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Up-Regulation of Endothelin Receptor Function and mRNA Expression in Airway Smooth Muscle Cells Following Sephadex-Induced Airway Inflammation

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Abstract: The hypothesis that up-regulation of bronchial constrictor endothelin receptors in airway smooth muscle cells may contribute to hyperreactivity during airway inflammation was tested in the present study by quantitative endothelin receptor mRNA analysis and functional responses in ring segments of rat trachea and bronchi. Real time reverse transcription polymerase chain reaction was used to quantify endothelin receptor expression in rat airway smooth muscle cells following Sephadex-induced inflammation. Compared with controls, Sephadex-induced airway inflammation caused a significant increase (3.9 times $P<0.05$) of endothelin receptor type B mRNA expression in bronchial smooth muscle cells, but not in tracheal smooth muscle cells. Functional myograph studies of bronchial and tracheal ring segments without epithelium (mechanically denuded) revealed an increase of the maximum contractile effects of endothelin-1 (a dual agonist for both endothelin type A and B receptors) and sarafotoxin 6c (a selective agonist for endothelin B receptors) in bronchial smooth muscle cells in Sephadex-induced inflammation, but not in tracheal smooth muscle cells. The enhanced maximal responses of bronchial smooth muscle cells to endothelin-1 and sarafotoxin 6c in Sephadex-induced inflammation support our molecular findings and hence imply a role for endothelin B receptors in airway hyperreactivity during airway inflammation.

Airway inflammation plays an important role for airway hyperreactivity in *asthma bronchiale*. Under basal conditions asthmatic patients, with or without inhaled steroids, seem to have normal endothelin-1 levels in the bronchi when measured in induced sputum (Chalmers *et al.* 1997a). Pro-inflammatory mediators such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) released during inflammatory processes are considered to directly activate airway smooth muscle cells to makes them more responsive to contractile agents (Hakonarson *et al.* 2001). Interestingly, TNF-α and IL-1β have been found to promote an increased production of endothelin-1 in cultured airway epithelial cells (Yang *et al.* 1997). Accordingly, a number of studies have revealed that endothelin-1 levels are significantly elevated in broncho-alveolar lavage fluid and in plasma during asthmatic attacks (Vittori *et al.* 1992; Michael & Markewitz 1996). Moreover, intratracheal instillation of Sephadex has been shown to create an eosinophilic airway inflammation with increased endothelin-1 levels in broncho-alveolar fluid (Andersson *et al.* 1996) and that this elevation is preceded by a rapid entothelin-1 mRNA synthesis (Finsnes *et al.*

1998). These studies have suggested that this peptide plays an important role in the initial phase of an eosinophilic airway inflammation.

The responses to endothelin-1 are mediated through endothelin type A and type B receptors with similar affinity for endothelin A and B receptors. In rat tracheal smooth muscle and peripheral lung endothelin A and B receptors exist in approximately equal numbers (Henry 1993; Goldie *et al.* 1996) while endothelin B receptors predominate in the human bronchial airway smooth muscle (Goldie *et al.* 1995). The endothelin A and B receptors are both present on airway smooth muscle cells where they mediate strong contractions, while only endothelin A receptors are present on airway epithelium to induce realxation via the release of nitric oxide (Naline *et al.* 1999). If endothelin-1 receptors are up-regulated during pulmonary inflammation such as that seen in blood vessels (White *et al.* 1999) the functional consequences of the enhanced levels of endothelin-1 would be an amplification of contractility. The present study was designed to examine the hypothesis that endothelin-1 receptors are up-regulated in airway smooth muscle in the early phase of *asthma bronchiale*. This was tested by using Sephadex-induced inflammation using real-time quantitative reverse transcription polymerase chain reaction to quantify endothelin-1 receptor mRNA levels and a sensitive myograph method to study functional responses of bronchial and tracheal segments to endothelin-1 and sarafotoxin 6c.

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The results suggest up-regulation of endothelin-B receptors in bronchial smooth cells.

Materials and Methods

Animals. Male Sprague Dawley rats (body weight 200–250 g, M& B, Denmark) were acclimatized for one week under standardised temperature (21–22°), humidity (50–60%) and light (12:12 light-dark) conditions in the animal department at AstraZeneca in Lund. Intratracheal Sephadex instillations were performed under brief enflurane (Efrane®, Abbott Scandinavia AB, Sweden) anaesthesia. Briefly, Sephadex G-200 (Pharmacia, Sweden) was suspended in sterile saline and given as an intratracheal instillation in the dose 5 mg ml^{-1}, 1 ml kg^{-1} body weight, while control animals received sterile saline, 1 ml kg^{-1} body weight. Twenty-four hours after intratracheal Sephadex instillation the animals were killed with an overdose of pentobarbithal (ACO, Sweden). The method using Sephadex to achieve an eosinophilic inflammation is the same procedure as has been described before (Andersson *et al.* 1992 & 1995) and done in the same laboratory. Macroscopically inflammatory changes were easily seen and the lung weight was higher. The trachea and bronchi were isolated for myograph studies and real-time quantitative reverse transcription polymerase chain reaction (PCR). The protocol was approved by the animal ethics committee at Lund University.

mRNA quantification.

Total RNA isolation and reverse transcription. Tracheal and bronchial smooth muscle cell layers were isolated under a light microscope on ice and rinsed with cold phosphate buffered saline (PBS) (GibcoBRL, USA) several times. The tissues were homogenized in the TRIzol reagent (GibcoBRL, USA) for extraction of total mRNA. Reverse transcription (RT) of total RNA to cDNA was carried out using the Gene Amp RT kit (PE Applied Biosystems, USA) in a Perkin-Elmer 2400 PCR machine at 42° for 30 min.

Quantification of the expression of endothelin type A (ET_A) and endothelin type B (ET_B) receptor mRNA.

The real-time quantitative RT-PCR was performed with the GeneAmp SYBR Green PCR kit (PE Applied Biosystems, USA) in a Perkin-Elmer real-time PCR machine (GeneAmp 5700 sequence detection system). The system automatically monitors the binding of a fluorescent dye to double-strand DNA by real-time detection of the fluorescence during each cycle of PCR amplification. Specific primers for rat ET_A and ET_B receptors were designed as below:

ET_A receptor forward: 5′-ATT GCC CTC AGC GAA CAC-3′
    reverse: 5′-CAA CAA AGC AGA AAG ACG GTC-3′
ET_B receptor forward: 5′-GAT ACG ACA ACT TCC GCT CCA-3′
    reverse: 5′-GTC CAC GAT GAG GAC AAT GAG-3′

The housekeeping gene, Elongation factor-1 (EF-1), mRNA continuously expressed to a constant amount in the cells, was compared with the house keeping gene β-actin in a pilot study by real-time PCR (data not shown). EF-1 was used as a reference in this study, but both were equally well constant in the tests. Rat EF-1 primers were designed as below:

EF-1 forward: 5′-GCA AGC CCA TGT GTG TTG AA-3′
  reverse: 5′-TGA TGA CAC CCA CAG CAA CTG-3′

The PCR reaction was performed in a 50 μl volume and started at a temperature of 50° for 2 min., 95° for 10 min. and the following 40 PCR cycles with 95° for 15 sec. and 60° for 1 min. Dissociation curves were run after the real-time PCR, and no non-specific amplification was detected in the present study. All primers were designed using the Primer Express 2.0 software (PE Applied Biosystems, USA) and synthesized by GibcoBRL Custom Primers (Life Techno-

logies, Inc., USA). The PCR products of ET_A (64 bp), ET_B (86 bp) and EF-1 (96 bp) were visualized with agarose gel electrophoresis.

To evaluate the amount of ET_A or ET_B receptor mRNA in a sample, EF-1 mRNA was assessed in the same sample simultaneously. The cycle threshold values of EF-1 mRNA was used as a reference to quantify the relative amount of ET_A or ET_B receptor mRNA. The relative amount of mRNA was calculated with the cycle threshold values of ET_A or ET_B receptor mRNA in relation to the cycle threshold values of EF-1 mRNA in the sample.

Functional studies. Circular segments were cut from trachea and bronchi. Each segment was denuded of its epithelium using a metal thread of 1 mm in diameter and a surface as a fine graded file to avoid confounding dilatory effects (Hadj Kaddour *et al.* 1995). They were then mounted on two L-shaped metal prongs. One prong was connected to a force displacement transducer attached to a computer for continuous registration of isometric tension and the other to a displacement device. The segments were then immersed into small (2.5 ml) temperature-controlled (37°) tissue baths containing a bicarbonate based buffer solution (see below). The solution was equilibrated with 5% CO_2 in O_2, resulting in a pH of 7.4. Initially the tracheal and bronchial segments were allowed to stabilise for 60 min. under a tension of 2 mN and 1 mN, respectively. The tone was chosen on the basis of pretension-contraction curves in Ca^{++} free and Ca^{++} containing solution as published before (Högestätt *et al.* 1983) and now modified for tracheal and bronchial ring segments (data not shown). The contractile ability of each segment was first examined by exposure to a potassium rich (60 mM) buffer solution (for composition, see below), which caused maximum effect at this concentration (data not shown). When maximum contraction was reached within a minute, the potassium solution was washed out with the buffer solution. Only after two strong (>1 mN) reproducible contractions (variation <10%) had been elicited the individual segments were used for further studies. The contraction induced by K$^+$-solution (60 mM) was used as contractility reference and maximum was defined as 100%. Endothelin-1 or sarafotoxin 6c was added to achieve cumulative concentration-response curves. The segments were allowed to stabilize at each contraction level before a higher concentration was added. Comparing experiments between epithelium-denuded and epithelium-intact segments proved that the ability to contract concerning potassium and acetylcholine was not influenced or harmed by denudation (data not shown). The maximal contractile force was also tested at the end of each experiment using acetylcholine (10^{-3} mM).

After the eosinophilic inflammation had been established (24 hr), the contractile effects of endothelin-1 and sarafotoxin 6c were studied at two levels of the rat airways, trachea and bronchi. All segments could be used in this study, i.e. no segment was damaged through removal of epithelium or from the Sephadex-induced inflammation. The result of the removal of epithelial cells was controlled by histological investigation after the experiments (haematoxylin-eosin staining), and also used to confirm the inflammation (Andersson *et al.* 1992).

Solutions. (A) Standard buffer solution (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2, and glucose 5.5.

(B) 60 mM K$^+$ buffer solution: as above, but substituting equimolar amounts of NaCl with KCl.

Drugs. Sarafotoxin 6c and endothelin-1 were obtained from Auspep (Parkville, Australia) and acetylcholine from Sigma (St. Louis, MO, USA). These agents were dissolved and further diluted in saline containing 0.1% bovine serum albumin (Beringwerke, Marburg, Germany) to avoid adhesion of peptides to vials.

Analysis. Contractile responses in the segments are expressed as a percentage of the contraction induced by 60 mM K$^+$. E_{max} values refer to the maximum contractile effect of an agonist. The pEC_{50} value (the negative logarithm of the molar concentration that pro-

duced half-maximum contraction) was calculated from the straight-line equation between the concentration above and below the mid-point of the concentration-response curve.

Statistics. Unpaired t-test was used for molecular studies and two-way ANOVA with Bonferroni post-test was used for functional responses to compare control and Sephadex groups. A P-value of <0.05 was regarded as significant.

Results

Real-time RT-PCR standard curves.

To make real-time RT-PCR standard curves for endothelin receptor mRNA, cDNA from reverse transcription of total RNA was diluted with the PCR buffer in three sequential log concentrations (1:0, 1:10 and 1:100). The log concentrations that relate to the PCR CT-values were found to be optimal. The PCR products of endothelin type A (64 bp), type B (86 bp) and endothelin-1 (96 bp) were visualized with agarose gel (2%) electrophoresis and corresponded to their predicted size (fig. 1A).

Endothelin receptor mRNA expression.

Endothelin receptor mRNA was assessed by the real-time RT-PCR of tracheal and bronchial smooth muscle in control and in Sephadex-induced inflammation rats (fig. 1B). The endothelin type B-receptor mRNA expression of bronchial smooth muscle increased 3.9 times in Sephadex-induced airway inflammation (P<0.05). No difference was seen in tracheal smooth muscle during induced inflammation. There was no alteration in endothelin type A-receptor mRNA expression (fig. 1B).

Airway smooth muscle responses to endothelin-1 and sarafotoxin 6c.

To examine if the molecular findings had been translated into functional receptors, tracheal and bronchial smooth muscle responses to concentration-dependent applications of endothelin-1 and sarafotoxin 6c were assessed in myograph studies (fig. 2A–D).

When Sephadex treated segments was compared to control segments, we found no change in contractile force from 60 mM K^+ buffer solution, neither in the tracheal, nor in the bronchial segments. The maximal contractile force was
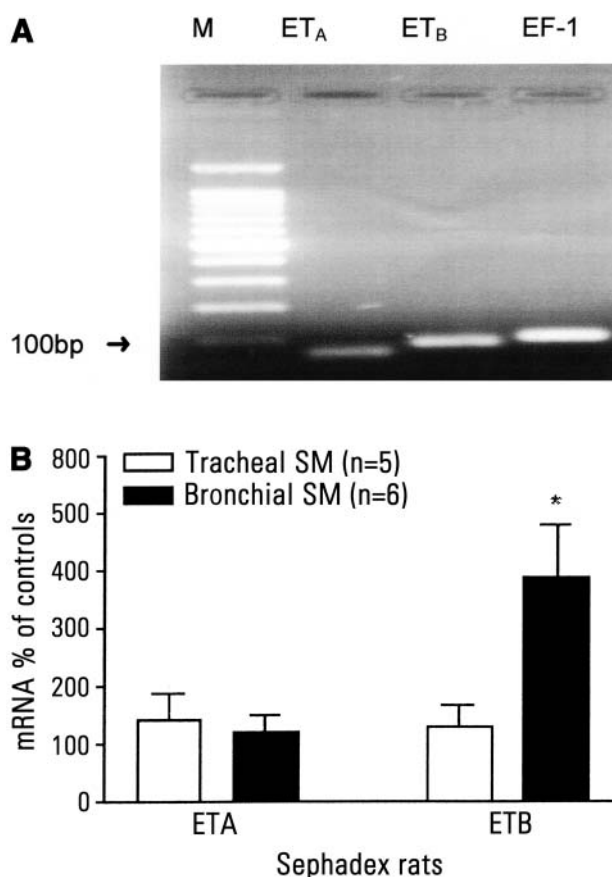

Fig. 1. A. Agarose gel (2%) electrophoresis of real-time RT-PCR products shows clear single bands with expected sizes for endothelin type A (ET_A) (64 bp), endothelin type B (ET_B) (86 bp) and endothelin-1 (96 bp). M=basepair ladder. 1B. Tracheal and bronchial smooth muscle cell ET_A and ET_B receptor mRNA were assessed by real-time PCR. Mean data with S.E.M. are represented as percent of sham-treated rats. n=number of rats. Two-way unpaired t-test was applied to compare the groups. *P<0.05.

also tested using acetylcholine (10^{-3} mM) at the end of each experiment and the results were equal in the two groups compared (table 2).

In tracheal smooth muscle, there was no increase in the responses to endothelin-1 or sarafotoxin 6c following Se-

Table 1.

The maximal contractile responses (% of maximal contraction to 60 mM potassium) and pEC_{50} of endothelin-1 and sarafotoxin 6c in tracheal and bronchial segments from Sephadex-provoked rats. Values are given as means±S.E.M, n=7 in each group.

	Trachea		Bronchi	
	Maximal contraction	pEC50	Maximal contraction	pEC50
Endothelin-1				
Control (n=7)	115.8±6.6	7.5±0.04	97.7±5.4*	7.1±0.04
Sephadex (n=7)	121.7±4.8	7.7±0.06	122.9±11.2*	7.3±0.10
Sarafotoxin 6c				
Control (n=7)	85.2±3.3	8.5±0.07	122.5±6.7#	7.7±0.15
Sephadex (n=7)	86.1±4.5	8.3±0.08	155.1±14.1#	7.8±0.09

n=number of rats. *,#=P<0.05.

phadex-induced inflammation compared to control at any concentration tested (fig. 2A and 2B).

In bronchial smooth muscle there was a significant increase ($P < 0.05$) in contractile responses to both endothelin-1 and sarafotoxin 6c following Sephadex-induced inflammation (table 1 and fig. 2C and D), however the pEC_{50} values of endothelin-1 and sarafotoxin 6c in bronchial and

tracheal smooth muscle following Sephadex-induced inflammation did not significantly differ from control.

Discussion

The present study has shown that Sephadex-induced inflammation for 24 hr up-regulates both the endothelin type
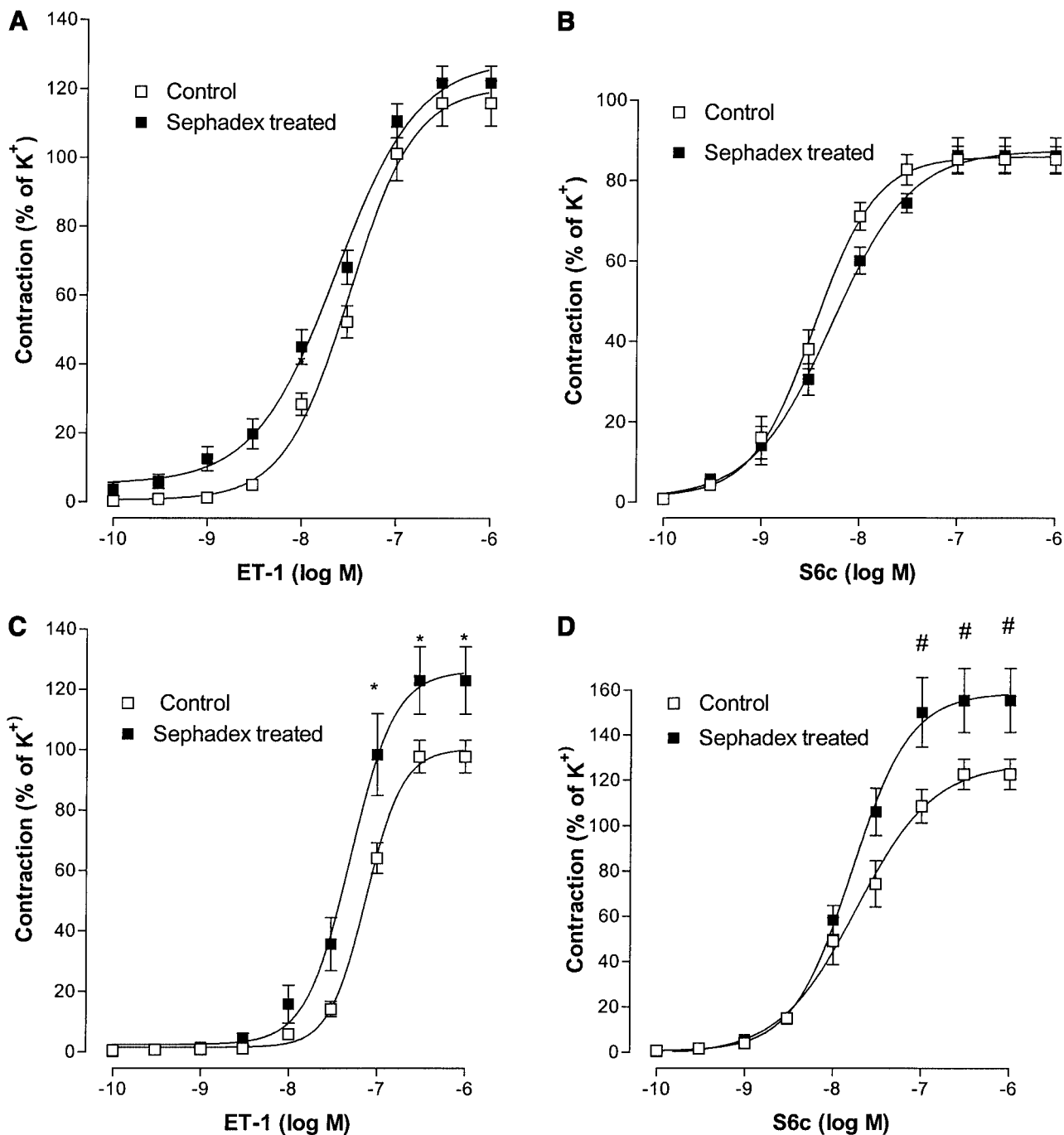
Fig. 2. Rat tracheal (A,B) and bronchial (C,D) smooth muscle cell responses to concentration-dependent application of endothelin-1 (A,C) and sarafotoxin 6c (B,D) following Sephadex-induced inflammation (filled symbol) compared to control (open symbol). Each data point was derived from 7 rats. Mean values with S.E.M. are given. Two way ANOVA with Bonferroni post-test analysis was performed to compare endothelin-1 or sarafotoxin 6c induced contraction in the two groups; *$P < 0.05$.

Table 2.

The maximal contractile responses and pEC50 to 60 mM potassium and acetylcholine (10^{-3} mM) in tracheal and bronchial segments from control and Sephadex-treated rats measured in mN.

	Trachea		Bronchi	
	Maximal contraction	S.E.M.	Maximal contraction	S.E.M.
K+, control	8.2	±0.6	9,0	±0.9
K+, Sephadex-treated	9.6	±0.9	8.1	±1.0
Acetylcholine, control	11.4	±0.9	11.6	±0.9
Acetylcholine, Sephadex-treated	11.5	±1.1	11.4	±1.2

There is no difference between control and Sephadex-treated segments.

B receptor mRNA level and the functional responses to the selective endothelin type B receptor agonist sarafotoxin 6c in bronchial smooth muscle cells. A real-time quantitative RT-PCR was used in the present study to quantify endothelin-receptor mRNA expression in rat airway smooth muscle cells from Sephadex-treated and control rats. We demonstrated for the first time that in Sephadex-induced airway inflammation endothelin type B receptor mRNA expression in bronchial smooth muscle cells is significantly increased but at the same time there was no difference in the expression of the endothelin type A receptor mRNA in tracheal or bronchial smooth muscle cells. The smooth muscle cell contractile responses to endothelin-1 and sarafotoxin 6c in functional studies support the molecular findings, i.e. an increase in the maximal contractile effects of endothelin-1 and sarafotoxin 6c were seen in bronchial but not in tracheal smooth muscle cells of Sephadex-treated rats.

Airway hyperreactivity is one of the important functional characteristics in asthmatic inflammation. The mechanisms of inflammation that induce airway hyperreactivity, i.e. an increased tendency of the smooth muscle cells in the airways to contract, are complex and confounding. Bronchial epithelial cells of asthmatic patients release high amounts of mature and biologically active endothelin compared to control donors (Vittori *et al.* 1992). Removal of epithelium from human bronchus significantly enhanced the contractile response to endothelins in an experimental situation (Candenas *et al.* 1992). In non-asthmatic healthy volunteers, inhaled endothelin-1 did not change lung function but asthmatic patients displayed a rapid-onset dose-dependent bronchconstriction (Chalmers *et al.* 1997b). A well-known feature during early asthmatic inflammation is disruption of the epithelium (Laitinen *et al.* 1993). This damaged physical barrier could be one mechanism for enhanced access of endothelin-1 to underlying bronchial smooth muscle cells. Two other processes that also could be impaired in the epithelium during asthmatic inflammation are changes in the clearance of endothelin-1 through the endothelin type B receptors (Fukuroda *et al.* 1994) and the production of relaxant factors as nitric oxide when endothelin-1 binds to endothelin type A receptors (Naline *et al.* 1999). To avoid the influence of epithelial cells on the contractile process and on the analysis of endothelin type B receptor mRNA in the present study, the epithelium was removed in all segments.

A well-known method to study early eosinophilic inflammation in airways is tracheal instillation of Sephadex (Andersson *et al.* 1992). During this kind of inflammation the concentration of endothelin-1 in broncho-alveolar fluid has been shown to be increased 30 times with its peak at 24 hr (Andersson *et al.* 1996). It has been suggested that endothelin-1 is paracrine in the airways during inflammation and hence may contribute to airway hyperreactivity. Support for the involvment of the endothelin system is a study which demonstrated significantly higher levels of endothelin type B receptor mRNA compared to endothelin type A receptor mRNA in bronchial biopsies from patients with asthma and chronic airway obstruction (Möller *et al.* 1999).

In the present study early Sephadex induced eosinofilic inflammation caused an up-regulation of endothelin type B receptor expression in airway smooth muscle cells. This finding is supported by the stronger responses of bronchial smooth muscle cells to endothelin-1 and sarafotoxin 6c. To address the question of receptor specificity the functional studies were designed to use endothelin-1 as an agonist of endothelin type A and endothelin type B receptors and sarafotoxin 6c is as a specific agonist of endothelin type B receptors (Williams *et al.* 1991). To ascertain the receptor characteristics we have performed antagonist experiments before this study, both in tracheal and bronchial segments, using FR 139317 and BQ 788 to block endothelin type A and endothelin type B receptors, respectively (unpublished results) as well as experiments on the isolated lung preparation (Granström *et al.* 2004). The experiments confirmed the observations from many other studies (Henry 1993). Thus, the increased responses to sarafotoxin 6c and endothelin-1 in bronchial tissue can be explained by up-regulation of endothelin type B receptors. During the eosinophilic inflammatory process the raised level of endothelin-1 probably causes a raised consumption of endothelin B receptors, i.e. a raised turnover, explaining varying results in different studies. The mechanism behind the upregulation of endothelin type B receptors, a *de novo* synthesis, in airways is not known but the phenomenon has been studied in detail for blood vessels. *De novo* transcription and translation (Möller *et al.* 1997 & 1998), via protein kinase C (Uddman *et al.* 2002) and mitogen-activated protein kinases (Henriksson *et al.* 2003; Uddman *et al.* 2003) have been shown. Puta-

tively, a similar regulatory mechanism could take place in bronchi but this requires further analysis. Another mechanisms for regulation of endothelin-receptors in airway inflammation may hypothetically occur via cytokines such as tumour necrosis factor-α and interleukin-1α. Our group has demonstrated that such a phenomenon can occur in vascular smooth muscle cells (Leseth *et al.* 1999; Uddman *et al.* 1999). During airway inflammation cytokines derived from inflammatory cells could induce both increased levels of endothelin-1 and up-regulation of endothelin type B receptors, which synergistically enhance contractility of the airway smooth muscle.

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