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

H. SYLVIN, E. WEITZBERG, AND K. ALVING

Department of Physiology and Pharmacology, Karolinska Institutet, SE-171 77; and Department of Anaesthesiology and Intensive Care, Karolinska Hospital, SE-171 76 Stockholm, Sweden

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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 ET

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 was 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).

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Address for reprint requests and other correspondence: K. Alving, Dept. of Physiology and Pharmacology, Karolinska Institutet, SE-171 77 Stockholm, Sweden (E-mail: kjell.alving@fyfa.ki.se).
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 (Allergen, 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 pentobarbitone 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; Organon, Boxtel, The Netherlands) to achieve muscle relaxation. After tracheostomy, the pigs were intubated and mechanically ventilated with a mixture of air and oxygen by using an automatic blood-gas analyzer (ILS 1610, Instrumentation Laboratory, Lexington, MA), and ventilator settings were adjusted to get normal blood gas and pH values.

A femoral vein was cannulated for fluid and drug infusions. Anesthesia was then changed to infusion of pentobarbitone sodium (10 mg·kg−1·h−1, allergen challenge) or infusion of midazolam (0.16 mg·kg−1·h−1, Dormicum; Hoffmann-La Roche AG, Basel, Switzerland) and fentanyl (12 μg·kg−1·h−1, Antigen Pharmaceuticals, Roscrea, Ireland) (ET-induced effects). Pancuronium bromide (0.6 mg·kg−1·h−1) and Ringer solution with 0.5% glucose at an approximate rate of 25 ml·kg−1·h−1 were given in all experiments. Femoral arteries were cannulated for blood sampling and measurements of mean arterial pressure (MAP) and heart rate (HR). Anesthesia was continuously checked by MAP and HR measurements. Tracheal airflow was measured with a pediatric pneumotachygraph (3500 series, Hans Rudolph, Kansas City, KS), connected to a pressure transducer (Kent Scientific, Litchfield, CT). Tracheal airflow and pressure signals were sent to a computer system (AP 200, ConMeTech, Uppsala, Sweden) for on-line calculations of airway resistance (Raw) and dynamic lung compliance (Cdyn). Raw was calculated with the following formula: peak pressure – pause pressure)/end inspiratory flow. The resistance of the tracheal tube was not subtracted for the calculation of Raw. Cdyn calculations were made as follows: expiratory tidal volume/(pause pressure – end expiratory pressure). After a right-side thoracotomy, an ultrasonic flow probe (Transonic System, Ithaca, NY) was placed around the bronchial artery for measurements of bronchial blood flow. Reliable blood flow measurements were achieved in 34 of 39 pigs. MAP, HR, Cdyn, Raw, and bronchial blood flow measurements were recorded on a Grass polygraph throughout the experiments (Grass Instrument, Quincy, MA). At the end of all experiments, the pigs were killed with an intravenous overdose of pentobarbital sodium.

Experimental Procedures

ET-induced effects. Three ET-receptor agonists were administered intravenously to eight pigs through the femoral vein as bolus doses in the following order: ET-3, sarafotoxin 6c (Sf6c), and ET-1 (American Peptide, Sunnyvale, CA). Sf6c is a snake venom that is very similar to ETs and acts selectively on ETA receptors (45). The doses administered were 0.4, 4, 40, and 200 pmol/kg in 1 ml of saline. There was at least a 15-min interval between injections of the same agonist. When agonist was changed, a longer interval was allowed. The nonpeptide ETs/ETB-receptor antagonist bosentan (Actelion; 10 mg/kg) in 20 ml of distilled water was subsequently infused intravenously during 10 min. Injections of ET agonists were then repeated by using the same doses. ET-1 and Sf6c (200 and 400 pmol/kg, respectively, in 1 ml of saline) were also given to the airways of seven additional pigs, starting by the ET-1 doses. Aerosols were generated by using an ultrasonic nebulizer (NB 108, Engstrom Medical, Stockholm, Sweden).

To establish the mechanism behind the ET-induced increase in bronchial vascular conductance (BVC), 10 pigs were administered ET-1 and ET-3 (40 pmol/kg iv). Five of them were given the prostaglandin synthesis inhibitor diclofenac (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.

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 µM in the control group and 15.9 ± 4.5 µM 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 µM).
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 ETA 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 (ETA, ETB1, and ETB2) (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. ETA 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 (ETA/ETB, 30:70), whereas in the bronchus, the relation is the opposite (ETA/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 (Cdyn; B), and arterial Po2 (Pao2; C) after allergen challenge in pigs sensitized to ascariis 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 ascariis 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 ascariis 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 ascariis 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, S66c, 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 S66c 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 S66c, when given in equimolar doses.

The ET-1-induced increase in MAP most likely depends on ETA/ETB2 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 vasodilation of the bronchial circulation. Bolus intravenous doses of agonists, as given in this study, might primarily affect ETB2 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 ETB1 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-NNA, a non-selective 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-NNA pretreatment. The latter finding indicates that ETB1-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 ETB1 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 S66c led to a decrease in BVC, opposite to the effect of intravenous administration. This is most likely an ETA- and/or ETB2-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 ETB1 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 ETB2 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 ETB2 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 constriction (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

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mediated primarily via the ET$_B$ receptor. However, ETs do not seem to contribute to the allergen-induced acute bronchoconstriction in the sensitized pig.

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