBR Green to double-stranded DNA by real-time detection of the fluorescence during each cycle of PCR amplification. The real-time PCR was performed in 20 µL reaction volume and carried out with heating 95 °C for 10 min followed by 45 PCR cycles with 95 °C for 10 s, 65 °C for 5 s and 72 °C for 5 s. The data were analysed with cycle threshold (C_T) method and the specific PCR products were analysed by running melting curve and visualized by agarose electrophoresis. Expected PCR products of GAPDH (102 bp), ET_A receptor (101 bp), ET_B receptor (106 bp) and ET-1 (101 bp) with a single band were seen.

The $\Delta\Delta C_T$ method was employed to calculate a relative amount of mRNA for ET-1, ET_A and ET_B receptors to GAPDH. This is the C_T value of mRNA for house-keeping gene GAPDH as a reference to normalize a relative amount of mRNA for ET-1, ET_A and ET_B receptors. The relative amount of mRNA was obtained by the C_T values of mRNA for ET-1 or ET_A or ET_B receptors in relation to the C_T values of mRNA for GAPDH in the same sample. A blank control (no template) was included in all the experiments for negative controls.

Chemicals and solutions

Recombinant murine IL-1 β (R&D Systems, Abingdon, UK); S6c and S6b (Neosystem, Strasbourg, France); SP600125 (Calbiochem, Bad Soden, Germany); SB203580 (Tocris-Cookson, Bristol, UK); phosphoramidon, PD98059, indomethacin, carbachol, L-NMMA, dimethylsulphoxide (DMSO), DMEM and Krebs–Henseleit Buffer (Sigma); Penicillin and streptomycin (Life Technologies, Gaithersburg, MD, USA). The stock solution of IL-1 β , S6c and S6b was prepared in 0.1% chicken serum albumin, carbachol, L-

NMMA and phosphoramidon were dissolved in the distilled water, indomethacin was dissolved in 95% ethanol and SP600125 (protected from light), SB203580 and PD98059 were dissolved in DMSO.

Statistical analysis

All data are expressed as mean values $\pm$ SEM. The responses of S6c and S6b are presented as percentage of contraction to carbachol. Agonist concentration–effect curves were fitted to the Hill equation using an iterative, least-square method (GraphPad Prism), to provide estimates of maximal contraction (α) and the pEC₅₀ values (negative logarithm of the agonist concentration that produces 50% of the maximal effect). Statistical analysis was performed using one-way ANOVA and Dunnet's post-test or unpaired student's *t*-test. $P < 0.05$ was considered to be significant.

Results

Contractile responses to S6c and S6b

As described in the methods, the S6c concentration–effect curve were performed first, and the segments remained in contact with S6c for a further 30 min for the contraction fading down to the baseline levels. The desensitization of ET_B receptors has been demonstrated by no response to further increase of S6c concentration to 300 nM (Fig. 1a). Thereafter the response to S6b concentration–effect curves was conducted. Thus, in the present study the contractile response to S6b was only mediated by ET_A receptors, although it is an agonist for both ET_A and ET_B receptors. A typical experiment is shown in Fig. 1a. To exclude the involvement of NO, NOS inhibitor L-NMMA (100 µM) was used. There were no effects of L-NMMA on the contractile responses to S6c or S6b (data not shown).

Contractile response in fresh tracheal segments

Both S6c and S6b induced fully defined concentration–effect curves in the fresh segments (Fig. 1b). S6b induced a significantly stronger maximal contraction than S6c ($P < 0.05$), ($\alpha = 45.7 \pm 4.9\%$ and $65.4 \pm 3.9\%$ of carbachol, respectively), whereas S6c had slightly higher pEC₅₀ values than S6b ($P > 0.05$) (pEC₅₀ = 8.16 ± 0.18 and 7.74 ± 0.12 , respectively) (Fig. 1B, Table 1).

Contractile response in organ cultured tracheal segments

The contractile responses to both S6c and S6b were increased after organ culture of the tracheal segments for 1, 2, 4 and 8 days, but reached statistical significances only at 4 days to S6b ($P < 0.05$) and at 8 days to the contractile response to S6c ($P < 0.05$) (Table 1, Figs 2a–d and 3a–d).

Different concentrations of IL-1 β on the contractile response

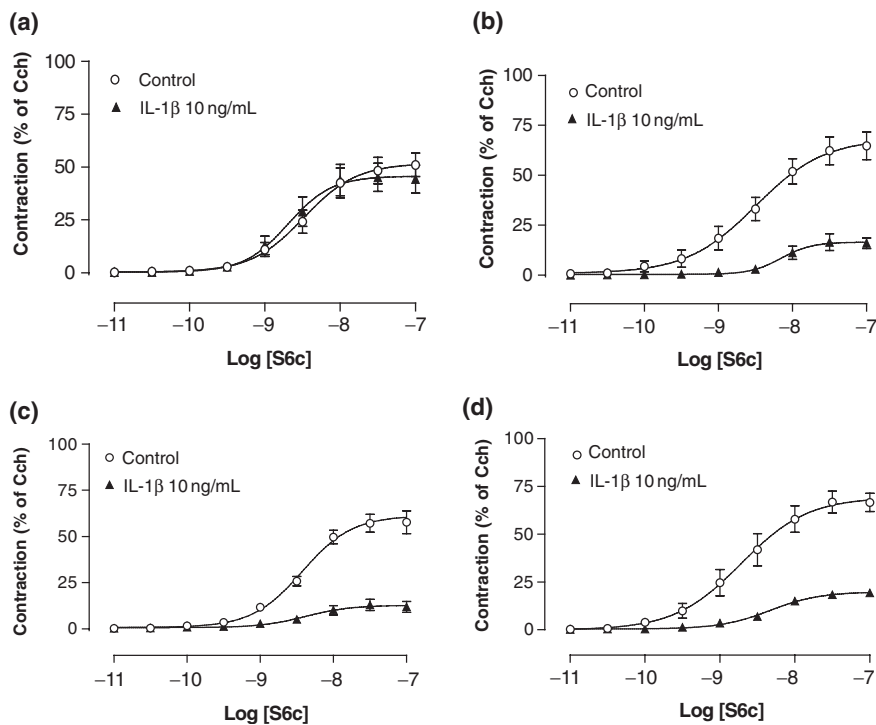
Organ culture of the tracheal segments with 0.1, 1 and 10 ng/mL of IL-1 β for 4 days produced concentration-dependent reductions of the maximal contraction to S6c, but no effects of IL-1 β on the contractile response to S6b (Table 1). The

Table 1. Maximal contractile response induced by Sarafotoxin 6c (S6c) and Sarafotoxin 6b (S6b) in mouse tracheal segments, fresh and following organ culture in absence and presence of IL-1 β

Incubation time	IL-1 β (ng/mL)	S6c			S6b		
		<i>n</i>	α (% of Cch)	pEC ₅₀	<i>n</i>	α (% of Cch)	pEC ₅₀
Day 0	0	6	45.7 $\pm$ 4.9	8.16 $\pm$ 0.18	6	65.4 $\pm$ 4.0§	7.74 $\pm$ 0.12
Day 1	0	8	53.5 $\pm$ 5.9	8.41 $\pm$ 0.12	8	65.2 $\pm$ 5.0	7.82 $\pm$ 0.10
	10	8	45.9 $\pm$ 6.8	8.62 $\pm$ 0.07	8	57.3 $\pm$ 5.5	7.73 $\pm$ 0.18
Day 2	0	8	66.6 $\pm$ 7.3	8.54 $\pm$ 0.11	7	65.4 $\pm$ 1.3	7.44 $\pm$ 0.11
	10	7	17.6 $\pm$ 3.0***	8.01 $\pm$ 0.10	7	58.4 $\pm$ 3.9	7.28 $\pm$ 0.08
Day 4	0	7	60.1 $\pm$ 6.5	8.47 $\pm$ 0.07	7	78.5 $\pm$ 2.7†	7.78 $\pm$ 0.08
	0.1	6	60.2 $\pm$ 9.4	8.61 $\pm$ 0.06	6	70.6 $\pm$ 4.3	7.71 $\pm$ 0.18
	1	6	29.2 $\pm$ 6.9**	8.75 $\pm$ 0.11	6	77.5 $\pm$ 2.6	7.86 $\pm$ 0.13
	10	6	13.2 $\pm$ 3.2***	8.42 $\pm$ 0.11	6	76.9 $\pm$ 2.6	7.62 $\pm$ 0.06
Day 8	0	8	70.9 $\pm$ 4.8†	8.62 $\pm$ 0.15	7	77.8 $\pm$ 1.2	7.87 $\pm$ 0.11
	10	8	21.3 $\pm$ 1.6***	8.25 $\pm$ 0.12	7	73.8 $\pm$ 3.5	7.59 $\pm$ 0.15

Values are expressed as mean $\pm$ SEM. Statistical analysis was performed with one-way ANOVA and Dunnet's post-test or unpaired Student's *t*-test. Cch, carbachol.

†Fresh vs. control (ANOVA). *Control vs. IL-1 β treated (*t*-test). §S6c (day 0) vs. S6b (day 0) (*t*-test). **P* < 0.05, ***P* < 0.01, ****P* < 0.001, †*P* < 0.05, §*P* < 0.05.

**Fig. 2.** Time course of IL-1 β on the contractile response to sarafotoxin 6c (S6c). Cumulative concentration-effect curves induced by S6c in absence (control) and presence of IL-1 β 10 ng/mL on mouse tracheal segments, which incubated for 1 day (a), 2 days (b), 4 days (c) and 8 days (d). Each point represents the mean of all segments $\pm$ SEM (*n* = 6–8).

potencies to both S6c and S6b were not different from control segments (*P* > 0.05, Table 1).

Time course of interleukin-1 β on the contractile response

Experiments were performed by organ culture of the tracheal segments with IL-1 β 10 ng/mL for 1, 2, 4 and 8 days. Significant reductions of the maximal contractile response to S6c by IL-1 β were obtained after 2, 4 and 8 days of the culture with a maximal reduction at 4 days (*P* < 0.001, respectively) (Figs 2a–d, Table 1). However, only a tendency of reducing the contractile response to S6b was seen during 1–8 days of the culture (*P* > 0.05, Figs 3a–d, Table 1). The potencies of the contractile responses to both S6c and S6b in

organ culture with IL-1 β were not different from organ culture only (Table 1).

Endothelin converting enzyme inhibitor on the reduction of the contractile response

The experiments were performed by 4 days of organ culture. The ECE inhibitor phosphoramidon 10 μ M could partly abolish IL-1 β -induced reduction of the contractile response to S6c and a maximal contraction increases from 17.9 $\pm$ 4.5% to 38.9 $\pm$ 6.5% (*P* < 0.01), and it had no effect on the control segments (without IL-1 β treatment) (α = 60.3 $\pm$ 5.6% vs. 55.1 $\pm$ 7.9%, *P* > 0.05) (Fig. 4a). No influence by phosphoramidon was observed in response to S6b in the control

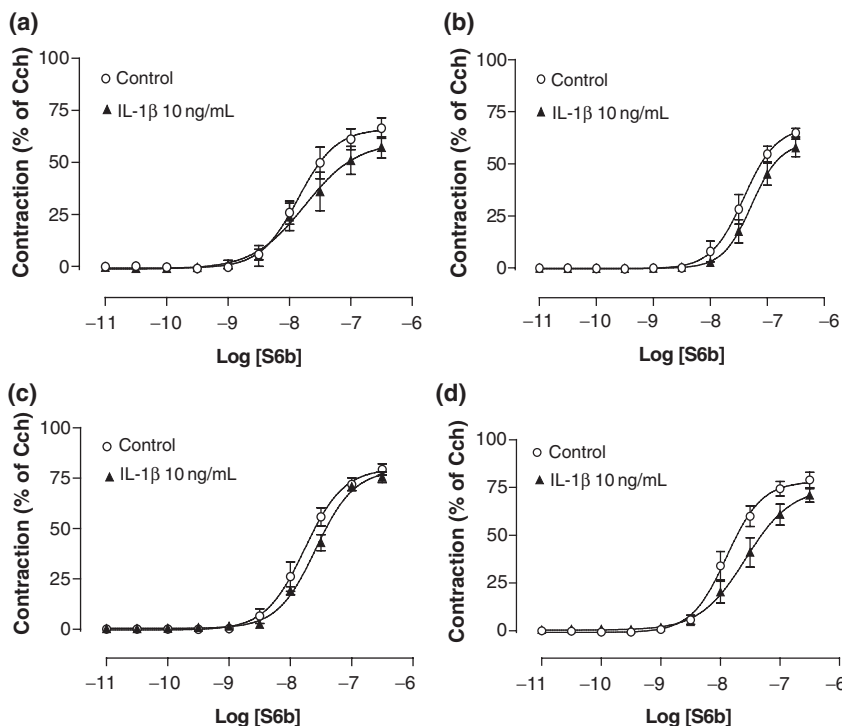


Fig. 3. Time course of IL-1 β on the contractile response to S6b. Cumulative concentration-effect curves induced by S6b in absence (control) and presence of IL-1 β 10 ng/mL on mouse tracheal segments, which incubated for 1 day (a), 2 days (b), 4 days (c) and 8 days (d). Each point represents the mean of all segments $\pm$ SEM ($n = 6-8$).

segment ($\alpha = 78.1 \pm 2.2\%$ vs. $76.8 \pm 1.0\%$, $P > 0.05$) and with IL-1 β treatment ($\alpha = 76.9 \pm 2.3\%$ vs. $74.9 \pm 2.1\%$, $P > 0.05$, Fig. 4b).

c-JUN N-terminal kinase, extracellular-signal regulated kinase 1/2 and p38 pathway inhibitors on the reduction of the contractile response

To find intracellular signal transduction links between IL-1 β and the contractile response to S6c, experiments were performed by 4 days of organ culture with IL-1 β in the absence/presence of c-JUN N-terminal kinase (JNK) inhibitor SP600125 (10 μ M) or/and extracellular-signal regulated kinase 1/2 (ERK1/2) inhibitor PD98059 (100 μ M) or p38 pathway inhibitor SB203580 (10 μ M). The SP600125 and PD98059 used separately could only partly reverse IL-1 β -induced reduction of the contractile response to S6c and a combination of SP 600125 (10 μ M) with PD98059 (100 μ M) could almost completely reverse the reduction. However, SB203580 only slightly reversed IL-1 β -induced reduction ($P > 0.05$, Fig. 5). No effects of SP600125 or PD98059 or SB203580 on the contractile response to S6b were observed (Fig. 5).

The expression of mRNA for ET-1 in the tracheal segments with epithelium and ET_A and ET_B receptors in the tracheal smooth muscle

The tracheal segments were cultured in the absence and presence of IL-1 β 10 ng/mL for 2 days for ET-1 mRNA (with epithelium) and 4 days for ET_A/ET_B receptor mRNA (smooth muscle). The amount of mRNA relative to GAPDH was quantified by the real-time quantitative PCR. The level of ET-1 mRNA was increased by IL-1 β treatment ($P < 0.05$), the ET_B receptor mRNA was significantly down-regulated by

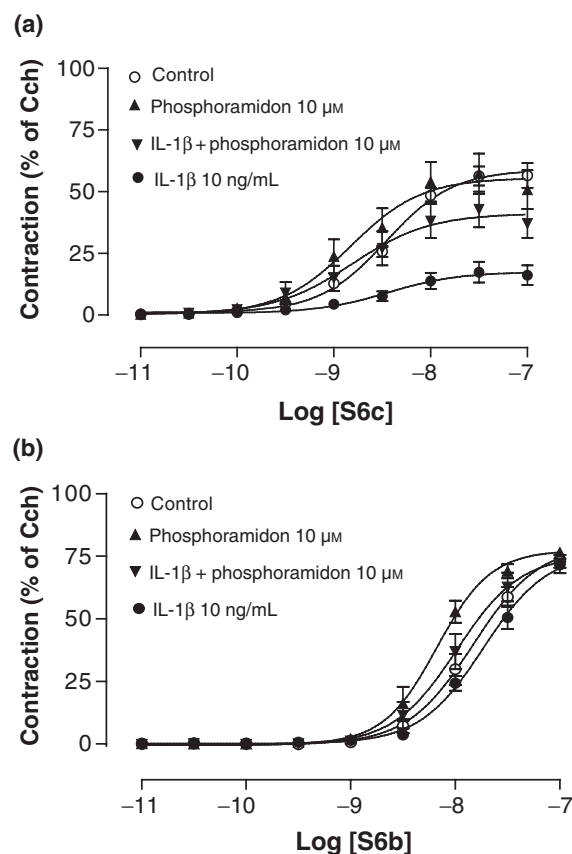


Fig. 4. Endothelin converting enzyme inhibitor phosphoramidon on the reduction of the contractile response to sarafotoxin 6c (S6c) (a) and sarafotoxin 6b (S6b) (b). Cumulative concentration-effect curves induced by S6c and S6b in isolated tracheal segments incubated for 4 days in presence/absence of IL-1 β (10 ng/mL) and phosphoramidon (10 μ M). Each point represents the mean of all segments $\pm$ SEM ($n = 5-7$).

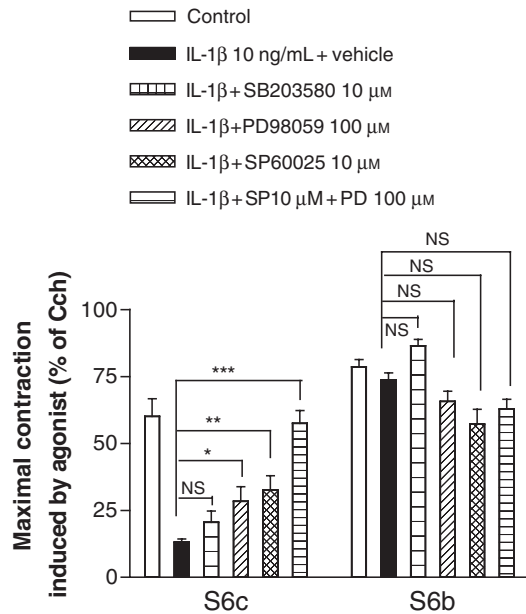


Fig. 5. JUN N-terminal kinase, extracellular-signal regulated kinase 1/2 and p38 pathway inhibitors (SP600125, PD98059 and SB203580) on the reduction of the contractile response to sarafotoxin 6c (S6c) and sarafotoxin 6b (S6b). Maximal contraction-induced by S6c and S6b in isolated tracheal segments incubated for 4 days in the presence of IL-1 β (10 ng/mL) with/without SP600125 (10 μ M) or/and PD98059 (100 μ M) or SB203580 (10 μ M). Data are mean $\pm$ SEM ($n = 5-7$). Statistical analysis was performed with unpaired Student's *t*-test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. *IL-1 β vs. inhibitors treated. NS, Not significant; PD, PD98059; SP, SP600125; vehicle, dimethyl sulphoxide.

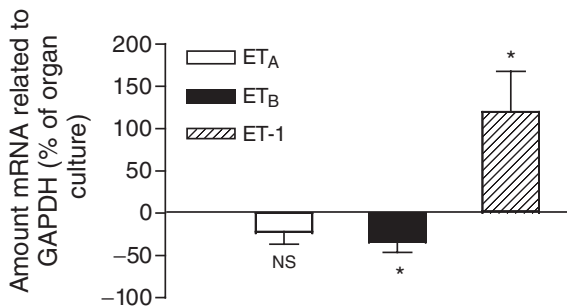


Fig. 6. The expression of mRNA for endothelin-1 (ET-1) in tracheal segments with epithelium and ET_A and ET_B receptors on the tracheal smooth muscle. The tracheal segments were incubated with or without IL-1 β 10 ng/mL for 2 days for ET-1 mRNA and 4 days for ET_A and ET_B receptor mRNA. The data were derived from four to six identical experiments and was presented as percent of control (organ culture without IL-1 β). Statistical analysis was performed with unpaired student's *t*-test. * $P < 0.05$. NS, not significant.

IL-1 β ($P < 0.05$), while only a tendency of down-regulation of ET_A receptor mRNA was observed ($P > 0.05$), compared with the control (without IL-1 β) (Fig 6). The ratio of ET_A/ET_B receptor mRNAs increased in IL-1 β group (1.79 ± 0.38), compared with organ culture group (1.29 ± 0.29), but this did not reach the statistical significance ($n = 6$, $P > 0.05$).

Discussion

The present study demonstrated that IL-1 β caused a significant reduction of the contractile responses to S6c, but not S6b, on mouse tracheal segments. In addition, ECE

activity, ERK1/2 and JNK, but not p38 pathway, were required for this reduction, as suggested by the inhibitory effects of the ECE inhibitor phosphoramidon, the JNK inhibitor SP600125, the ERK1/2 inhibitor PD98059 and the p38 pathway inhibitor SB203580. The individual inhibitor phosphoramidon, SP600125 and PD98059, but not SB203580, could only partly abolish the reduction of the contractile response to S6c. A combination of SP600125 and PD98059 had an additional effect. Interestingly, the expression of ET-1 mRNA in the tracheal segments with epithelium was up-regulated by IL-1 β and the expression of ET_B receptor mRNA in the tracheal smooth muscle was down-regulated.

In human bronchus, ET_B receptor was demonstrated in smooth muscle to mediate contraction, while ET_A receptor was present in epithelium to induce an epithelium-dependent relaxation via the release of NO [26]. However, the present study revealed both functional and mRNA of ET_A and ET_B receptors in mouse tracheal smooth muscle and both type of receptors mediate contraction. Interestingly, IL-1 β only affected the contraction mediated by ET_B receptors, but not ET_A receptors.

The up-regulation of ET-1 mRNA expression by IL-1 β suggests an increase of ET-1 paracrine locally by airway cells in our organ culture. The increase of ET-1 paracrine may lead to the reduction of the contractile response to selective ET_B receptor agonist S6c via the ET_B receptor internalization mechanism. Phosphoramidon, a dual ECE/neutral endopeptidase inhibitor blocks ECE converting big-ET into a fully active ET-1 peptide [27, 28]. The generation of ET-1 from big-ET is phosphoramidon sensitive, but not NEP in airway [29, 30]. Studies revealed that pro-inflammatory cytokine IL-1 β up-regulates the release of ET-1 and the expression of ECE mRNA in human bronchial epithelial cells and vascular smooth muscle cells, further more phosphoramidon could block the production of ET-1 [31, 32]. In this study IL-1 β results in a reduction of the contractile response in mouse tracheal segments to S6c and this reduction was partly blocked by ECE inhibitor phosphoramidon, together with the finding of the increase of ET-1 mRNA expression by IL-1 β revealed that the increase of ET-1 paracrine may at least partly contribute to the reduction of the contractile response to S6c. The results discussed above strongly supports the *in vivo* study by Henry and colleagues who found that virus-infected airway in mice had the hyporesponsiveness to S6c and ET-1. The decrease of ET_B receptor densities on airway smooth muscle was found to be responsible for the hyporesponsiveness [23]. The decrease of ET_B receptor densities in tracheal smooth muscle may occur, when the expression of ET_B receptor mRNA was down-regulated or ET_B receptor was internalized into cells and degraded in lysosome. This mechanism seems to serve a feedback in response to the elevated level of ET-1 locally, since it is a strong airway constrictor.

A slight increase of contractile response induced by S6c and S6b was seen during organ culture in the absence of IL-1 β in the present study. The mechanisms behind this are unclear. Putatively, an up-regulation of ET receptors could be involved as reported in organ culture of arteries [33, 34] or it might be generally increased responses to stimuli as seen in airway inflammation [35].

The present study was designed to investigate the mechanisms responsible for the reduction of contractile response to S6c by IL-1 β . IL-1 β is known as an agonist for the activation of MAPK pathways that consist of ERK1/2, p38 and JNK signal transduction pathways as well as the down stream of transcription factor nuclear factor kappa B and activating protein 1 (AP-1) [36, 37]. These transcription factors influence many biological functions such as receptor expressions and peptides synthesis in airway [37–39]. It has been reported that (i) PD98059, but not SB203580, inhibited angiotensin II-induced ET-1 expression and (ii) AP-1 binding site was an important cis-element in the induced ET-1 gene expression [40]. Inhibitors for JNK (SP600125), ERK1/2 (PD98059) and p38 pathway (SB203580) were used in the present study to find links between IL-1 β and ET receptors in airway smooth muscle. We found that both ERK1/2 and JNK, but not p38 pathway, are responsible for the reduction of the contractile response to S6c by IL-1 β . Blockage of ERK1/2 and JNK pathways may reduce the release of ET-1 by airway cells via the down stream transcription factors. The reduction of the release of ET-1 will prevent ET_B receptor internalization and thus can enhance the contractile response to S6c.

The two ET-receptor subtypes have different intracellular fates after ET-1 has bound to ET_A and ET_B receptors, i.e. ET_A receptors follow a recycling pathway and reappear to the plasma membrane of cell surface while ET_B receptors are directed to lysosome for degradation [21]. The increase of ET-1 paracrine by airway cells may therefore result in the desensitization of ET_B receptors in the smooth muscle cells through the internalization mechanism, while this cannot occur on ET_A receptors [41].

Reduction of the contractile response to S6c by IL-1 β was found at days 2, 4 and 8 with a maximum at day 4 in the present study. It seems that the release of ET-1 from the airway cells needs time to reach the effective and the maximal levels. Our finding agrees well with an *in vivo* study showing that parainfluenza-1 viral infection in mice reduced the contractile response to S6c at day 2 with a maximal reduction at day 4 [42]. Henry et al. [41] has revealed that the mechanism for the internalization of ET_B receptors by viral infection in mice airway occurred via the elevated levels of ET-1 locally.

Interestingly, the down-regulation of ET_B receptor mRNA by IL-1 β was also demonstrated in the present study. This gives an additional mechanism for IL-1 β to reduce the contractile response to S6c. In fact, ECE inhibitor could only abolish a part of the reduction of the contractile response to S6c in our study. Blockage of both ERK1/2 and JNK pathways could almost completely abolish the reduction of the contractile response to S6c by IL-1 β , which suggest MAPK pathways are responsible for the up-regulation of ET-1 expression, and other factors may be responsible for the down-regulation of ET_B receptors.

In conclusion, the prolonged presence of the pro-inflammatory mediator IL-1 β within the murine airways, attenuate the contractile response to the potent bronchoconstrictor ET. This effect is mediated via ET_B receptors and dependent on ECE, ERK1/2 and JNK MAPK pathways. Thus, understanding the mechanisms behind this type of interactions might allow us to find a new way to target the asthmatic inflammation.

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