Role of endothelin-converting enzyme, chymase and neutral endopeptidase in the processing of big ET-1, ET-1(1–21) and ET-1(1–31) in the trachea of allergic mice

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ABSTRACT

The present study examined the roles of endothelin-converting enzyme (ECE), neutral endopeptidase (NEP) and mast cell chymase as processors of the endothelin (ET) analogues ET-1(1–21), ET-1(1–31) and big ET-1 in the trachea of allergic mice. Male CBA/CaH mice were sensitized with ovalbumin (10 μg) delivered intraperitoneal on days 1 and 14, and exposed to aerosolized ovalbumin on days 14, 25, 26 and 27 (OVA mice). Mice were killed and the trachea excised for histological analysis and contraction studies on day 28. Tracheae from OVA mice had 40% more mast cells than vehicle-sensitized mice (sham mice). Ovalbumin (10 μg/ml) induced transient contractions (15 ± 3% of the Cmax) in tracheae from OVA mice. The ECE inhibitor CGS35066 (10 μM) inhibited contractions induced by big ET-1 (4.8-fold rightward shift of dose-response curve; P < 0.05), but not those induced by either ET-1(1–21) or ET-1(1–31). The chymase inhibitors chymostatin (10 μM) and Bowman-Birk inhibitor (10 μM) had no effect on contractions induced by any of the ET analogues used. The NEP inhibitor CGS24592 (10 μM) inhibited contractions induced by ET-1(1–31) (6.2-fold rightward shift; P < 0.05) but not ET-1(1–21) or big ET-1. These data suggest that big ET-1 is processed predominantly by a CGS35066-sensitive ECE within allergic airways rather than by mast cell-derived proteases such as chymase. If endogenous ET-1(1–31) is formed within allergic airways, it is likely to undergo further conversion by NEP to more active products.

INTRODUCTION

In addition to being a potent spasmogen of airway smooth muscle, endothelin-1 (ET-1) is able to stimulate microvascular leakage, airway oedema, smooth muscle hyperplasia and airways hyper-responsiveness [1]. These effects implicate ET-1 as an important mediator in asthma. Endothelin-converting enzyme (ECE) converts inactive big ET-1 to a biologically active 21-amino-acid peptide ET-1(1–21)[2]. Interestingly, in studies using ECE knockout mice, significant levels of mature ET-1 peptide were detected [3]. This raises the possibility that other non-ECE pathways may exist for the processing of big ET-1.

Recent evidence indicates that purified human mast cell chymase is also able to convert big ET-1 to a

Key words: allergic inflammation, chymase, endothelin-converting enzyme, big endothelin.

Abbreviations: BBI, Bowman-Birk inhibitor; ET, endothelin; ECE, endothelin-converting enzyme; NEP, neutral endopeptidase; OVA mice, mice sensitized and challenged with aerosolized ovalbumin.

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biologically active 31-amino-acid peptide ET-1(1–31) [4]. Allergic challenge stimulates the release of mast cell degranulation products such as chymase into the surrounding tissue. Using an ovalbumin-driven model of allergic inflammation, the present study investigates the possibility that increased levels of chymase in the airways after mast cell degranulation will shunt the processing of big ET-1 to ET-1(1–31). This pathway may be important in the generation of biologically active ETs in allergic asthmatics. In addition, we compared the potencies of big ET-1, ET-1(1–21) and ET-1(1–31) and examined the possibility that ET-1(1–31) may be processed further by other mast cell-derived chymotrypsin-like enzymes or by metalloproteases such as ECE and neutral endopeptidase (NEP).

**METHODS**

**Ovalbumin-induced allergic inflammation in mice**

Mice were randomly allocated into two equally sized groups. One group was sensitized and challenged with ovalbumin (OVA mice). The sensitization phase consisted of an intraperitoneal injection of 10 μg of ovalbumin and 1 mg Al(OH)₃ (adjuvant) in 0.3 ml sterile saline on day 1 and day 14, and a 20 min exposure to aerosolized ovalbumin on day 14. Animals were placed in a perspex box and exposed to aerosolized ovalbumin, generated by a Wright’s nebulizer (rate of air flow, 5 litres/min; concentration of ovalbumin; 10 mg/ml). On days 25, 26 and 27, allergic mice were challenged with further 20 min periods of exposure to aerosolized ovalbumin administered by nebulizer. The second group of animals were treated identically except that the intraperitoneal injections and aerosols did not contain ovalbumin (sham). Mice were killed on day 28 and tracheal tissue taken for contraction studies and histological analysis.

**Histological analysis of mouse tracheae**

Tracheae were excised and cut into 1–2 mm-length rings that were then rinsed in macrodex. Tracheal rings were mounted in 0.5 ml aluminium foil pans and submerged with macrodex. Pans were snap-frozen in isopentane quenched with liquid nitrogen. Random non-serial sections (10 μm thick) were cut from each of the upper, lower and middle levels of each preparation using a cryostat (–20 °C) and mounted on to slides. Preparations were fixed by immersion in absolute ethanol for 1 min and stained by immersion in Toluidine Blue for 30 s. Slides were cover-slipped using DePeX as a mounting medium. Mast cells, which stain deep purple, were visualized by light microscopy.

**Measurement of the contraction of mouse tracheae**

Tracheal ring preparations (1–2 mm in length) were suspended between two stainless steel hooks at a tension of 0.3–0.4 g in a 2 ml bath containing Krebs bicarbonate solution (mM: NaCl 117, glucose 11.1, NaHCO₃ 25, KCl 5.4, MgSO₄ 7H₂O 1.3, KH₂PO₄ 1.0, CaCl₂ 2.5 and indomethacin 0.0025), maintained at 37 °C and bubbled with 5% CO₂ in O₂. Preparations were equilibrated for 45 min during which time the bathing medium was replaced and the tension readjusted to 0.3–0.4 g every 15 min.

Each preparation was then exposed to two cumulatively administered concentrations of carbachol (0.2 μM and 10 μM) to test tissue viability. Ten minutes after washout, this was repeated. Ovalbumin (10 μg/ml) was administered to all preparations 10 min after the final carbachol administration and 10 min prior to commencing inhibitor addition. Preparations from sham and OVA mice were incubated with CGS35066 (10 μM; a gift from Novartis, Summit, NJ, U.S.A.), CGS24592 (10 μM; a gift from Novartis), chymostatin (10 μM; Sigma Chemical Co., St Louis, MO, U.S.A.), Bowman-Birk inhibitor (BBI; 10 μM; Sigma) or vehicle for 20 min prior to addition of an ET analogue in a half-log cumulative dosing regimen. In all experiments, only a single concentration–effect curve to an ET analogue [ET-1(1–21) (1–100 nM; Auspep), big ET-1 (10–300 nM; Auspep), ET-1(1–31) (1–300 nM; Peptide Institute, Minoh-Shi, Osaka, Japan), big ET-1 (10–300 nM; Auspep)] was obtained from each preparation. Contractions were measured by an FT03C isometric force displacement transducer (Grass Instruments, West Warwick, RI, U.S.A.) coupled to a pre-amplifier and visualized on an IBM computer using custom-built software. All stated concentrations refer to final bath concentrations.

**Data analysis**

Graphical dose-response data is expressed as mean±S.E.M. –log EC₅₀ values were used to measure potency for the ET analogues. –log EC₅₀ values for the different groups were compared using two-way ANOVA. Direct comparisons between any two of the groups was then made using the modified t-statistic. Data obtained by counting mast cells histologically were compared using the t test. The level of significance was set at P < 0.05.

**RESULTS**

**Histological analysis of mouse tracheae**

Tracheal preparations from sham mice had, on average, 7.5 ± 1.0 mast cells/section (n = 6), whereas those from OVA mice had 10.6 ± 0.7 mast cells/section (n = 6). Hence, tissue sections of the trachea of OVA mice had 40% more mast cells than sham mice (P < 0.05).
Contraction studies

The potencies of ET-1(1–21), ET-1(1–31) and big ET-1 were similar [EC₅₀ values of 16 nM (95% confidence limits, 5.1–51 nM; n = 5 mice), 28 nM (21–38 nM; n = 4 mice) and 32 nM (19–52 nM; n = 7 mice) respectively], although big ET-1-induced contractions developed more slowly.

Ovalbumin (10 μg/ml) induced a transient contraction [15 ± 3 % of the maximum contraction induced by 10 μM carbachol (Cₘₕₜ)] in trachea from OVA mice, but not sham mice. CGS35066 (10 μM) inhibited contractions induced by big ET-1 (4.8-fold rightward shift of dose-response curve, P < 0.05; Figure 1a), but not those induced by ET-1(1–21) and ET-1(1–31) (n = 4–6 mice). Chymostatin (10 μM) and BBI (10 μM) had no effect on contractions induced by any of the ET analogues (Figure 1b). CGS24592 inhibited contractions induced by ET-1(1–31) (6.2-fold rightward shift, P < 0.05; Figure 1c), but not those induced by big ET-1 or ET-1(1–21) (n = 4–7 mice; Table 1).

**DISCUSSION**

Amino acid sequence alignment indicates that mouse mast cell chymase 5 is structurally homologous with human α-chymase [5], which is able to cleave big ET-1 at the Tyr⁴¹–Gly⁴² bond to yield ET-1(1–31) in vitro [4]. Histological data from this study indicate a 40% increase in mast cell numbers within the trachea of OVA mice. Furthermore, contraction studies indicate that ovalbumin induced mast cell degranulation in tracheal preparations from OVA mice. Under these conditions, we were able to examine whether mast cell-derived chymases released upon degranulation would shunt the processing of big ET-1 to yield ET-1(1–31). However, we found that exogenous big ET-1-induced contractions were insensitive to chymase inhibition in OVA mice. In addition, we found that big ET-1-induced contractions were attenuated by inhibition of a CGS35066-sensitive ECE. These functional data suggest that mast cell-derived chymases do not significantly process big ET-1 in allergic airways.

Exogenous ET-1(1–31) induced dose-dependent contractions in the trachea of both sham and OVA mice. This is consistent with previous studies which indicate that ET-1(1–31) is a spasmogen of both monkey and rat trachea [4,6]. These studies reported that ET-1(1–31) is directly active, without further conversion. However, we found that ET-1(1–31)-induced contractions were attenuated by NEP inhibition via CGS24592 in both sham and OVA mice. Therefore, if endogenous ET-1(1–31) is...
present within the allergic airways of mice, it is probably processed further by NEP to more active products such as ET-1(1–21).

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