Endothelin-induced vascular and bronchial effects in pig airways: role in acute allergic responses

Sylvin, H., E. Weitzberg, and K. Alving. Endothelin-induced vascular and bronchial effects in pig airways: role in acute allergic responses. J Appl Physiol 93: 1608–1615, 2002; 10.1152/japplphysiol.00426.2002.—The effects of endothelin (ET) agonists on airway mechanics and bronchial blood flow were studied as well as the effects of mixed ET-receptor antagonist bosentan on allergen-induced airway reactions in the pig. ET agonists [ET-1, ET-3, and the ETB receptor-selective agonist Sarafotoxin 6c (Sf6c)] were given as intravenous injections (0.4–200 pmol/kg) to eight anesthetized pigs. Bosentan (10 mg/kg iv) was then administered, and the injections were repeated. Only Sf6c caused a significant increase in airway resistance, and this response was blocked by bosentan. Sf6c and ET-1 (200 and 400 pmol/kg, respectively) were also given as aerosols to five pigs. Sf6c, but not ET-1, caused bronchoconstriction via this route. All agonists (intravenous) caused increases in bronchial vascular conductance, an effect that was blocked by an NO-synthase inhibitor (Nω-nitro-L-arginine) but unaffected by a cyclooxygenase inhibitor (diclofenac). Fourteen pigs were sensitized with ascaris suum antigen. Under anesthesia, eight pigs were pretreated with bosentan, and six pigs were controls. They were all challenged with allergen aerosol resulting in acute bronchoconstriction and elevation of ET-1 in bronchoalveolar lavage fluid. Bosentan did not affect the maximal acute airway obstruction but markedly increased baseline bronchial vascular conductance, suggesting a basal vascular tone regulated by ETs. In conclusion, ETs induce bronchoconstriction primarily via the ETB receptor in the pig. However, ETs are probably not involved in the allergen-induced acute bronchoconstriction in this model.

SEVERAL ENDOTHELIN (ET) isoforms have been described (ET-1, -2, -3) (23), of which ET-1 is the most abundant in humans because it is the only isoform produced in endothelial cells (5). ET-1 is one of the most potent vaso- and bronchoconstrictors known (1, 40). In mammals, ETs act by binding to two receptor subtypes found: the ETA and ETB receptors (34). The ETA receptor has a higher affinity for ET-1 and ET-2 than for ET-3, whereas the ETB receptor has equal affinity for the three ET ligands (26). ETA receptors are found on smooth muscle cells and mediate vasoconstriction and cell proliferation. For the ETB receptor, a further division into ETB1 and ETB2 receptors has been suggested (17). The ETB1 receptor is primarily found on endothelial cells and causes vasodilation because of formation of nitric oxide (NO) and/or prostacyclin. The ETB2 receptor is present on smooth muscle cells where it mediates vaso- and bronchoconstriction. However, there is not yet any molecular evidence for this subdivision (4, 20).

Elevated levels of ETs have been found in bronchoalveolar lavage (BAL) fluid from asthmatics, indicating a role for ET in this disease (28). The expression of ET is increased in bronchial epithelial cells and vascular endothelium in asthmatic subjects (29, 32). ET-1-induced bronchoconstriction is seen in asthmatic subjects but not in healthy controls (6). In a sheep model, specific blocking of ETA receptors using peptide antagonist BQ-123 causes a small reduction of the acute allergen-induced bronchoconstriction and a marked reduction of the late-phase obstruction (32). ETB-selective antagonists BQ-788 and RES-710-1 block the acute asthmatic response in sensitized guinea pigs, whereas BQ-123 suppresses the late response in this model (39). Thus there are several implications for ETs being important mediators in asthma, and it would be of interest to also study a potent nonpeptide, nonselective ET antagonist in allergic responses.

The bronchial circulation may be important in acute airway responses (8). For instance, bronchial blood flow is important for the washout of bronchoconstrictors and proinflammatory mediators from the airway wall (26, 21). Systemic administration of low doses of ET-1 is known to cause an increase in bronchial blood flow in the pig (27), but the mechanism for this is unknown. Because there is an increased expression of ETs in the vascular endothelium of asthmatic patients (36), it would be of interest to study what effect an ET-receptor antagonist has on bronchial blood flow in the sensitized pig. The pig has previously been shown to be suitable for integrative studies on vascular and bronchial airway changes in allergic responses (for examples, Refs. 3 and 37).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
The aim of this study was thus to characterize the response to ETs in pig airways in vivo and to evaluate the role of ETs in the acute allergic response in sensitized pigs by administering bosentan (Ro 47-0203). Bosentan is a nonpeptide competitive ET-receptor antagonist that acts on both ETA and ETB receptors.

**MATERIALS AND METHODS**

The regional Ethics Committee for animal research approved the experiments in this study, and the experiments were performed according to regulations issued by the Swedish National Board for Laboratory Animals.

**Animals**

In the first part of the study (ET-induced effects), 25 barrier-bred pigs (Seropig; Persbo Gård, Ransta, Sweden) were used. In the second part of the study (allergen challenge), 21 barrier-bred pigs were sensitized to ascaris suum antigen (Allergon, Angelholm, Sweden) in a suspension of Al(OH)3 injected two times subcutaneously at a 4-wk interval, starting at 3 wk of age.

**Surgical Preparation**

All pigs were premedicated with ketamine hydrochloride (20 mg/kg im, Parke-Davis, Barcelona, Spain) and atropine (0.5 mg, im), and subsequently anesthesia was induced with pentobarbital sodium (16 mg/kg iv, Apoteksbolaget, Umeå, Sweden). Interdigital skin was pinched to assess anesthesia. Pancuronium bromide was given (0.2 mg/kg iv, Pavulon; Astra, Sweden) and subsequently anesthesia was induced with pentobarbitone sodium (16 mg/kg iv, Apoteksbolaget, Umeå, Sweden). Anesthesia was then changed to infusion of pentobarbitone sodium (10 mg/kg iv over 5 min). After diclofenac and L-NNA (Sigma-Aldrich, Steinheim, Germany; in saline, 3 mg/kg iv over 5 min) and five other pigs were given the NO synthase inhibitor Nω-nitro-L-arginine (L-NNA; Sigma-Aldrich; in saline, 50 mg/kg iv over 5 min). After diclofenac and L-NNA administration, ET-1 and ET-3 injections were repeated.

**Allergen challenge.** All 21 sensitized pigs were skin tested with a 50-μl subcutaneous injection of ascaris suum extract in a 10-fold dilution series (in saline), to estimate the degree of sensitivity to the allergen. There was no significant difference in sensitivity between the two groups.

Bosentan (10 mg/kg) was given to eight pigs intravenously during 10 min at time (t) = −40. These pigs and six controls were challenged with antigen at t = 0. Ascaris suum antigen was diluted in saline to a volume of 2 ml and nebulized over 5–10 min to the airways by using an ultrasonic nebulizer until the tracheal pressure was increased by ~5 cmH2O. Control pigs received 1.7 ± 0.1 ml, and the bosentan-treated pigs received 1.2 ± 0.2 ml (no significant difference between the groups).

Seven additional pigs were challenged with ovalbumin (OVA; 20 mg in 2 ml of saline solution) instead of ascaris suum. A BAL was performed in all pigs at +45 min by using a fiber-optic bronchoscope (Olympus). Twenty milliliters of saline (37°C) was infused into the right middle lobe and then aspirated. All pigs were monitored for 2 h after challenge. Pao2 pressure data was regularly recorded.

**Sample Analyses**

BAL fluid samples were centrifuged at 170 g for 20 min at 4°C. ET-1 concentrations were measured in the supernatants by using a radioimmunoassay as described by Hemsen (18). Total protein content was measured by using the Pierce BCA protein assay (Pierce Chemical, Rockford, IL).
Calculations

Data are presented as means ± SE. BVC is presented as bronchial blood flow divided by MAP. For changes in ET-induced vasodilatory effects before and after diclofenac and l-NNA, total blood flow increase (integrated area under the curve) was calculated instead of changes in BVC. This is to better compare the two situations because basal BVC was markedly reduced by l-NNA. Statistical comparisons were performed by using the Mann-Whitney U-test for detecting significant differences between the two groups at different time points. Wilcoxon matched-pairs test was used to detect significant changes within the same group by comparing data before and after treatment. For ET levels in BAL fluid that were below the detection limit of the assay (2.0 pM), the levels were set to 2.0 pM for statistical analyses. Data were considered to be significant if P < 0.05. For the analysis of changes from the lowest dose in the injections studies or from baseline in the allergy studies, Friedman test and Dunn’s multiple-comparison test were used. Analyses were performed with Prism software (Graphpad, San Diego, CA) on a Macintosh computer.

RESULTS

ET-induced Effects

Raw was significantly increased by injection of the highest dose of Sf6c (200 pmol/kg; Fig. 1). The bronchoconstriction induced by this dose lasted for ~7–10 min. ET-1 and ET-3 did not affect Raw significantly within this dose-range, although some increases were seen in both cases. The bronchoconstrictor effect of the 200 pmol/kg dose of Sf6c was significantly blocked by bosentan. When given as aerosols, Sf6c (200 and 400 pmol/kg) induced significant bronchoconstriction, whereas ET-1 did not cause bronchoconstriction at these doses (Fig. 2A). Aerosolized ET-1 and Sf6c (200 and 400 pmol/kg) caused a reduction of BVC, but these effects were not significant compared with baseline (Fig. 2B).

When given intravenously, on the other hand, all agonists caused concentration-dependent increases in BVC, with threshold doses of 200 pmol/kg (ET-1) and
40 pmol/kg (ET-3, Sf6c) (Fig. 3). ET-3 caused the most profound increase in BVC (~181% from baseline) within the given dose range. The maximal effects were reached after 3–5 min, and the responses lasted for 10–15 min. Bosentan blocked all these responses significantly. ET-1 infusion caused a significant increase in MAP at the highest dose (from 120 ± 7 to 142 ± 7 mmHg for 200 pmol/kg) before bosentan treatment. ET-3 and Sf6c did not significantly affect MAP (not shown). The increase in MAP induced by the highest agonist dose was in some cases followed by a decrease in HR (not shown).

Diclofenac had no effect on the total increase in bronchial blood flow after ET-1 and ET-3 injections (40 pmol/kg iv; Fig. 4). L-NNA, on the other hand, significantly reduced the blood flow increase in response to ET-1 and ET-3 injections. Diclofenac did not affect baseline BVC or MAP and did not alter the nonsignificant effect of ET injections on MAP. In contrast, l-NNA markedly reduced BVC (from 0.11 ± 0.02 to 0.02 ± 0.01 ml · min⁻¹ · mmHg⁻¹), increased baseline MAP (from 118 ± 3 to 147 ± 8 mmHg), and also unveiled an acute ET-induced effect on MAP (from nonsignificant to +47 ± 5 and +26 ± 5 mmHg for ET-1 and ET-3, respectively).

**Allergen Challenge**

The bosentan infusion did not affect the baseline values of Raw or Cdyn (Fig. 5, A and B). Both bosentan-pretreated and nonpretreated control pigs responded to allergen challenge with a significant increase in Raw by ~140% and a significant decrease in Cdyn by 30% 15 min after ascaris suum challenge. No significant effect of bosentan on acute airway obstruction or the decrease in PaO₂ was observed (Fig. 5C).

MAP was significantly lowered after bosentan infusion, and basal BVC was increased in this group (Fig. 6). The corresponding increase in bronchial blood flow after bosentan infusion was from 9.0 ± 1.4 to 11.5 ± 2.5 ml/min. In control pigs, BVC remained almost unchanged during the same period (Fig. 6B). However, the allergen-induced acute increase in BVC was similar in both groups, although a marked elevation of the basal level was seen throughout the observation period in the bosentan group (Fig. 6B).

There was no significant difference between the groups in total protein content in the BAL fluid sampled at 45 min (322 ± 131 and 379 ± 152 µg/ml for controls and bosentan-treated pigs, respectively). ET-1 concentration in the BAL fluid was 7.4 ± 1.0 pM in the control group and 15.9 ± 4.5 pM in the bosentan-treated group (nonsignificant difference between the groups). Both ascaris suum-challenged groups had significantly higher levels compared with BAL fluid from the OVA-challenged animals (P < 0.05). In OVA-challenged animals, ET-1 concentrations were below the detection limit (<2.0 pM).
DISCUSSION

The results of the present study show that ETs cause bronchoconstriction in pigs in vivo, primarily via ETB-receptor activation. However, ETs do not seem to be important in acute allergen-induced bronchoconstriction, although a role for ET in the regulation of the bronchial circulation is suggested.

In this study, the endogenous ET agonists ET-1 and ET-3, and Sf6c were administered intravenously and as aerosols. As yet, no selective ETA agonist is available. ET-1 is the endogenous agonist with the highest affinity for the ETB receptor (34). ET-3 is the endogenous agonist with highest preference for ETB, and Sf6c, derived from snake venom, is a selective ETB receptor agonist (45). Bosentan is a mixed, nonpeptide ET receptor antagonist specific for ET receptors, and it acts on all ET receptor subtypes (ET\textsubscript{A}, ET\textsubscript{B\textsubscript{1}} and ET\textsubscript{B\textsubscript{2}}) (7). Intravenous injection of Sf6c (200 pmol/kg) caused a significant increase in Raw, and pretreatment with bosentan blocked this effect. Aerosol challenge with Sf6c also induced marked bronchoconstriction. The Sf6c-induced effect on Raw when given intravenously is about eight times higher than after intravenous administration of an equimolar dose of histamine (unpublished data). In a sheep model, the ET-1-induced bronchoconstriction is ETA-receptor mediated. ET\textsubscript{A} is the predominating ET receptor in ovine airway smooth muscle (13), whereas in mice, guinea pig, rabbit, and human bronchi, the ETB receptor dominates (12, 13, 18, 25, 27). In the pig, receptor-binding studies show that ETB predominates in the trachea (ET\textsubscript{A}/ETB, 30:70), whereas in the bronchus, the relation is the opposite (ET\textsubscript{A}/ETB, 70:30) (12). In vitro studies on porcine bronchi suggest that both ETA- and ETB-receptor mediated bronchoconstrictor effects are present, whereas our in vivo data suggest primarily ETB-mediated bronchoconstriction in the pig.

The fact that ET is bronchoconstrictive in asthmatic subjects but not in healthy controls might partly be

Fig. 5. Effect of bosentan on Raw (A), dynamic compliance (C\textsubscript{dyn}; B), and arterial PO\textsubscript{2} (P\textsubscript{O2}; C) after allergen challenge in pigs sensitized to ascaris suum. Bosentan (10 mg/kg iv) was administered to 8 pigs (●) over 10 min at time (t) = −40. Control animals (n = 6; ○) received vehicle. At t = 0, all pigs were challenged with ascaris aerosol. Values are means ± SE. *Significantly different from control (P < 0.05, Mann-Whitney U-test). #Significantly different from baseline (P < 0.05, Friedman and Dunn’s multiple-comparison test).

Fig. 6. Effect of bosentan on mean arterial blood pressure (MAP; A) and BVC (B) after allergen challenge in pigs sensitized to ascaris suum. Bosentan (10 mg/kg iv) was administered to 8 pigs (●) over 10 min at t = −40. Control animals (n = 6; ○) received vehicle. At t = 0, all pigs were challenged with ascaris aerosol. Values are means ± SE. *Significantly different from control (P < 0.05, Mann-Whitney U-test). #Significantly different from baseline (P < 0.05, Friedman and Dunn’s multiple-comparison test).
explained by the fact that an intact epithelium down-regulates the response to ET-1 (44). Neutral endopeptidase in the epithelial cells is responsible for the breakdown of released ET (40). In the nonsensitized pig, no response to ET-1 could be detected when nebulized to the airways, similar to healthy human controls (6). Interestingly, in the present study, Sf6c, but not ET-1, induced bronchoconstriction in nonsensitized pigs. This result can be explained by the fact that sarafotoxins seem to be more resistant to degradation by neutral endopeptidase than by ETs (35). Because Sf6c is selective for the ETB receptor, whereas ET-1 and ET-3 also bind to the ETA receptor, this may be an additional explanation why ET-1 and ET-3 had weaker effects on the ETB receptors in the airways than Sf6c, when given in equimolar doses.

The ET-1-induced increase in MAP most likely depends on ET_A/ET_B2 receptor-mediated vasoconstriction in a majority of peripheral vascular beds (19). In contrast, BVC was markedly increased in response to all three agonists given intravenously, indicating a local vasodilatation of the bronchial circulation. Bolus intravenous doses of agonists, as given in this study, might primarily affect ET_B1 receptors found on the vascular endothelium because these are most easily accessed via this route. Bosentan could block this effect. The vasodilatory effect of ETs via the ET_B1 receptor is thought to be mediated by NO and/or prostacyclin formation. In the pig, our results show that this vasodilation is mediated by NO because l-NAME, a nonselective NO synthase inhibitor, could abolish the increase in bronchial blood flow in response to ET-1 and ET-3 despite the fact that a marked increase in MAP was seen in response to ET injection after l-NAME pretreatment. The latter finding indicates that ET_B-mediated NO release normally counteracts vasoconstrictor effects of ETs at the systemic level. Diclofenac did not affect the ET-induced increase in BVC, suggesting no role of arachidonic acid metabolites in the ET_B1 receptor-mediated bronchial vasodilation. Thus the bronchial circulation seems to be under marked endothelial control and mediated by NO release. This is supported by previous studies, in which the pig bronchial circulation was shown to be highly sensitive to both NO synthase inhibitors and exogenous NO (2).

Aerosol challenge with ET-1 and Sf6c led to a decrease in BVC, opposite to the effect of intravenous administration. This is most likely an ET_A- and/or ET_B2-mediated effect directly on vascular smooth muscle. When ET agonists are administered locally (in aerosol form in the present study), the vasoconstrictor receptors on the smooth muscle cells are more easily reached than the vasodilatory ET_B1 receptors on the vascular endothelium. ETs also seem to control basal vascular tone of the bronchial circulation in the pig. These basal ET-mediated vascular effects are probably regulated by abuminally released ET-1 acting on the ETA receptor and/or on the ET_B2 receptor (41). The decrease in MAP seen in the bosentan-treated pigs is a result of the blockage of these receptors.

Plasma levels of ET-1 in humans are low (~3 pg/ml) (4). In the pigs in this experimental setup, plasma levels are ~17–30 pg/ml (43). In humans during surgery, the plasma levels can be increased up to 15 times (4). The high plasma ET concentrations under these conditions might lead to a more pronounced basal vasoconstrictor tone.

In the present study, ET-1 concentrations in BAL fluid were significantly elevated in ascaris-challenged pigs compared with pigs challenged with OVA. Similarly, ET-1 levels in BAL fluid are significantly elevated in humans during asthma attacks (38). In bosentan-treated pigs, ET-1 BAL fluid levels seemed to be further elevated compared with nonbosentan-treated animals. This might be due to displacement of ET-1 from ETA/ETB receptors. A parallel observation has been described in humans, where bosentan increases the plasma concentration of circulating ET-1 (42). This increase in ET-1 concentration can be described as a decreased clearance of ETs, which normally is mediated by the ETB receptor (20). The increase in circulating ETs does not have any apparent physiological consequences, and bosentan has little or no effect on basal hemodynamics in normal subjects (7), whereas in the anesthetized pig with raised plasma ET levels, bosentan reduces MAP.

Bosentan did not significantly reduce the acute airway obstruction to allergen challenge. Thus ETs are probably not involved in allergen-induced acute bronchoconstriction in the pig. However, ET-1 has been suggested to potentiate cholinergic nerve-mediated bronchial contraction (9). In the present study, cholinergic reflexes might be suppressed by the use of barbiturates or benzodiazepines (15, 22). ET-1-induced potentiation has only been shown in vitro, however, and the effects were minor (9).

There was, however, a tendency to faster recovery in Raw in the pigs of the control group. This might be because the higher bronchial blood flow in this group leads to a more rapid clearance of bronchoconstrictors and other mediators, or possibly because of a late (1–2 h) formation of ETs. In sheep, blocking the ETA receptor (BQ-123) had only a minor effect on the acute antigen-induced bronchoconstriction, but a significant reduction of the late-phase reaction was seen (32). BQ-123 also blocked antigen-induced airway hyperresponsiveness in sheep. Elevated levels of ET-1 have been found in BAL fluid and bronchial tissue in rats during acute airway inflammation. Bosentan treatment inhibited the cellular inflammatory response in these animals (11). Bosentan treatment also resulted in a decrease in proinflammatory cytokines in the rat model (10). Thus several animal studies implicate a role for ETs in late airway responses, and we see some indications of this also in our study.

In conclusion, the ET system seems to play a role in the regulation of basal vascular tone in the pig bronchial circulation. In response to exogenous ETs, bronchial circulation is either dilated or constricted, depending on the route of administration. Furthermore, ETs cause bronchoconstriction in the pig, an effect
mediated primarily via the ET$_B$ receptor. However, ETs do not seem to contribute to the allergen-induced acute bronchoconstriction in the sensitized pig.

We thank Margareta Stendotter for expert technical assistance.

This study was supported by the Swedish Medical Research Council (no. 10354), the Swedish Heart-Lung Foundation, the Swedish Foundation for Health Care Science and Allergy Research, the AGA cil (no. 10354), the Swedish Heart-Lung Foundation, the Swedish Asthma and Allergy Association’s Research Foundation, and Karolinska Institutet. Boesentan was a kind gift from Dr. Martine Clozel (Actelion).

REFERENCES


