ET-1-induced bronchoconstriction is mediated via ET$_B$ receptor in mice

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Nagase, Takahide, Tomoko Aoki, Teruaki Oka, Yoshinosuke Fukuchi, and Yasuyoshi Ouchi. ET-1-induced bronchoconstriction is mediated via ET$_B$ receptor in mice. J. Appl. Physiol. 83(1): 46–51, 1997.—Endothelin (ET)-1 is one of the most potent agonists of airway smooth muscle and can act via two different ET receptor subtypes, i.e., ETA and ETB. To determine the effects of ET-1 on in vivo pulmonary function and which ET receptors are involved in murine lungs, we investigated 1) the effects of ET and sarafotoxin S6c (S6c), a selective ET$_B$ agonist, on pulmonary function and 2) the effects of BQ-123 and BQ-788, specific ET$_A$- and ET$_B$-receptor antagonists, on ET-1-induced bronchoconstriction. ICR mice were anesthetized and mechanically ventilated (frequency = 2.5 Hz, tidal volume = 8 ml/kg, positive end-expiratory pressure = 3 cmH$_2$O). Intravenous ET-1, ET-2, and ET-3 increased lung resistance similarly and equipotently, whereas S6c elicited a greater degree of bronchoconstriction. Mice were then pretreated with saline (Sal), BQ-123 (0.2, 1, and 5 mg/kg), or BQ-788 (0.2, 1, and 5 mg/kg) before administration of ET-1 (10$^{-7}$ mol/kg iv). No dose of BQ-123 blocked ET-1-induced constriction, whereas pretreatment with each dose of BQ-788 significantly inhibited ET-1-induced responses. There were significant differences in morphometrically assessed airway constriction between Sal and BQ-788 and between BQ-123 and BQ-788, whereas no significant difference was observed between Sal and BQ-123. There were no significant morphometric differences in the airway wall area among the three groups. These observations suggest that the ET$_B$- but not ET$_A$-receptor subtype may mediate the changes in murine pulmonary function in response to ET-1. In addition, the ET$_B$-receptor antagonist reduces ET-1-induced airway narrowing by affecting airway smooth muscle contraction in mice.

BQ-123; BQ-788; airway resistance; wild-type mice; endothelin$_{1}$; endothelin$_{6}$ receptors; endothelin$_{6}$ receptors

ENDOTHELIN (ET)-1 is a 21-amino acid peptide isolated from vascular endothelial cells (30). ET-1 has been demonstrated to be one of the most potent agonists of both vascular and airway smooth muscle in various species, including mice (5, 15, 27, 28, 30). Recent studies suggest that ET-1 may be involved in the pathogenesis of asthma (6, 16, 25), pulmonary inflammation (18), and pulmonary vascular disease (3). To examine the roles of ET in the pathogenesis of various pulmonary diseases, including asthma, these transgenic mice are expected to be employed as appropriate animal models. However, even in genetically wild-type mice, the roles of ET receptors in airway physiology remain to be clarified.

We hypothesized that ET-1 might affect wild-type murine lung via either ET-receptor subtype in vivo. To test this hypothesis, we obtained dose-response curves for ET-1, ET-2, ET-3, and sarafotoxin S6c (S6c), a selective ET$_B$ agonist (29), in ICR mice. We then investigated the effects of ET-1 on airways of ICR mice by using ET$_A$- and ET$_B$-selective antagonists, i.e., BQ-123 (22) and BQ-788 (9), respectively. In addition, we questioned whether and how ET-receptor antagonists would modify ET-1-induced constriction, i.e., whether the ET$_A$ or ET$_B$ antagonist would affect airway smooth muscle shortening or airway wall thickening. To answer this question, we performed morphometric analysis of airways.

MATERIALS AND METHODS

Animal preparation. Male ICR mice (35–42 g) were studied. Animals were anesthetized with pentobarbital sodium (25 mg/kg ip) and ketamine hydrochloride (25 mg/kg ip) in combination. Then they were paralyzed with pancuronium bromide (0.3 mg/kg ip). A jugular venous line was placed for fluid and drug administration. Anesthesia and paralysis were maintained by supplemental administration of 10% of the initial dose every hour. After tracheostomy was performed, an endotracheal metal tube (1 mm ID, 8 mm long) was inserted in the trachea. Animals were mechanically ventilated (model 683, Harvard Apparatus, South Natick, MA) with a tidal volume of 8 ml/kg and a frequency of 2.5 Hz. The thorax was widely opened by means of midline sternotomy, and a positive end-expiratory pressure (PEEP) of 3 cmH$_2$O was applied by placing the expired line underwater. During the experiments, O$_2$ was continuously supplied to the ventilatory system. Under these ventilatory conditions, arterial pH, PO$_2$, and PCO$_2$ were 7.35–7.45, 100–180 Torr, and 30–45 Torr, respectively, at the end of experiments. A heating pad was used to maintain the body temperature of the animals at 37°C.

Tracheal pressure (Ptr) was measured with a piezoresistive microtransducer (Endevco 8510B-2, San Juan Capistrano, CA) placed in the lateral port of the tracheal cannula. Tracheal flow was measured by means of a Fleisch pneumotachograph (model no. 00000; Metabo, Lauzanne, Switzerland). All signals were amplified, filtered at a cutoff frequency of 100 Hz, and converted from analog to digital with a converter (DT2801-A; Data Translation, Marlborough, MA).
The signals were sampled at a rate of 200 Hz and stored on an IBM-AT compatible computer.

Calculation. The $P_{\text{tr}}$ was corrected for both the tube resistance and the Bernoulli effect (17). From flow, volume ($V$), and corrected $P_{\text{tr}}$, lung elastance ($E_{L}$) and total lung resistance ($R_{L}$) were calculated by finding the best fit for the equation of motion

$$P_{\text{tr}} = R_{L} \cdot \frac{dV}{dt} + E_{L} \cdot V + K$$

$K$ is a constant having a value that was also estimated by multiple linear regression and a function that was to take into account any error made in estimating zero volume (2, 24).

In the present experiment, $K$ was $<0.5$ cmH$_2$O different from the real value of PEEP.

Effects of ET-1, ET-2, ET-3, and S6c on pulmonary function. Synthetic ET-1, ET-2, ET-3, and S6c (Peptide Institute, Osaka, Japan) were dissolved in phosphate-buffered saline (PBS) at doses of $10^{-9}$ to $10^{-7}$ mol/kg. In a preliminary experiment, the administration of $>10^{-7}$ mol/kg ET-1, ET-2, or ET-3 caused severe arrhythmia or cardiac failure.

After two deep inflations (peak $P_{\text{tr}}$ of 30 cmH$_2$O) were performed, 0.1 ml of PBS and peptide solutions were given intravenously in half-log increasing doses to obtain cumulative dose-response curves ($n = 5$ for each group).

Effects of BQ-123 and BQ-788 on ET-1-induced bronchoconstriction. Two minutes before the bolus of 0.1 ml of a solution containing $10^{-7}$ mol/kg ET-1, animals were pretreated with one of the following solutions: 1) saline as controls ($n = 7$, Sal group); 2) 0.2, 1, or 5 mg/kg BQ-123 ($n = 5$, respectively, BQ-123 group); or 3) 0.2, 1, or 5 mg/kg BQ-788 ($n = 5$, respectively, BQ-788 group). Saline was used as the vehicle for BQ-123, whereas BQ-788 was dissolved in 1% polyoxyethylene hydrogenated castor oil (HCO 60; Nikko Chemicals, Tokyo, Japan) in saline (9). After the pretreatment of each solution, we made a measurement as baseline. After the bolus of $10^{-7}$ mol/kg ET-1, measurements were made at intervals up to 7 min.

Morphometric study. In groups pretreated with Sal, BQ-123 (5 mg/kg), and BQ-788 (5 mg/kg) ($n = 4$ for each group), we studied the ET-1-induced responses by using morphometric techniques. During ET-1-induced responses (5 min after ET-1 administration), the lungs were removed intact and frozen with liquid nitrogen. By delivering a constant flow into the trachea, we maintained a constant $P_{\text{tr}}$ of 3 cmH$_2$O during freezing. Frozen lungs were fixed in Carnoy solution (60% ethyl alcohol, 30% chloroform, and 10% acetic acid) at $-70^\circ$C for 18 h. Progressively increasing concentrations of ethanol at $-20^\circ$C were then substituted for the Carnoy solution until 100% ethanol was reached. The tissue was maintained at $-20^\circ$C for 4 h, warmed to $4^\circ$C for 12 h, and then allowed to reach and remain at room temperature for 2 h. After fixation, the tissue blocks obtained from midsagittal slices of the lungs were embedded in paraffin. Blocks were cut 4-µm thick by using a microtome. Slides were stained with hematoxylin and eosin. We assessed tissue shrinkage, and subsequent measurements were corrected for shrinkage.

We assessed airway constriction by measuring the length of the epithelial basement membrane (Pbm) and the area ($A_{\text{bm}}$) it circumscribed by projecting microscopic images onto a digitizer by means of a drawing attachment fixed to the microscope. The ideal area of the lumen of the relaxed airway ($A_{\text{bm}}^*$) was then calculated as

$$A_{\text{bm}}^* = \frac{\text{Pbm}}{4\pi}$$

and the degree of constriction ($A_{\text{bm}}/A_{\text{bm}}^*$) was derived (10). The area circumscribed by the outer border of adventitia ($A_{\text{o}}$) was also measured, and the area of the airway wall (WA) was calculated by the difference between $A_{\text{o}}$ and $A_{\text{bm}}$. We normalized WA to the relaxed area to adjust for differences in airway size. To assess whether airways were cut in cross section, the maximal diameter of the airway ($D_2$) and the diameter at the widest point perpendicular to this axis ($D_1$) were measured. We analyzed airways with a ratio of $D_2/D_1 > 0.33$.

Data analysis. Comparisons of physiological and morphometric data among the experimental groups were carried out with analysis of variance (Fisher's least significant difference test). Data are expressed as means ± SE. $P$ values <0.05 were taken as significant.

RESULTS

Effects of ET-1, ET-2, ET-3, and S6c on pulmonary function. Dose-response curves for ET-1, ET-2, ET-3, and S6c are shown in Fig. 1. ET-1, ET-2, and ET-3, and sarafotoxin S6c (SX6c) in mice ($n = 5$ for each group). PBS, phosphate-buffered saline. +$P < 0.01$ vs. PBS baseline; #+$P < 0.001$ vs. ET-1, ET-2, and ET-3 groups.

Figure 2 demonstrates a representative time course of the responses to ET-1 bolus administra-
tion in mice. ET-1 provoked a sustained increase in $R_L$ and elicited a maximal response 3–5 min after bolus administration, suggesting induced bronchoconstriction without initial bronchodilation in vivo.

Figure 3 summarizes the responses to ET-1 in each experimental group. There were no significant differences in baseline $R_L$ values among each group. In the saline-pretreated group, $10^{-7}$ mol/kg ET-1 caused increases in $R_L$ from $0.415 \pm 0.035$ to $1.320 \pm 0.124$ cmH$_2$O·ml$^{-1}$·s. As shown in Fig. 3, no dose of BQ-123 blocked ET-1-induced constriction. In a marked contrast, pretreatment with >0.2 mg/kg BQ-788 significantly inhibited ET-1-induced constriction.

Morphometric results demonstrate that BQ-788 reduces the bronchial responses to ET-1 in mice in vivo. Morphometric study. Table 1 and Fig. 4 summarize the morphometric data. There were no significant differences in Pbm or $D_2/D_1$ among the Sal, BQ-123, and BQ-788 groups. These results indicate that there were no significant biases among the three groups in terms of airway selection. There were significant differences in $A_{bm}/A_{bm,*}$ between Sal and BQ-788 and between BQ-123 and BQ-788 ($P < 0.001$), whereas no significant difference in $A_{bm}/A_{bm,*}$ was observed between Sal and BQ-123 (Fig. 4). There were no significant differences in WA/$A_{bm,*}$ among the three groups.

Photomicrographs of representative airways from Sal, BQ-123, and BQ-788 groups (Fig. 5) show substantial airway narrowing in the Sal and BQ-123 groups. On the other hand, airway narrowing is minimal in the BQ-788 group.

**DISCUSSION**

The results of the present experiments show that ET-1, ET-2, and ET-3 are equipotent as bronchoconstrictor agonists and that S6c, a specific ET$_B$ agonist, is more potent than ETs. BQ-788, but not BQ-123, reduces the bronchial responses to ET-1 in mice in vivo. Morphometric results demonstrate that BQ-788 reduces ET-1-induced airway narrowing by affecting airway smooth muscle contraction but not airway wall thickening. These findings suggest that the ET$_B$ but not ET$_A$-receptor subtype may have important physiological roles in airways in mice in vivo.

**Table 1. Morphometric data during endothelin-1-induced responses**

<table>
<thead>
<tr>
<th></th>
<th>Sal (n = 4)</th>
<th>BQ-123 (n = 4)</th>
<th>BQ-788 (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of airways</td>
<td>66</td>
<td>67</td>
<td>64</td>
</tr>
<tr>
<td>No. of airways per animal</td>
<td>16.5 ± 0.6</td>
<td>16.8 ± 0.8</td>
<td>16.0 ± 0.4</td>
</tr>
<tr>
<td>Pbm, mm</td>
<td>0.891 ± 0.030</td>
<td>0.901 ± 0.038</td>
<td>0.890 ± 0.038</td>
</tr>
<tr>
<td>$D_2/D_1$</td>
<td>0.793 ± 0.013</td>
<td>0.795 ± 0.013</td>
<td>0.781 ± 0.018</td>
</tr>
<tr>
<td>$A_{bm,*}$</td>
<td>0.249 ± 0.016</td>
<td>0.247 ± 0.017</td>
<td>0.250 ± 0.014</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of mice. ET-1, endothelin-1; Sal, ET-1-challenged group pretreated with saline; BQ-123, ET-1-challenged group pretreated with 5 mg/kg BQ-123; BQ-788, ET-1-challenged group pretreated with 5 mg/kg BQ-788; Pbm, length of basement membrane; $D_2/D_1$, index of airway roundness; WA, airway wall area; $A_{bm,*}$, ideally relaxed area.

Several technical issues warrant consideration before discussion of the results. It has been demonstrated that airway resistance is lung-volume dependent even after induced constriction (14, 20, 21). Therefore, to compare airway resistance before and after constriction, it is important to be sure that lung volumes are similar under all experimental conditions. In the present experiment, we maintained PEEP at 3 cmH$_2$O for all experimental groups. To make comparisons of functional and structural measures, it is also important that lung volume is maintained at the same level during freezing as during the physiological measurements. Therefore, we delivered constant flow into the trachea during freezing, maintaining Ptr equal to 3 cmH$_2$O. Potentially, artifacts could arise during the freezing process, which was very rapid (26), much faster than the time constant of relaxation after constriction induced by intravenous administration of ET-1. Although constriction might have exaggerated any freezing artifact, no topographic or systematic distribution of atelectasis was observed among the groups.

Dose-response curves for ET-1, ET-2, and ET-3 indicate that these three ETs are equipotent as bronchoconstrictor agonists. S6c, a specific ET$_B$ agonist, induced a greater degree of bronchoconstriction and was more potent than ETs. Based on the observations that the
The ET$_B$ receptor is nonisopeptide selective (23), this rank order of potency (S6c > ET-1 = ET-2 = ET-3) suggests that the bronchoconstrictor action of ETs is mediated via the ET$_B$ receptor in mice in vivo.

In ICR mice, BQ-123 did not block ET-1-induced constriction. In contrast, pretreatment with BQ-788 significantly inhibited ET-1-induced constriction. These data suggest that ET-1-induced contraction is an ET$_B$-mediated response.
mediated response. White et al. (28) have reported that in guinea pigs ET-1-induced airway contraction is potentially mediated through the release of products of cyclooxygenase from the epithelium. The present observations in ICR mice may be explained by the possible mechanism that the ET₆ receptor may reside on the epithelium of the airways and that the ET₆-receptor antagonist BQ-788 may inhibit ET-1-induced bronchoconstriction by blocking the activation of cyclooxygenase in the epithelium.

The present observations in mice are somewhat different from the previous studies in other species. In pigs, blood vessels and bronchi are rich in the ETA receptor, and lung parenchyma is rich in the ET₆ receptor (22). In guinea pigs, both ET₆ and ET₆ receptors are involved in ET-1-induced bronchoconstriction in vivo (19), whereas differences in the relative distribution of ET-receptor subtypes exist between trachea (ETA) and bronchus (ET₆) in vitro (7). On the other hand, in rats in situ (13) and in humans in vitro (4, 7), ET₆ receptor may play an important role in ET-1-elicited bronchoconstriction, compatible with the present findings in mice in vivo. In human bronchial smooth muscle, it has been reported by Goldie et al. (4) that ~82–88% of ET-1 binding sites are ET₆ receptors.

Indexes of airway constriction (Aₐ/ₐ/ₐₐ/ₐₐ) and WA thickening (WA/ₐ/ₐ/ₐₐ) were calculated to assess airway narrowing and WA edema formation. There were significant differences in the degree of airway constriction between Sal and BQ-788 and between BQ-123 and BQ-788 groups, whereas no significant difference was observed between Sal and BQ-123 groups. There were no significant differences in the WA among the three groups. These results suggest that antagonism of ET₆ attenuates ET-1-induced airway narrowing by affecting airway smooth muscle contraction.

Recently, the pathophysiological importance of ET-1 has been reported in experiments using transgenic mice. Kurihara et al. (12) have disrupted the mouse Edn-1 locus encoding ET-1 and observed that resultant mice homozygous for ET-1 null mutation represent morphological abnormalities of the pharyngeal arch-derived craniofacial tissues and organs, indicating that ET-1 is essential to normal embryonic development. They have also reported (11) that ET-1 knockout homozygous mice display cardiovascular malformations, including aortic arch malformations and ventricular septal defect, and that the frequency and extent of these abnormalities are increased by antagonism of ETA receptor by using BQ-123. It has also been demonstrated (8) that a targeted disruption of the mouse ET₆-receptor gene results in aganglionic megacolon and pigmentary disorders resembling human Hirschsprung’s disease.

The present observations in genetically wild-type mice may provide useful information to study the roles of ET ligand-receptor systems in murine airway biology. To investigate the roles of ETs in the pathogenesis of ET-related diseases, including bronchial asthma, transgenic mice such as ET-1 knockout mice (12) could be employed as animal models in future studies. In such studies, it is essential to understand the basic roles of ET ligands and receptors in genetically wild-type mice. In addition, in both humans and mice, ET₆-receptor subtypes have crucial roles in ET-1-induced airway responses (4, 7). This resemblance between humans and mice may suggest that mice are one of the most appropriate animal models for study of the roles of ET-1 in the etiology of bronchial asthma.

In conclusion, the order of bronchoconstrictive potency was S6c > ET-1 = ET-2 = ET-3 in mice in vivo. Only BQ-788 reduced the pulmonary responses to ET-1 in ICR mice. BQ-788 reduced ET-1-induced airway narrowing, although it did not affect WA thickening. These observations suggest that the ET₆ receptor subtype may have important roles in airway physiology in ICR mice, one of the most common wild-type murine species.

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