Effects of Endothelin-1 on Tracheal Submucosal Gland Secretion and Epithelial Function in the Ferret*

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Endothelin-1 (ET-1) is one of a family of novel peptides recently discovered in the human genome. It was originally isolated from cultured porcine aortic endothelial cells and is a potent constrictor of isolated blood vessels. ET-1 is also the most potent of the endothelins at contracting bronchial smooth muscle.

Immunofluorescent staining for ET-1 has been found in epithelial cells from the hilum to the periphery of the conducting airways of mice, and epithelial cells from human bronchi form and release endothelin-like material. However, the effects of ET-1 on airway mucosal tissues, including submucosal glands and the epithelium itself, have not been studied.

The ferret trachea in vitro is a preparation which allows the simultaneous measurement of a number of airway functions, and we have used this preparation to assess the activity of ET-1 on baseline smooth muscle tone, submucosal gland secretion (lysozyme secretion from serous cells), and epithelial function and integrity (active epithelial albumin transport and transepithelial potential difference [PD]). We have also examined the effect of ET-1 on responses to the muscarinic agonist methacholine and the α-adrenoreceptor agonist, phenylephrine. The contraction of bronchial smooth muscle by ET-1 may be mediated through activation of dihydropyridine-sensitive Ca²⁺ channels. The role of these Ca²⁺ channels in the responses to ET-1 in the present study was assessed using the Ca²⁺ channel blocker, nifedipine.

METHODS

The trachea was removed from the ferret, cannulated at both ends, and mounted laryngeal end downwards in an organ bath. The trachea was bathed on the submucosal side with Krebs buffer...
containing BSA (4 mg/ml) and fluorescent BSA (40 µg/ml). This buffer was maintained at 37°C and gassed with 95% O₂/5% CO₂. The lumen of the trachea remained air-filled. Secretions were carried by gravity and mucociliary transport to the lower or laryngeal cannula where they were pooled and could be periodically withdrawn into catheters. Secretions were washed out of the catheters into vials with 0.5 ml of distilled H₂O. These vials were stored frozen for use in albumin and lysozyme assays. Secretion volumes were estimated by the differences in weights of the catheters with secretions and dried without secretions. The upper or carinal cannula was attached to a digital manometer to measure changes in intraluminal pressure reflecting changes in tracheal smooth muscle tone. The electrical potential difference across the trachea was measured using 2 calomel reference electrodes filled with 3.8M KCl and placed in separate beakers of the same solution. Electrical contact was made across the preparation using 2 agar bridges filled with 3.8M KCl in 2.5% w/v agar solution. One bridge was placed in the submucosal buffer and the second inserted into a second hole in the cannula used to collect the mucus. Output from the 2 electrodes was into a high input impedance buffer amplifier and displayed on a digital voltmeter. All drugs were added to the submucosal buffer.

Lysozyme and Albumin Assays

Lysozyme was measured using a turbidimetric assay which relies on its ability to break down the cell wall of the bacterium Micrococcus lysodeikticus. Addition of lysozyme to a solution of the bacterium reduces the turbidity of the solution leading to a fall in optical density (OD) at 450 nm. The lysozyme concentration of the mucus samples was estimated by reference to a standard curve. Albumin was assayed by measuring the fluorescence of the mucus samples (excitation 490 nm, emission 550 nm) and comparing this to a standard curve relating fluorescence to the concentration of fluorescent albumin. The total concentration of albumin was obtained by multiplying the fluorescent albumin concentration by the ratio of nonfluorescent to fluorescent albumin used in the experiment. The lysozyme or albumin output was obtained by dividing the total lysozyme or albumin in a mucus sample by the time over which the sample accumulated.

Protocols

Baseline Effects of ET-1: Tracheas were exposed to 3 or 4 concentrations of ET-1 in a random sequence. Each concentration was left in contact with the trachea for 30 min and 2 control periods of 30 min were allowed between each addition. The maximum change in luminal pressure produced by each concentration of ET-1 was noted, and PD was measured 5 min before the end of each period.

Effects of ET-1 on Responses to Methacholine and Phenylephrine: Tracheas were exposed to methacholine (20 µM) or phenylephrine (100 µM) until a maintained constant output of mucus was obtained and then exposed to 5 concentrations of ET-1 in ascending order. Each concentration of ET-1 was added with the secretagogue and left in contact with the trachea for 30 min.

Effects of Nifedipine on Responses to ET-1: Tracheas were set-up in pairs, one acted as a control (not exposed to nifedipine) and the other as a test (nifedipine present throughout). The protocols described above were repeated on these control and test tracheas.

Results

Effects of ET-1 on Baseline Variables

ET-1 (0.1 to 100 nmol) had no significant effect on baseline lysozyme or albumin output. ET-1 produced a concentration-dependent tracheal smooth muscle contraction (Fig 1, upper). The baseline PD before the addition of ET-1 was -8.8±1.1mV; ET-1 (0.1 to 100 nmol) produced small but significant concentration-dependent increases in the negativity of this PD (Fig 1, lower).

Effects of ET-1 on Responses to Methacholine and Phenylephrine

Methacholine (20 µM) and phenylephrine (100 µM) produced maintained lysozyme and albumin outputs of 526±69 and 429±137ng·min⁻¹, and 2.2±0.52 and 2.04±0.31 µg·min⁻¹, respectively.

ET-1 concentration dependently reduced maintained methacholine and phenylephrine-induced lysozyme and albumin outputs (Fig 2). In the case of lysozyme, ET-1 at concentrations greater than 0.01 nmol was significantly more potent at inhibiting the responses to methacholine than to phenylephrine (Fig 2, lower). In the case of albumin, ET-1 was approximately equipotent on methacholine and phenylephrine-induced responses (Fig 2, upper). All concentration-response curves to ET-1 were shallow occurring over a 10,000-fold concentration range. For inhibition of
ET-1 increased intraluminal tracheal pressure indicating contraction of the tracheal smooth muscle which is consistent with previous studies. Furthermore, the concentrations of ET-1 required to induce contraction in the present study are similar to those used previously on other in vitro preparations. The contraction produced by ET-1 was significantly reduced by nifedipine, the inhibition being about 45% at the highest concentration of ET-1 (10 nmol). This suggests that the contraction to ET-1 is mediated in part by activation of dihydropyridine-sensitive Ca\(^{2+}\) channels in the smooth muscle cell membranes. However, at least part of the response was insensitive even to the high concentration of nifedipine used (10 \( \mu \)mol) and may reflect an effect of ET-1 on other Ca\(^{2+}\) channels, or mobilization of intracellular Ca\(^{2+}\) stores. Alternatively, ET-1 may release a contractile cyclooxygenase product.

ET-1 also produced a concentration-dependent increase in the negativity of the transepithelial PD. The size of this PD is determined by active ion transport across the epithelium (Na\(^{+}\) reabsorption from or Cl\(^{-}\) secretion into the lumen) and the size of the paracellular pathways between epithelial cells. The increased negativity of PD produced by ET-1 could be due to stimulation of Na\(^{+}\) reabsorption or of Cl\(^{-}\) secretion into the lumen, or a reduction in the size of the paracellular pathways. It is not clear which of these effects or combination of effects ET-1 is having. The effects of ET-1 on epithelial ion transport have not been studied. The increase in PD, like the smooth muscle contraction to ET-1, was only partially blocked by nifedipine, again suggesting this effect is only partially due to activation of dihydropyridine-sensitive Ca\(^{2+}\) channels and partly due to some other mechanism such as described above.

ET-1 had no significant effect on baseline lysozyme or albumin outputs, suggesting no effect on serous cell secretion or active epithelial albumin transport. In contrast, ET-1 reduced methacholine- and phenylephrine-induced lysozyme and albumin outputs indicating inhibition of serous cell secretion and epithelial albumin transport produced by muscarinic and \( \alpha \)-adrenoceptor stimulation. In the case of lysozyme, but not albumin, ET-1 was more potent at inhibiting the response to methacholine than phenylephrine.

The reason for this difference is not clear, however methacholine and phenylephrine act through different intracellular pathways to contract airway smooth muscle and may do the same on serous cells, but work through similar mechanisms on epithelial cells. ET-1 may more readily interfere with the signal transduction mechanisms involved with the serous cell response to methacholine than those associated with the response to phenylephrine.

The concentration-response curves for ET-1-induced inhibition of lysozyme and albumin outputs were all shallow, occurring over a 10,000-fold concentration range, and in many cases, the curves appeared to be somewhat biphasic.
This suggests that the action of endothelin in inhibiting stimulated serous cell secretion and epithelial albumin transport is complex and involves more than one mechanism. This hypothesis is supported by the results with nifedipine. The inhibition of methacholine- and phenylephrine-induced albumin output by high concentrations of ET-1 was inhibited by nifedipine, but the responses to lower concentrations of ET-1 were not affected. This suggests that ET-1 at higher concentrations, at least in part, is inhibiting methacholine- and phenylephrine-induced mucus volume and albumin outputs by activating dihydropyridine sensitive Ca\textsuperscript{2+} channels, whereas at lower concentrations, ET-1 acts by a different mechanism. The results for lysozyme output are harder to interpret since the inhibition of methacholine-induced lysozyme output by ET-1 was not affected by nifedipine, but the inhibition of phenylephrine-induced output was significantly reduced by nifedipine at each concentration of ET-1. Thus, it seems that ET-1 possibly activates dihydropyridine-sensitive Ca\textsuperscript{2+} channels to inhibit methacholine-induced lysozyme output, but this influx of extracellular Ca\textsuperscript{2+} with ET-1 has no effect on the response to phenylephrine. The reason for this difference is unclear, but again, may reflect a difference in the signal transduction pathways for methacholine and phenylephrine-induced lysozyme outputs. The fact that ET-1 increases Ca\textsuperscript{2+} influx and that this inhibits serous cell secretion and epithelial albumin transport is interesting in itself since a rise in intracellular Ca\textsuperscript{2+} is usually associated with a positive response of the cell, such as contraction of smooth muscle cells or secretion from glandular cells. The reason for a negative effect with increased intracellular Ca\textsuperscript{2+} in the present study is not clear, and a mechanism is hard to envisage, however, there may be some complex intracellular negative feedback system.

In summary, ET-1 contracts ferret tracheal smooth muscle and increases the negativity of the transepithelial PD at least in part by activating dihydropyridine-sensitive Ca\textsuperscript{2+} channels. ET-1 has no effect on baseline serous cell secretion or epithelial albumin transport but inhibits the gland and epithelial responses to muscarinic and \alpha-receptor stimula-
tion. At high concentrations of ET-1, these inhibitory effects might be mediated by activation of dihydropyridine-sensitive Ca$^{2+}$ channels, whereas at lower concentrations, other mechanisms are involved. These mechanisms may involve other Ca$^{2+}$ channels, intracellular Ca$^{2+}$ stores, or release of other mediators.

REFERENCES