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Analysis of ET-A and ET-B receptors using an isolated perfused rat lung preparation

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Abstract

Aims and Methods: The pulmonary and vascular effects of endothelin-1 receptor activation were studied in isolated perfused and ventilated lung preparations from rat. The responses to endothelin-1 (ET-1) and the endothelin B (ET_B) receptor agonist sarafotoxin 6c (S6c) were characterized using the endothelin A (ET_A)-receptor antagonist FR 139317, the ET_B-receptor antagonist BQ 788 and the combined ET_A/ET_B-receptor antagonist Bosentan. The respiratory parameter airway conductance (G_{aw}) and the vascular parameter perfusion flow were analysed simultaneously.

Results: Concentration–response curves for ET-1 administered intra-arterially revealed that its most potent effect was on the vascular side while S6c had a more potent effect on airway conductance. ET-1, given as a bolus dose intra-arterially (100 μL of 0.2 nM), induced a strong- and long-lasting contraction of the vasculature while only a less pronounced contraction was seen in the airways. Neither of the antagonists had a significant effect *per se* on G_{aw} or perfusion flow. FR 139317 reduced the effect of ET-1 on perfusion flow by about 50%, while airway conductance was augmented. BQ 788 enhanced the decrease in perfusion flow by ET-1 while G_{aw} was not influenced. The combined ET_A/ET_B antagonist Bosentan powerfully prevented the ET-1-induced decrease in G_{aw} but did not alter its reduction in perfusion flow.

Conclusions: The potent effect of ET-1 on the vascular side of the lung is mediated mainly through ET_A receptors, whereas both ET_A and ET_B receptors are involved in G_{aw} in the rat lung.

Keywords Bosentan, BQ 788, endothelin-1, ET_A receptor, ET_B receptor, FR 139317, rat bronchus.

Endothelin-1 (ET-1) is a potent vasoconstrictor, one of the three endothelin isoforms ET-1, ET-2 and ET-3 (Yanagisawa *et al.* 1988, Masaki *et al.* 1992). Their effects are mediated through two types of receptors: ET_A and ET_B (Arai *et al.* 1990, Sakurai *et al.* 1990). ET-1 has the same affinity for ET_A and ET_B receptors while Sarafotoxin 6c (S6c) preferentially activates ET_B receptors (Masaki *et al.* 1992). The distribution of the receptor subtypes varies between species, between vascular regions and depends on size of vessels within the same species (Cardell *et al.* 1990, Adner *et al.* 1998). ET-1 is synthesized, stored, released and metabolized in the lungs of many species including rat (Rozengurt *et al.* 1990) and man (Mattoli *et al.* 1990), suggesting a role both in normal physiology and in pathophysiological processes. Thus, a number of studies in asthma have reported that the levels of ET-1 are increased both within the bronchial tree and in the plasma (Vittori *et al.* 1992, Redington *et al.* 1995). ET_A and ET_B receptors have been identified in lung tissue of

man and animals (Cardell *et al.* 1990, Adner *et al.* 1996). In rat airways there are equal proportions of contractile ET_A and ET_B receptors (Henry 1993). The other component of the lung, the vascular bed, shows a complex response to ET-1; in pulmonary arteries the constriction is mediated through ET_A receptors and balanced by the release of endothelium-derived nitric oxide generating a relaxation via ET_B receptors. However, ET_B receptors mediate constriction in the intrapulmonary arteries (MacLean *et al.* 1994). Thus, the localization and response to ET-receptor activation in the pulmonary circulation is unclear.

The aim of the present study was to develop an *ex vivo* perfused lung set-up and to investigate the functional role of ET_A and ET_B receptors by studying the responses of ET-1 and S6c alone and in combination with specific antagonists in parallel on vascular perfusion and on respiratory parameters (Shennib *et al.* 1998). The ET_A antagonist FR 139317 (Cardell *et al.* 1993), the ET_B antagonist BQ 788 (Ishikawa *et al.* 1994) and the combined ET_A and ET_B antagonist Bosentan (Clozel *et al.* 1994) were used alone or in combination to allow a more precise pharmacological characterization.

Materials and methods

Isolated lung preparation

Male Sprague–Dawley rats, weighing 200–250 g (Möllegaard, Ejby, Denmark), were used for the lung preparation. The study was approved by the Animal Ethics Committee, Lund University, Sweden. After arrival, the rats were acclimatized for 1 week under standardized temperature (21–22 °C), humidity (50–60%) and light conditions (12 : 12 light–dark) in the animal department (AstraZeneca R&D, Lund, Sweden) until used. Five animals were kept in each cage (Macrolon type IV) with litter (B&K Universal, Sollentuna, Sweden) with tap water and free access to pelleted food (lactamin R70; Lactamin, Vadstena, Sweden).

A modified isolated and buffer perfused rat lung model (IPL) with negative pressure ventilation was used for the study (Fig. 1). Experiments were randomized, one group for each treatment with ET-1/S6c with or without either antagonist. Animals were anaesthetized by an intraperitoneal injection of a mixture of Hypnorm® (fluanisone 8.25 mg kg^{-1}, fentanyl citrate 0.26 mg kg^{-1}; Janssen Animal Health, Buckinghamshire, UK), Dormicum® (midazolam 4.13 mg kg^{-1}; Roche AB, Stockholm, Sweden) and water in volume ratio 1 : 1 : 2. Heparin® (500 IU; Lövens, Malmö, Sweden) was injected into the jugular vein to prevent thrombosis. An airway cannula (PE-240; Becton Dickinson, Parsippany, NJ, USA) was inserted into the trachea and connected to a small animal ventilator (TG-Instrument AB, Mölndal, Sweden). During the surgical procedure the lungs were ventilated with room air at a frequency of 80 breaths min^{-1}. The animals were killed, the chest opened and the pulmonary artery and vein cannulated (PE-240). The lungs were carefully perfused with a modified Krebs–Ringer buffer solution (37 °C), dissected-free together with the heart and suspended by the trachea in a humidified jacketed artificial 'thoracic' glass chamber maintained at 37 °C. Ventilation was carried out using alternating negative pressure inside the chamber (−0.1 to −1.2 kPa, 80 breaths min^{-1}), relating the ambient atmosphere, with the aid of a Rodent Ventilator type 7025 (Ugo Basile Biological Research Apparatus, Varese, Italy). The lungs were perfused at a flow rate of about 10–15 mL min^{-1} using the hydrostatic force and single-pass perfusion. The pH of the buffer solution was maintained at 7.35–7.45 by adjustment with CO_2. The tracheal airflow was measured with a pneumotachygraph type 8420 (Hans Rudolph, Kansas City, KS, USA) and a pressure transducer (AstraZeneca R&D). A second pressure transducer was used to measure the alternating negative pressure in the artificial 'thoracic' chamber for measurement of the transpulmonary pressure. Perfusion flow rate was measured with a T106 small animal flow meter with in-line flow probe 2N648 (Transonic Systems, Ithaca, NY, USA). The signals from the pressure transducers and the perfusion flow meter were amplified, transformed and recorded in a computer. Data on lung mechanics (tidal volume, dynamic compliance, airway conductance) were calculated in real time by

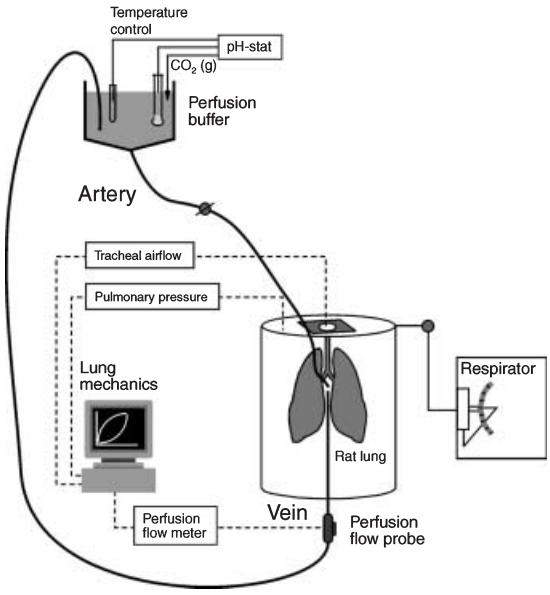

Figure 1 Schematic picture of the method used to study the perfused and ventilated lung preparation from the rat.

using data from the whole breath cycle, monitored and stored using an in-house designed PC program.

Study design

After isolation, the lung was allowed to stabilize for at least 15 min during single-pass perfusion with buffer before administration of drugs. During this stabilization period, data was collected every 5 min from 10 successive breath cycles. The system was thereafter switched to a recirculating system (50 mL perfusion buffer).

In the first study, the agonists ET-1 and S6c were examined in a dose–response manner in the concentration ranges 0.01–1 and 0.01–0.3 nmol, respectively, added to the vascular perfusion buffer.

In the second study, the antagonists (BQ 788, FR 139317 or Bosentan) were added to the vascular perfusion buffer 20 min before either of the agonists ET^{-1} (0.2 nmol) or S6c (0.1 nmol) were administered as single doses. Thereafter, the lungs were perfused for 2 h and the vascular parameters were measured every 15 min.

In the third study, the antagonists were examined during a prolonged exposure time in order to evaluate a possible tonic influence of ET on the pulmonary vasculature or airways. The antagonistic concentrations were selected on previous experiments (Cardell *et al.* 1993, Adner *et al.* 1998, Szok *et al.* 2001).

Solutions and drugs

The perfusion medium for the lung experiments was a modified Krebs–Ringer buffer with a pH of 7.4, saturated with 95% O_2 and 5% CO_2 and included NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 24.9 mM, KH_2PO_4 1.2 mM, HEPES 10 mM, D-glucose 11 mM and 4.5% w/v bovine serum albumin (BSA; Beringwerke, Marburg, Germany). All chemicals above were of analytical grade (Sigma Aldrich Sweden AB, Stockholm, Sweden).

The following drugs were studied: ET-1, S6c, BQ 788 (Auspep, Parkville, Australia), FR 139317 (Fujisawa Pharmaceuticals, Osaka, Japan), Bosentan (La Roche). All agents were dissolved and further diluted in saline containing 1% BSA to avoid adhesion of peptides to vials. The peptides were used in the experiments within 60 min to avoid any possible degradation. Analytical-grade chemicals and twice-distilled water were used for preparing all solutions.

Statistics

Results are expressed as mean ± SEM. Significance analysis was calculated with Student's *t*-test. Statistical significance was assumed when $P < 0.05$.

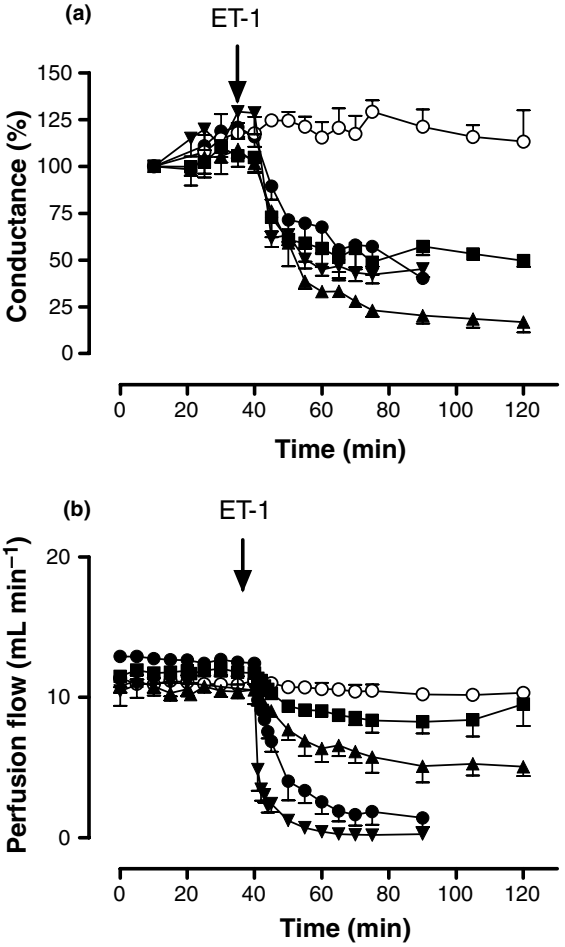

Figure 2 Control (○) with no substance in the perfusate. ET-1 (●) was given at the arrow as a bolus dose (100 μL of 0.2 nmol) intraarterially. Airway conductance (a) and perfusion flow (b) were followed over time. In separate experiments either of the antagonists FR 139317 (▲), BQ 788 (▼) or Bosentan (■) was given 20 min before ET-1 administration. Data are given as mean ± SEM, $n = 3$.

Results

Agonists

Perfusion with the solvent for up to 2 h did not significantly alter either airway conductance or the vascular perfusion flow (Fig. 2a, b). ET-1, given as a bolus dose (0.2 nmol) into a pulmonary artery, caused a very strong and persistent drop in the perfusion flow, followed by a smaller drop in the conductance in the airways (G_{aw}) (Fig. 2a, b). Similarly, infusion of ET-1 in increasing doses into the pulmonary artery caused a more potent drop in the perfusion flow than in G_{aw} (Fig. 3).

The selective ET_B agonist S6c, given in increasing doses into the pulmonary artery, did not diminish the

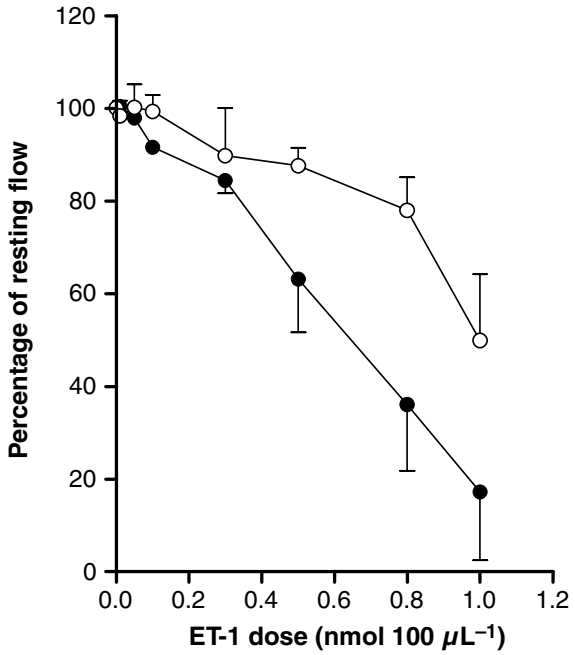

Figure 3 Comparison of increasing doses of ET-1 on the conductance (○) and perfusion flow (●) expressed in per cent of resting values. Data are given as mean ± SEM, $n = 3$.

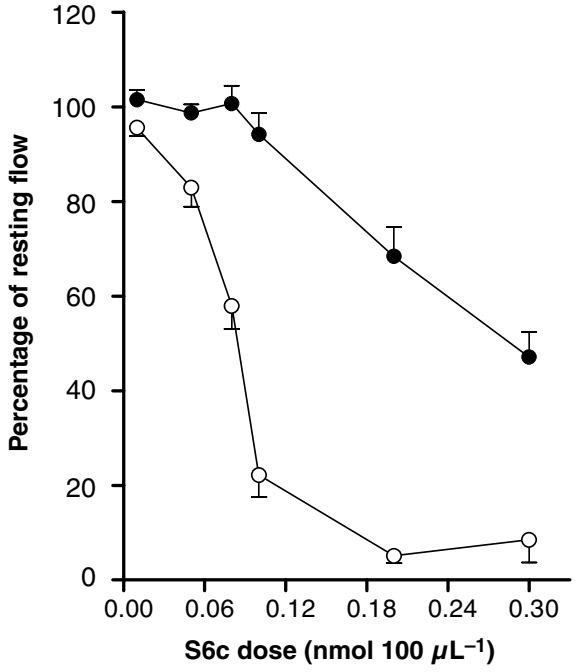

Figure 4 Comparison of increasing doses of S6c responses following simultaneous measurements of conductance (○) and perfusion flow (●) expressed in per cent of resting value. Data are given as mean ± SEM, $n = 3$.

perfusion flow until the higher doses were given. S6c resulted in a powerful drop in G_{aw}, which was more potent than when ET-1 was administered (Fig. 4).

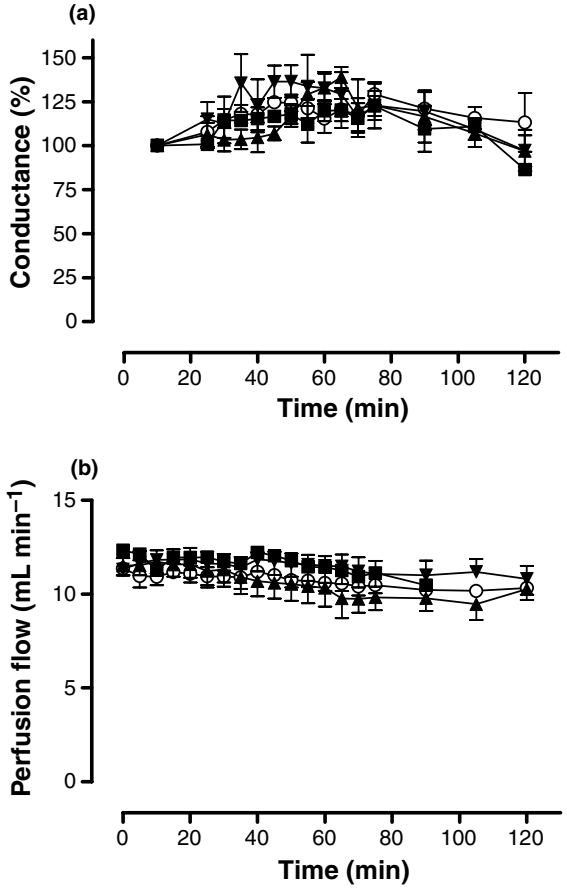

Figure 5 Effect of antagonists used on conductance in the airways (a) and perfusion flow in the vascular system (b), they were added in separate experiments: FR 139317 (▲, $n = 5$), BQ 788 (▼, $n = 4$), Bosentan (■, $n = 2$), control (○). Data are given as mean ± SEM.

Antagonists

None of the antagonists alone had any effect on the vascular tone or on G_{aw} in the doses studied (Fig. 5a, b).

The ET_A-receptor antagonist FR 139317 diminished the ET-1-induced fall in perfusion flow ($P < 0.05$), however, the fall in G_{aw} was augmented (Fig. 2a, b). On the other hand, the selective ET_B-antagonist BQ 788 augmented the fall in perfusion caused by ET-1 ($P < 0.05$) while the G_{aw} was not influenced. Bosentan, the combined ET_A- and ET_B-receptor antagonist, almost totally blocked the fall in perfusion flow induced by ET-1 ($P < 0.05$). The effect was more pronounced than the response seen following FR139317 administration, while the G_{aw} was only slightly affected (Fig. 2a, b).

Discussion

Due to high concentration of predominantly ET_A receptors on the smooth muscle cells in the pulmonary

vascular system (Bonvallet *et al.* 1993), ET-1 has a strong effect on the vascular bed. Consequently, ET-1 administrated into the pulmonary artery resulted in strong, almost irreversible, binding to the receptors on the smooth muscles in the vascular wall (Westcott *et al.* 1990), which induced a persistent contraction, i.e. a profound and long-lasting decrease in the lung perfusion flow. An essential requirement for the effect of a substance is an uncompromised access to the receptor. When ET-1 is administered on the vascular side, the binding primarily takes place in the ET receptors on the endothelial and smooth muscle cells within the vascular walls. However, there is a rapid reduction in the circulation, which possibly compromises the further access of ET-1 to the pulmonary side. In this situation, the putatively reduced amounts of ET-1 may pass less well to the receptors in the airway system, explaining the weaker bronchial contraction than what would be expected. Furthermore, attenuation of ET-1 binding and vasoconstriction by the ET_A antagonist FR 139317 should improve the possibility for ET-1 binding to the ET_B receptors in the airways resulting in a stronger decrease in airway conduction.

In our study, ET-1 given either as a bolus dose or in a dose–response manner induced a fast and persistent drop in perfusion flow. The decrease in flow was persistent during 2 h after administration (until experiments were finished, data not shown). The long-lasting decrease in perfusion flow may be the main factor why no reduction of the G_{aw} was seen later in time and when higher doses of ET-1 were given. However, S6c, which is a highly selective ET_B agonist (Adner *et al.* 1998), resulted primarily in a more potent effect on G_{aw} than in diminishing the perfusion flow. The same observations were made using another ET_B agonist, IRL 1620, where only a minor effect was seen on the vascular perfusion (Uhlig *et al.* 1995). In this situation, the lung circulation is not compromised and only small amounts of S6c will bind to the vascular epithelium. In addition, it is likely that the endothelial effect of ET_B receptor stimulation results in relaxation of the underlying smooth muscle cells (Szok *et al.* 2001). The contractile ET_B receptors in the airways can then be reached and the main effect observed was a powerful drop in the G_{aw}; this was more potent than the response noted upon ET-1 administration. The contractile effect of S6c acting via ET_B receptors has a different mode of action when compared with the ET_A-mediated contraction. The contraction appears within minutes but with an obvious tachyphylaxia (O'Donnell & Kay 1995) and after about 2 h the contractile response following S6c has disappeared.

Considering that the ET_A receptors are the dominant endothelin receptor in the vessels (90% vs. 10%) and the proportions of the ET receptors are equal in the airways, the most apparent effect of ET_A antagonists was seen in the vessels (Lal *et al.* 1995). The ET_A-receptor antagonist FR 139317 was added to the recirculating perfusion 20 min before administration of ET-1. The vasoconstriction elicited, compared with control, was attenuated but the bronchial constriction was rapid and strong. This may be explained by the dominant ET_B receptors in the airways and blocking of dilatory ET_A receptors by FR 139317 (Granstrom *et al.* 1997).

The ET_B antagonist BQ 788 had an opposite effect when compared with FR 139317 on the perfusion flow induced by ET-1; the ET-1-induced reduction in flow was more marked (Fig. 2), suggesting that a week ET_B-induced relaxation had been blocked (Szok *et al.* 2001). There was no obvious effect of BQ 788 on the ET-1-induced effect on airway conductance. These results suggest less contractile activity of ET_A than of ET_B receptors in the rat airways. Bosentan inhibited most of the effects of ET-1 on the perfusion flow. The reason why Bosentan had a significantly stronger blockage effect than FR 139317 on the ET-1 effect of the perfusion flow might simply be that the dose given was somewhat higher in terms of antagonistic effect. The antagonism of ET receptors in the bronchial tree seems to be partial. The persistent almost normal circulation gives good access for ET-1 to the still remaining non-antagonized receptors in the bronchial tree and results in a response that is similar to that of ET-1 without any antagonist used.

Many studies have indicated that ET-1 is an important neuropeptide in lung diseases such as asthma bronchiale (Goldie *et al.* 1996). It is important to recognize that in lung diseases ET receptors are upregulated (Möller *et al.* 1997, Granström *et al.* 2004), which further adds to their importance in pulmonary obstructive disease. The present study is a description of an isolated lung model in which complex interactions between the airways and the vascular tree can be characterized. We have shown that the use of isolated, perfused and ventilated lungs from the rat is a useful model for studying both the vascular and pulmonary effects of ET receptor agonists and antagonists. The vasculature contained mainly a contractile ET_A receptor while the airway conductance involves both ET_A and ET_B receptors. The results demonstrate the importance of how drugs are administrated.

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