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Formation of endothelin by cultured airway epithelial cells

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Immunoreactivity to endothelin was detected in conditioned culture medium from both canine and porcine tracheal epithelial cells. Gel permeation chromatography and fast protein liquid chromatography were used to confirm the identity of the endothelin. The two peaks demonstrated on fast protein liquid chromatography co-eluted with endothelin 1 and endothelin 3 respectively.

Endothelin; (Airway epithelial cell)

1. INTRODUCTION

Endothelin is a potent vasoconstrictor peptide which was first isolated from the conditioned medium of porcine aortic endothelial cells [1]. Endothelin 1 contracts isolated blood vessels from different vascular beds in a variety of species [1,2]. It also promotes the release of prostaglandins from isolated lungs [3], endothelium-derived relaxing factor from blood vessels [3] and atrial natriuretic peptide from atrial myocytes [4] but inhibits the release of renin from isolated glomeruli [5]. In addition it stimulates the proliferation of vascular smooth muscle [6]. Two other forms of endothelin have now been characterised. Endothelin 3 shares 15 of its 21 residues with porcine endothelin and is also a potent vasoconstrictor [7].

More recently it has become apparent that endothelin can exert effects on the airways. Endothelin 1 contracts isolated rat trachea [8] and when aerosolised it causes bronchoconstriction in anaesthetised and ventilated guinea-pigs [9]. Because there is evidence that airway epithelial cells can release constrictor substances [10] we have investigated the possibility that cultured airway epithelial cells form endothelin.

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2. MATERIALS AND METHODS

2.1. *Cell culture*

Segments of canine trachea were obtained immediately after death. The segments were filled with digestion medium (143.4 mM Na^+, 5.9 mM K^+, 123.1 mM Cl^-, 1.2 mM $H_2PO_4^-$, 5 mM HCO_3^-, 5.9 mM glucose, 20 mM Hepes, 10^5 U/l penicillin, 100 mg/l streptomycin, 100 mg/l gentamicin, 1% bovine serum albumin and 0.1% pronase), the ends were sealed with parafilm and the segments incubated at 4°C for 24 h. The digestion medium was then spun, the supernatant removed and the cell pellet resuspended in a 1:1 mixture of Ham's F12 medium and Dulbecco's Modified Eagle's Medium (with sodium pyruvate and 1000 mg/l glucose) containing 5% foetal calf serum (FCS), 10^5 U/l penicillin, 100 mg/l streptomycin, 100 mg/l gentamicin and 10^5 U/l mycostatin. The cell suspension was then incubated at 37°C for 1 h in plastic wells to allow any contaminating fibroblasts to adhere to the plastic. The cell suspension was then removed and added to 25 cm^2 collagen-coated plastic flasks and incubated at 37°C. Cells took 3–4 days to reach confluence. Once confluent cells were incubated with either serum-free medium or medium containing 5% FCS for 48 h. Medium incubated at 37°C for 48 h in the absence of cells served as a control. Conditioned medium was stored at −70°C until it was assayed. We confirmed that the cells were epithelial by performing immunohistochemical staining using a mouse anti-human antibody for cytokeratin. 98% of the cells were positive for cytokeratin. In contrast we were unable to demonstrate any staining for Von Willebrand Factor using a mouse anti-human antibody known to cross-react with canine

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tissue, showing that there was no evidence of contamination with endothelial cells. Porcine epithelial cells were cultured in the same way.

Culture medium, foetal calf serum, penicillin-gentamicin and streptomycin were purchased from Gibco (Uxbridge, England). Mycostatin was obtained from E.R. Squibb and Sons (Hounslow, England) and pronase from Sigma (Poole, England). Monoclonal antibodies were purchased from Dako (High Wycombe, England).

2.2. *Extraction procedure*

Conditioned media were extracted on Sep-Pak C18 cartridges (Waters Associates, CA, USA) following Sep-Pak activation by 10 ml acetonitrile, 10 ml methanol and then 10 ml of 4% (v/v) acetic acid. 8 ml of each sample were acidified with 4 ml of 4% acetic acid, loaded onto the cartridge and the cartridge washed with 10 ml of 4% acetic acid. Endothelin-like immunoreactivity was eluted with 2 ml of 60% (v/v) acetonitrile/water containing 0.026 M ammonium acetate. The eluate was dried in a Savant vacuum centrifuge, the resulting pellet reconstituted with assay buffer and the aliquot assayed. With this extraction procedure the recovery of synthetic endothelin added to culture medium was 86 ± 4% ($n = 4$).

2.3. *Radioimmunoassay*

The assay was performed in 60 mM phosphate buffer, pH 7.4, containing 10 mM EDTA, 7 mM sodium azide and 0.3% (w/v) bovine serum albumin. The antiserum raised in rabbits to endothelin 1 was obtained from the Peptide Institute, Osaka, Japan. It was used at a final dilution of 1:14000 and cross-reacted 30% with endothelin 3. Radiolabelled tracer was prepared by conventional chloramine-T oxidation and purified by HPLC. In a typical experiment 50 μl of endothelin solution (4 nmol) in 0.4 M phosphate buffer, pH 7.4, was added to 10 μl (1 mCi) of $Na^{125}I$. The reaction was initiated with 10 μl of 0.5 mg/ml of chloramine-T and was stopped 10 s later by addition of 25 μl of 2.4 ng/ml of sodium metabisulphite. The reaction mixture was diluted with 20% (by vol.) aqueous acetonitrite containing 0.1% (by vol.) and trifluoroacetic acid (TFA) and loaded on a reverse phase Techsill 5 C18 column which was equilibrated with the same solution. Optimal separation of [^{125}I]endothelin was achieved by elution with an isocratic gradient of 36% aqueous acetonitrile/0.1% TFA. The endothelin tracer had a specific activity of 60 Bq/fmol as assessed by self displacement. Endothelin tracer (1800 cpm) and antiserum were added to each tube to a total volume of 700 μl and samples (10–100 μl) were assayed in duplicate. After 5 days incubation at 4°C, antibody-bound and free fractions were separated by charcoal adsorption of the free fraction. The standard used was synthetic endothelin 1. The assay could detect changes of 10 fmol/assay tube with 95% confidence and the intra- and inter-assay variation was less than 10%.

2.4. *Gel permeation and fast protein liquid chromatography*

Endothelin-like immunoreactivity was characterised by gel permeation chromatography on a column containing Sephadex G-25 (Pharmacia Chemicals, Uppsala, Sweden) and by fast protein liquid chromatography with a PEPRPC HR5/5 reverse-phase C18 high resolution column (Pharmacia). Samples after being extracted and dried in a Savant vacuum centrifuge were reconstituted in phosphate buffer and were loaded onto the gel column (0.9 × 60 cm). This was eluted with 60 mM phosphate buffer, pH 7.4, containing 0.2 M sodium chloride, 10 mM EDTA, 7 mM sodium azide and 0.3% (w/v) bovine serum albumin at 0.6 ml per 12 min per fraction. With each sample run, dextran blue 2000 (mol. wt. 2×10^6), cytochrome c (mol. wt. 12384) and $Na^{125}I$ were included as markers for the void volume, molecular size and total volume. Samples of each fraction were assayed for endothelin immunoreactivity and the elution fraction was determined.

Samples for FPLC were reconstituted in water containing 0.1% TFA, centrifuged in a microfuge and the supernatant loaded onto the column. This was equilibrated with 10% (v/v) of 10% (v/v) acetonitrile with 0.1% TFA, from 10% to 45% (v/v) in water over 1 h followed by an isocratic gradient of 45% (v/v) acetonitrile with 0.1% TFA for a further 10 min at 1 ml/min per fraction. Samples of each fraction were assayed for endothelin-like immunoreactivity. Both columns were calibrated with synthetic endothelin before and after a series of sample runs.

3. RESULTS AND DISCUSSION

We have detected immunoreactive endothelin in conditioned medium from cultured canine tracheal epithelial cells (table 1). In contrast, levels of endothelin in control medium were below the limits of detection, regardless of whether the medium contained foetal calf serum or not. Nor was there any significant difference between endothelin levels in conditioned medium, containing foetal calf serum, and medium which was serum-free. These findings indicate that the endothelin was formed by the epithelial cells and was not a component of foetal calf serum which was added to the medium. We have also detected immunoreactive endothelin (45 pmol/l) in pooled medium from 5 different cultures of porcine tracheal epithelial cells. Serial two-fold dilution curves with conditioned medium and two immunoreactive peaks eluted on FPLC were parallel with a standard curve for endothelin (fig.1).

We confirmed the identity of the immunoreactive endothelin in the conditioned medium from cultures of canine tracheal epithelium using gel permeation chromatography and by fast protein li-

Table 1

Concentration of endothelin (pmol/l) in conditioned culture medium

Control ($n = 6$)	Conditioned medium with FCS ($n = 7$)	Conditioned medium serum-free ($n = 10$)
<2	285 ± 48	240 ± 17

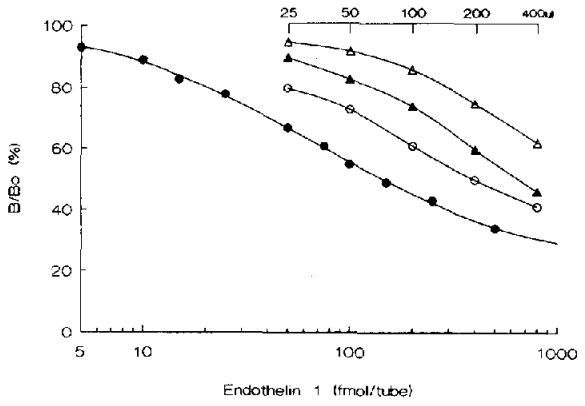

Fig.1. A standard curve of endothelin 1 (open circles) and serial 2-fold dilution curves of the conditioned medium extract of canine tracheal epithelial cells (●), the immunoreactive peak eluted in the position of endothelin 1 on FPLC (▲) and the immunoreactive peak eluted in the position of endothelin 3 on FPLC (△).

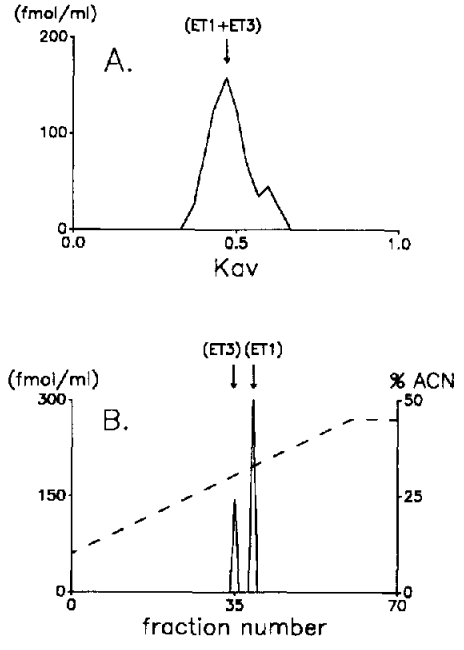

Fig.2. (A) Sephadex G-25 column chromatography and (B) FPLC of the immunoreactive endothelin in the conditioned medium of canine tracheal epithelial cells. ET1, ET3, the elution positions of endothelin 1 and endothelin 3, respectively; ACN, acetonitrite.

quid chromatography (FPLC) (fig.2). A broad peak which co-eluted with endothelins 1 and 3 was seen on gel permeation chromatography. Two peaks were demonstrated on FPLC. The first peak contained 25% of the immunoreactivity and co-eluted with endothelin 3. The rest of the immunoreactivity was present in the second peak which co-eluted with endothelin 1. The antibody to endothelin 1 used in the radio-immunoassay had 30% cross-reactivity with endothelin 3. This means that similar amounts of endothelin 1 and endothelin 3 were in effect present in the culture supernatant.

This is the first evidence that endothelin can be formed by non-endothelial cells. This finding is consistent with endothelin having actions outside the cardiovascular system. Certainly binding sites for endothelin are found not only in blood vessels but in a wide variety of tissues including the airways [11,12]. Cell culture, however, is an artificial situation and we do not yet know whether endothelin is formed in the airways in vivo. Indeed it could be argued that culturing cells on an artificial surface represents a form of cell injury. One of the characteristic features of asthma is epithelial injury and one could speculate that this is a situation where endothelin might be released from airway epithelial cells. However, although endothelin is a very potent vasoconstrictor agent, it is two orders of magnitude less potent in causing bronchoconstriction. Threshold contractions for isolated trachea in the rat [8], guinea-pig, dog and pig (unpublished observations) range from 10^{-8} to 10^{-7} M. Nonetheless if endothelin were released in vivo, it is not impossible that concentrations of this magnitude could occur in the airways close to the smooth muscle. There is also evidence that in the guinea-pig endothelin causes bronchoconstriction through the release of secondary mediators such as PAF and prostaglandins. If this were the case in man, endothelin might cause bronchoconstriction more readily in patients with asthma whose airways are infiltrated with inflammatory cells capable of generating mediators such as PAF. Endothelin could also have other effects in the airways. There is evidence that it has anti-inflammatory actions [13]. It is also a mitogen for vascular smooth muscle but it remains to be seen whether it is a mitogen for airway smooth muscle. Further work will be necessary to determine whether endothelin is a mediator in asthma.

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