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Expression, secretion, and inhibition of angiotensin-converting enzyme in cultured human bronchial epithelial cells

Misato Takimoto *, Hironobu Mitani, Seiji Hori, Masaaki Kimura, Tsutomu Bandoh, Toshikazu Okada

Takarazuka Research Institute, Novartis Pharma K.K., 10-66 Miyuki-cho, Takarazuka, 665-8666, Japan

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Abstract

The purpose of this study was to determine whether angiotensin-converting enzyme is present in cultured human bronchial epithelial cells and which types of epithelial cells possess this enzyme. It is well known that serum promotes squamous differentiation of airway epithelial cell culture in vitro. We found that whole-cell homogenates of both basal (serum-untreated) and squamous-differentiated bronchial epithelial cells degraded hippuryl-L-histidyl-L-leucine, a synthetic substrate for angiotensin-converting enzyme. Analysis of RNA expression by reverse transcription-polymerase chain reaction (RT-PCR) showed the presence of mRNA for angiotensin-converting enzyme in both types of cells. In addition, we found that squamous cells secreted the enzyme into the culture medium more than basal cells did. Angiotensin-converting enzyme inhibitors (imidaprilat, enalaprilat) inhibited the enzyme activity in bronchial epithelial cells with an IC_{50} of 0.9–3.6 nM. Exogenously added bradykinin was degraded to bradykinin-(1–5), an inactive fragment, in the squamous cell cultures. Our data indicate the presence of angiotensin-converting enzyme in cultured human bronchial epithelial cells and also that the enzyme is secreted by squamous differentiated cells.

Keywords: Angiotensin-converting enzyme; Airway; Bronchial epithelial cell; Angiotensin II; Bradykinin

1. Introduction

Angiotensin-converting enzyme (EC 3.4.15.1, Kininase II) is a peptidyl dipeptidase which converts the inactive decapeptide angiotensin I to the potent vasopressor octapeptide angiotensin II by hydrolytic removal of the C-terminal dipeptide histidyl leucine. It has been localised in endothelial cells and macrophages (Soffer, 1976; Sugiyama et al., 1988). Therefore, this enzyme is thought to play an important role in the regulation of blood pressure. Inhibition of angiotensin-converting enzyme has been documented to decrease blood pressure, an action primarily mediated by blockade of the conversion of angiotensin I to angiotensin II.

In the past decade, the treatment of essential hypertension has focused on the suppression of the angiotensin II level by angiotensin-converting enzyme inhibitors. A dry cough is the most common adverse effect in patients receiving angiotensin-converting enzyme inhibitors as therapy for heart failure or hypertension. The cough is considered to be caused by the suppression of kininase II activity, which may be followed by the accumulation of kinins, substance P and prostaglandins (Overlack, 1996).

It is considered that the major physiological role of angiotensin-converting enzyme in the lung is to hydrolyse circulating bradykinin, a potent proinflammatory mediator, and to convert angiotensin I to angiotensin II (Bhoola et al., 1992). Bradykinin is a basic peptide that is rapidly produced from kininogen in plasma and is released into bronchoalveolar lavage fluid during many inflammatory processes and exerts several physiological actions. It is well recognised that bradykinin causes bronchoconstriction and increases capillary permeability. Angiotensin-converting enzyme activity has also been detected in bronchoalveolar and nasal lavage fluid obtained from humans (Specks et al., 1990; Ohkubo et al., 1994). However, the cellular origin of the enzyme in bronchoalveolar and nasal

* Corresponding author. Tel.: +81-797-74-2339; Fax: +81-797-74-2455; E-mail: misato.takimoto@pharma.novartis.com

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lavage fluid and its physiological significance are still unclear. It is conceivable that airway epithelial cells express angiotensin-converting enzyme, which then can be retrieved in bronchoalveolar and nasal lavage fluid. Airway epithelium is composed of heterogeneous cells and, in trachea and large bronchi, it includes at least three distinct cell types: basal, ciliated columnar, and non-ciliated secretory cells (Jetten, 1991). Previous studies have demonstrated the presence of angiotensin-converting enzyme in cultured bovine bronchial epithelial cells (Müns et al., 1993), but no detectable expression of the enzyme was reported in the human bronchial epithelial cell line, BEAS-2B (Proud et al., 1994).

The present study was designed to determine whether angiotensin-converting enzyme is present in cultured human bronchial epithelial cells and which types of epithelial cells possess this enzyme. We demonstrate that both basal (serum-untreated) and squamous (serum-differentiated) bronchial epithelial cells express angiotensin-converting enzyme, and that the enzyme is released from squamous differentiated cells in much higher amounts than it is from basal cells. Also, the effect of the angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonist on the enzyme activity and on the degradation of bradykinin in bronchial epithelial cell cultures was examined.

2. Materials and methods

2.1. Materials

Bradykinin and hippuryl-L-histidyl-L-leucine (HHL) (Peptide Institute, Osaka, Japan); enzyme-immunoassay (EIA) kits for bradykinin and bradykinin-(1–5) (Dainippon Pharma, Osaka, Japan); Ham's F-12 medium, foetal bovine serum, and amphotericin B (Life Technologies, Grand Island, NY); and gentamicin (Sigma, St. Louis, MO) were obtained from the indicated manufacturers. Valsartan was synthesised (Ciba-Geigy, Basle, Switzerland). Poly $(A)^+$ RNA from human placenta was purchased from Clontech (Palo Alto, CA). Imidapril was extracted from Novalok® (Nard Institute, Amagasaki, Japan), and hydrated to obtain imidaprilat, an active metabolite of imidapril (Novartis Pharma. K.K., Takarazuka, Japan). Enalaprilat was synthesised in Nard Institute. All other reagents used were of analytical grade.

2.2. Methods

2.2.1. Cell culture

Primary cultures of normal human bronchial epithelial cells were obtained from Clonetics (Walkersville, MD). Almost all of the cells were basal cells morphologically, and this type of cell has the capacity for self-renewal. Cells were cultured at 37°C in a CO_2 incubator (5% CO_2-95% air) in serum-free, hormone-supplemented growth medium containing bovine pituitary extract (50 μg/ml), hydrocortisone (0.5 μg/ml), human recombinant epidermal growth factor (0.5 ng/ml), epinephrine (0.5 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), retinoic acid (0.1 ng/ml), triiodothyronine (6.5 ng/ml), gentamicin (50 μg/ml), and amphotericin B (50 μg/ml). For serum treatment of the cells, the culture medium of 5- to 7-day cultures of semi-confluent cells was changed to Ham's F-12 containing 10% foetal bovine serum, and the cells were cultured for 24 h (Takimoto et al., 1996). After serum treatment for 24 h, the cells were washed twice and incubated further with fresh Ham's F-12 medium (serum-free) for 24 h. The cells and conditioned medium were then collected to measure angiotensin-converting enzyme activity and the bradykinin and bradykinin-(1–5) content. All experiments were carried out with subcultured cells between the 2nd to 4th passage.

2.2.2. Reverse transcription–polymerase chain reaction (RT-PCR) amplification

Total RNA was prepared from serum-treated and untreated bronchial epithelial cells by the guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). Detection of mRNA for angiotensin-converting enzyme was performed by reverse transcription followed by polymerase chain reaction (RT-PCR). Total RNA (1 μg) was reverse transcribed in a total volume of 20 μl with an oligo dT primer and amplified by the primer oligonucleotides using the RNA LA PCR Kit (Takara, Shiga, Japan). Forward and reverse primer oligonucleotides were designed from the human cDNA sequences of angiotensin-converting enzyme (forward primer: 1717-1736 5′-AGGGGCCAAGCTCCGGAAGG-3′, reverse primer: 2269-2288 5′-CAGTGGCCACGCTGTAGGTG-3′). As previously described (Studer et al., 1994), another set of primers was also used (forward primer: 1656-1675 5′-GCAAGGAGGCAGGCTATGAG-3′, reverse primer: 2412-2431 5′-CGGGTAAAACTGGAGGATGG-3′). Both sets of primers were selected to detect the large endothelial type of angiotensin-converting enzyme mRNA and not the testicular type of angiotensin-converting enzyme mRNA. The PCR reaction was performed in a total volume of 100 μl, containing 20 μl of the reverse transcription reaction mixture with *Thermus aquaticus* DNA polymerase (Takara). The reaction mixtures were subjected to 40 cycles of PCR amplification using the GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, CT). The cycle profile included denaturation for 30 s at 94°C, annealing for 30 s at 58°C and extension for 45 s at 72°C. As these primer oligonucleotides span several splice junctions (Hubert et al., 1991), the PCR product from priming with contaminated genomic DNA can be clearly distinguished from that from spliced mRNA. For a

positive control, poly $(A)^+$ RNA (10 ng) from human placenta was used. To control the amounts of mRNA 456 base-pair fragments of GAPDH cDNA were amplified with primer oligonucleotides (forward primer: 5′-GTCCATGCCATCACTGCCACCCAGAAGACT-3′, reverse primer 5′-GGCCATGAGGTCCACCACCCTGTTGCTGTA-3′) from the same RNA preparations. The reaction mixtures were subjected to 30 cycles of PCR amplification. The cycle profile included denaturation for 30 s at 94°C, annealing for 30 s at 58°C and extension for 90 s at 72°C.

The PCR products (25 μl for angiotensin-converting enzyme, 10 μl for GAPDH) were subjected to agarose gel electrophoresis (1.5% gel). The bands for angiotensin-converting enzyme were found to be of the expected size.

In addition, the sequences of the amplified products for angiotensin-converting enzyme were confirmed by DNA sequencing. Briefly, the bands in agarose gels were picked out and purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Then the purified PCR products were treated with the Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) following analysis with the ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA).

2.2.3. Angiotensin-converting enzyme activity

The cells were seeded in 100-mm culture dishes at a density of 4×10^3 cells/cm^2 and cultured to semiconfluence (5 to 7 days). After the cells were treated with or without serum for 24 h, the cells were washed twice with phosphate-buffered saline (PBS) and incubated with fresh Ham's F-12 medium (serum-free) for an additional 24 h. Then the culture medium was collected, centrifuged at $1000 \times g$ for 20 min at 4°C to remove cell debris, and directly used for the measurement of angiotensin-converting enzyme activity. The cells were washed twice with ice-cold PBS, scraped off in PBS, and centrifuged at $200 \times g$ for 10 min. The cell pellet was resuspended (0.5 ml/dish) in homogenisation buffer consisting of 20 mM Tris–HCl (pH 8.3), 5 mM $Mg(CH_3COO)_2$, 30 mM KCl, and 0.25 M sucrose, and homogenised by sonication at 4°C. The homogenates were directly used to measure the enzyme activity.

Angiotensin-converting enzyme activity in the cell homogenate and conditioned medium was determined by the rate of production of hippuric acid from the synthetic tripeptide substrate hippuryl-L-histidyl-L-leucine (HHL) as previously described (Mitani et al., 1996). Assay buffer (200 μl), composed of 100 mM potassium phosphate buffer (pH 8.3), 600 mM NaCl, and 6.5 mM HHL, was mixed with 100 μl of sample solution, and incubated for 20 h at 37°C. This incubation period was used to ensure that the amount of product consistently fell in a range detectable by high-performance liquid chromatography (HPLC). The reaction was stopped by addition of 3% (final concentration) metaphosphoric acid. After centrifugation of the reaction mixture for 10 min at $2000 \times g$, 100 μl of the supernatant was applied to a reverse-phase column (TSK-GEL, ODS-80TM, Tosoh, Tokyo, Japan) and eluted at 38°C with 10 mM KH_2PO_4-methanol (1:1, pH 3.0) at a rate of 0.7 ml/min.

The hippuric acid concentration was determined by measuring absorbance at 228 nm. The enzyme activity is expressed as picomoles per hour per 10^6 cells.

2.2.4. Effect of angiotensin-converting enzyme inhibitor and angiotensin II receptor antagonist on enzyme activity

To examine the effect of the angiotensin-converting enzyme inhibitors, imidaprilat and enalaprilat, and an angiotensin II receptor antagonist, valsartan, on the enzyme activity in the conditioned medium, the inhibitors and antagonist were added at various concentrations to the conditioned medium of squamous bronchial epithelial cells 10 min prior to the addition of the substrate. Assay buffer (200 μl), composed of 100 mM potassium phosphate buffer (pH 8.3), 600 mM NaCl, and 6.5 mM HHL, was mixed with 100 μl of the conditioned medium and various concentrations of the drugs, and then incubated for 20 h at 37°C (in cell-free system). HPLC analysis was performed as described in Section 2.2.3.

To investigate the effect of these agents on HHL hydrolysis in living cells, bronchial epithelial cells were seeded into 6-well plates at 4×10^3 cells/cm^2. After 5–7 days, semiconfluent cultures were used for experiments. We examined the direct effect of the agents and HHL on both the cells and conditioned medium. After serum treatment for 24 h, valsartan, imidaprilat, enalaprilat, and HHL (final 0.1 mM) were added to squamous differentiated cells with fresh Ham's F-12 medium for 24 h. Then the conditioned medium was collected, centrifuged to remove cell debris, added to metaphosphoric acid (3%) to terminate the reaction, and then directly subjected to HPLC as described in the Section 2.2.3.

2.2.5. Measurement of bradykinin and bradykinin-(1–5)

Degradation of exogenously added bradykinin was measured by EIA with the kits of Markit-M bradykinin and Markit-M (1–5)-bradykinin (Dainippon Pharmaceutical, Osaka, Japan). The cross-reactivity is less than 0.1% between bradykinin and bradykinin-(1–5), an inactive fragment degraded by angiotensin-converting enzyme. The cells were cultured in 100-mm culture dishes to semiconfluence, incubated with 10% foetal bovine serum in Ham's F-12 medium for 24 h, and incubated further with serum-free Ham's F-12 medium (5 ml/dish) for an additional 24 h. Then, the conditioned medium was removed and centrifuged to remove cell debris. The conditioned medium was incubated with bradykinin in the presence or absence of angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonist. The reaction was stopped by addi-

tion of trichloroacetic acid (final 33 mg/ml), and the sample was centrifuged to eliminate proteins, neutralised, and stored at −80°C until analysis. The concentrations of immunoreactive bradykinin and bradykinin-(1–5) were measured by EIA.

2.2.6. Statistical analysis

All data are expressed as means ± S.E.M. Statistical analysis was performed using One-way analysis of variance followed by Dunnett's multiple comparisons test. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. RT-PCR for angiotensin-converting enzyme

To investigate whether angiotensin-converting enzyme is expressed in human bronchial epithelial cells, RT-PCR was performed with two sets of primers: forward (1656–1675) and reverse (2412–2431), and forward (1717–1736) and reverse (2269–2288) (Fig. 1). Both sets of primers produced DNA bands of the expected size on agarose gel electrophoresis. Although the set of forward (1656–1675) and reverse (2412–2431) primers was reported to work well in samples of human heart (Studer et al., 1994) and optimisation of RT-PCR was performed thoroughly, multiple bands were observed with the samples from bronchial epithelial cell cultures. We could not explain these multiple bands in the samples. However, the bands for angiotensin-converting enzyme were pre-eminently observed when using poly $(A)^+$ RNA from human placenta. Then, another set of forward (1717–1736) and reverse (2269–2288) primers was used. The expected bands (572 base pairs) for the enzyme were predominantly observed in placenta and in basal and squamous bronchial epithelial cells. Sequencing of these 572-base pair bands of PCR products was able to produce significant alignment with human angiotensin-converting enzyme (data not shown). Thus, RT-PCR demonstrated that angiotensin-converting enzyme mRNA is expressed in basal (non-differentiated) and squamous (serum-differentiated) bronchial epithelial cells, as well as human placenta.

3.2. Angiotensin-converting enzyme activity in bronchial epithelial cells

Hippuryl-L-histidyl-L-leucine (HHL) was hydrolysed by cell homogenates of basal (non-differentiated) and squamous differentiated bronchial epithelial cells with an activity of 420 ± 1 and 380 ± 7 pmol/h/10^6 cells, respectively (Fig. 2). The enzyme activity was also detected in the 24-h-conditioned medium of squamous cells (2700 ± 70 pmol/h/10^6 cells). However, in the conditioned medium of basal cells, the enzyme activity was only approximately 14% (390 ± 5 pmol/h/10^6 cells) of the activity found in squamous cells. These results indicate that bronchial epithelial cells express angiotensin-converting enzyme and that the enzyme is actively secreted from squamous differentiated bronchial epithelial cells.

3.3. Effects of angiotensin-converting enzyme selective inhibitors on enzyme activity

The effects of the angiotensin-converting enzyme selective inhibitors, imidaprilat and enalaprilat, and the angiotensin II receptor selective antagonist, valsartan, on the enzyme activity in the conditioned medium of squamous cells are shown in Fig. 3 (cell-free system). There was an almost complete and concentration-dependent inhibition of HHL hydrolysis with both of the inhibitors, with IC_{50} values of 0.9 nM for imidaprilat and 3.6 nM for enalaprilat. Valsartan did not inhibit HHL hydrolysis in concentrations up to 10 μM.

To determine HHL hydrolysis by living squamous cells, the enzyme inhibitors, angiotensin II receptor antagonist, and HHL (0.1 mM) were co-incubated with squamous cells for 24 h in serum-free medium. Enalaprilat inhibited HHL hydrolysis in a concentration-dependent manner (5.7, 40.1, 63.6% inhibition by 0.1, 1, 10 nM of enalaprilat,

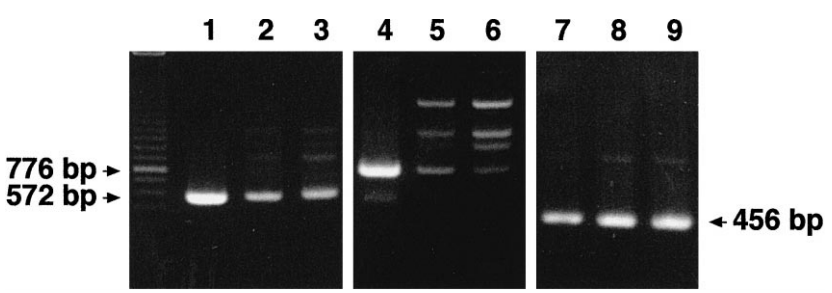

Fig. 1. Agarose gel electrophoresis of RT-PCR products using poly $(A)^+$ RNA from human placenta (lanes 1, 4, 7), total RNA from basal (serum-untreated) bronchial epithelial cells (lanes 2, 5, 8), and total RNA from squamous differentiated bronchial epithelial cells (lanes 3, 6, 9). cDNA fragments for angiotensin-converting enzyme with 572 base pairs were amplified by the primer set of forward (1717-1736) and reverse (2269–2288) primers (lanes 1–3). The 776-base pair fragments obtained were amplified by the primer set of forward (1656–1675) and reverse (2412–2431) primers (lanes 4–6). GAPDH cDNA fragments with 456 base pairs were amplified (lanes 7–9). On the left-hand side, a molecular weight marker (100 base-pair ladder) was loaded.

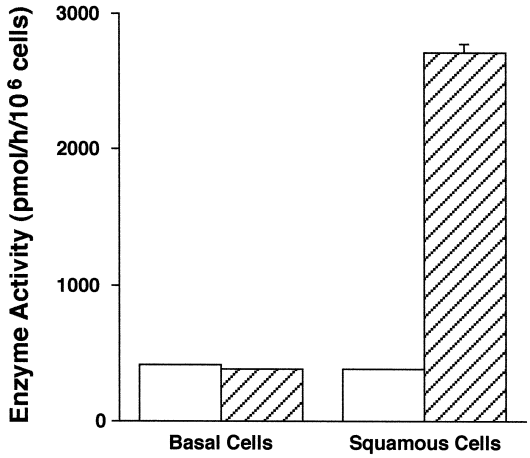

Fig. 2. Angiotensin-converting enzyme activity in the cell homogenate (open bars) and the conditioned medium (hatched bars) of the basal (serum-untreated) and squamous differentiated bronchial epithelial cells. Both types (basal and squamous) of cells were incubated with serum-free medium for 24 h, and then the cell homogenate and the conditioned medium were prepared. The activity was measured as the rate of production of hippuric acid from HHL (4.3 mM). Values shown are the means ± S.E.M. ($n = 3$).

respectively). Imidaprilat also inhibited HHL hydrolysis with a potency stronger than that of enalaprilat (30.2, 60.4, 67.6% inhibition by 0.1, 1, 10 nM of imidaprilat, respectively). However, valsartan did not inhibit HHL hydrolysis in concentrations up to 10 nM (Fig. 4). These concentrations of valsartan (~10 nM), imidaprilat (~10 nM), enalaprilat (~10 nM), and HHL (0.1 mM) did not affect cell number, viability, or morphology.

3.4. Degradation of bradykinin in bronchial epithelial cells

The degradation of bradykinin in the conditioned medium of bronchial epithelial cells for the indicated

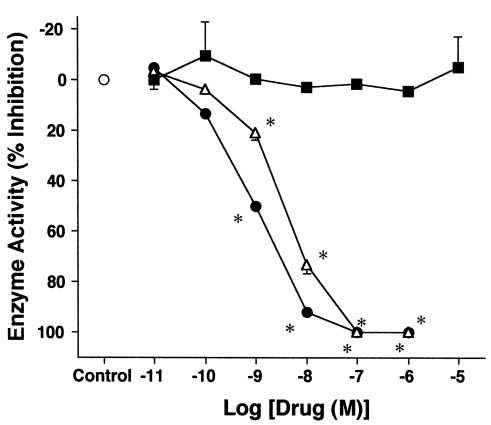

Fig. 3. Effects of valsartan (■), enalaprilat (△), or imidaprilat (●) on angiotensin-converting enzyme activity in the conditioned medium of squamous bronchial epithelial cells (cell-free system). The activity was measured as the rate of production of hippuric acid from HHL (4.3 mM). The control (○) value of the enzyme activity was 2580 ± 35 pmol/h/10^6 cells. *Significantly different from the control value, $P < 0.05$. Values shown are the means ± S.E.M. ($n = 3$).

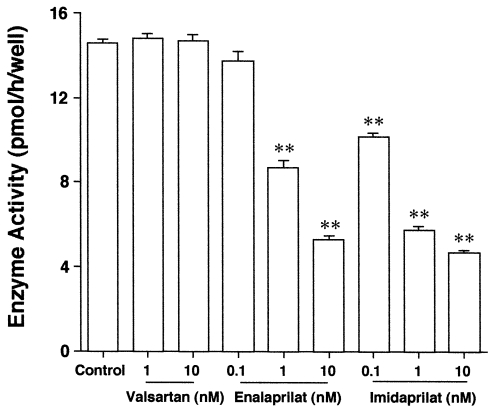

Fig. 4. Effects of valsartan, enalaprilat, or imidaprilat on angiotensin-converting enzyme activity in living squamous bronchial epithelial cells (cells and medium). The substrate HHL (0.1 mM) was co-incubated with the cells in the presence or absence of valsartan, enalaprilat, or imidaprilat (0.1–10 nM) for 24 h. *Significantly different from the control value, $P < 0.01$. Values shown are the means ± S.E.M. ($n = 4–8$).

periods (0–8 h) is shown in Fig. 5A. Conditioned medium of bronchial epithelial cells and bradykinin (10 nM; 10.6 ng/ml) were incubated for the indicated periods. Exoge-

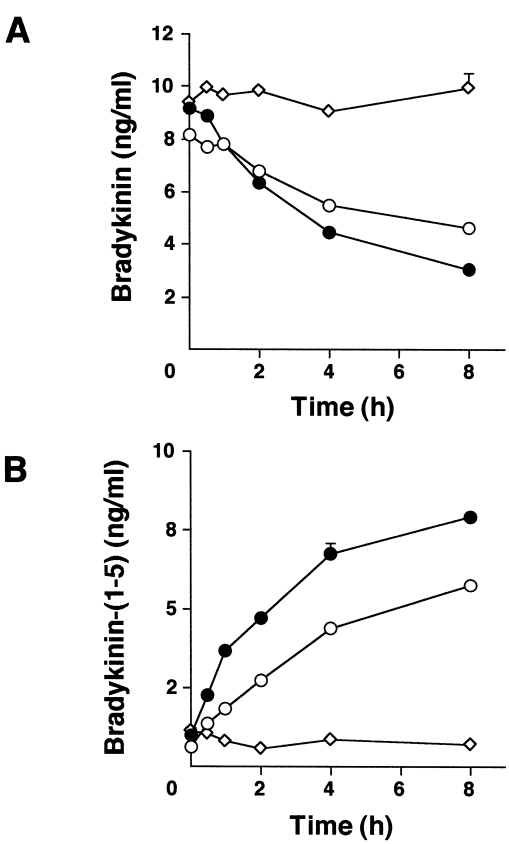

Fig. 5. Degradation of bradykinin (A) and formation of bradykinin-(1–5) (B) in the conditioned medium of basal (○) and squamous bronchial epithelial cells (●). Bradykinin (10 nM; 10.6 ng/ml) was added to the medium and incubated at 37°C for the indicated periods. Bradykinin was not degraded to bradykinin-(1–5) in the control serum-free Ham's F12 medium (◇). Values shown are the means ± S.E.M. ($n = 3$).

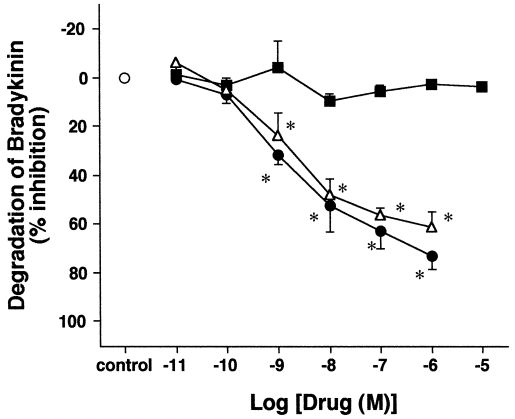

Fig. 6. Effects of valsartan (■), enalaprilat (△), or imidaprilat (●) on the degradation of bradykinin in the conditioned medium of squamous bronchial epithelial cells (cell-free system). Bradykinin (5 nM; 5.3 ng/ml) was added to the medium and incubated at 37°C for 3 h. The control (○) value after a 3-h incubation was 2.65 ± 0.04 ng/ml of bradykinin. * Significantly different from the control value, $P < 0.05$. Values shown are the means ± S.E.M. ($n = 3$).

nously added bradykinin was gradually degraded in the medium of squamous differentiated cells. The medium of basal cells also degraded bradykinin, but to a lesser extent than did squamous cell medium. Non-conditioned medium (Ham's F12) did not degrade bradykinin.

We also measured the content of bradykinin-(1–5), an inactive fragment of bradykinin, after the incubation of the conditioned medium of bronchial epithelial cells with bradykinin (10 nM; 10.6 ng/ml), just as we did for bradykinin (Fig. 5B). Bradykinin-(1–5) was time dependently produced in the conditioned medium of both basal and squamous cells. However, the conditioned medium of squamous cells degraded bradykinin more actively than did the medium of basal cells. This production of bradykinin-(1–5) corresponded well with the decrease in the content of bradykinin shown in Fig. 5A.

3.5. Effects of angiotensin-converting enzyme inhibitors on degradation of bradykinin

The effects of the angiotensin-converting enzyme inhibitors, imidaprilat and enalaprilat, and the angiotensin II receptor antagonist, valsartan, on the degradation of bradykinin in the conditioned medium of squamous cells are shown in Fig. 6. The enzyme inhibitors caused a concentration-dependent inhibition of bradykinin degradation, with IC_{50} values of 7.0 nM for imidaprilat and 13 nM for enalaprilat. Valsartan did not show any effect in concentrations up to 10 μM.

4. Discussion

Angiotensin-converting enzyme activity in tissue homogenates of human foetal airway and nasal mucosa was found in previous studies (Johnson et al., 1985; Ohkubo et al., 1994). However, in these studies the enzyme activity was derived from a mixture of cells, such as vascular endothelial cells, epithelial cells, and interstitial materials. It is well established that significant angiotensin-converting enzyme activity is present in endothelial cells and tissue macrophages. It is conceivable, therefore, that the enzyme activity measured in their investigation was partially derived from these cells. We previously demonstrated that proliferating cultures of normal human bronchial epithelial cells can be induced to undergo growth arrest and subsequent squamous differentiation by exposure to serum (Takimoto et al., 1996). Various agents, including transforming growth factor-β1, protein kinase C activators and Ca^{2+}, also induce squamous differentiation of bronchial epithelial cells (Jetten, 1991; Grunenert et al., 1995). The results of our present study clearly indicate the presence of angiotensin-converting enzyme in cultured human bronchial epithelial cells. Although the levels of angiotensin-converting enzyme mRNA of basal and squamous cells could not be compared in this study, RT-PCR demonstrated that angiotensin-converting enzyme mRNA is expressed by basal and squamous bronchial epithelial cells, as well as by human placenta. In addition, we found that cultured squamous cells secreted the enzyme into the culture media. Serum is a cocktail of various factors that may directly induce the expression and release of the enzyme from bronchial epithelial cells. The causal relation between squamous differentiation and secretion of angiotensin-converting enzyme still remains unclear. However, we demonstrated the ability of human bronchial epithelial cells to produce this enzyme. Our results suggest the possibility that the source of angiotensin-converting enzyme activity in bronchoalveolar and nasal lavage fluid obtained from humans is, at least in part, airway epithelial cells.

Metalloprotease-mediated cleavage secretion of pulmonary angiotensin-converting enzyme from vascular endothelial and kidney epithelial cells has been reported, and this secretion is enhanced by treatment of the cells with phorbol ester (Ramchandran et al., 1996). As in their study, in our study angiotensin-converting enzyme activity in the cell homogenate of bronchial epithelial cells could be due to a cell-associated enzyme and that in the conditioned medium could be derived from a soluble form produced by cleavage remodelling of the carboxyl terminal membrane-anchoring tail by a metalloprotease. The enhanced secretion of the enzyme in response to serum treatment in our study might be equivalent to the response to treatment with phorbol ester in their study.

Angiotensin-converting enzyme was also present in foetal bovine serum in a soluble form. Heat-inactivated serum (autoclaved at 120°C for 60 min) did not show any angiotensin-converting enzyme activity, but it could promote squamous differentiation of bronchial epithelial cells. Therefore, to confirm that the enzyme activity is not taken

up from serum added to the conditioned medium via sticking of proteins to the cell membrane, we also used denatured serum for serum treatment. Subsequently, the enzyme activity was also observed in the conditioned medium quite similar to that measured when non-denatured serum was used (data not shown).

The main enzymes thought to degrade bradykinin in vivo are angiotensin-converting enzyme (kininase II), carboxypeptidase N (kininase I), neutral endopeptidase, and aminopeptidase (Linz et al., 1995). Kininase I removes the carboxyl terminal Arg to form Des-Arg^9-bradykinin, an active metabolite. Neutral endopeptidase removes the carboxyl terminal Phe-Arg to form bradykinin-(1–7), an inactive fragment. Angiotensin-converting enzyme exhibits the same activity as neutral endopeptidase, but also cleaves the bond between Phe^5 and Ser^6 to form bradykinin-(1–5), an inactive fragment. It is conceivable that angiotensin-converting enzyme may regulate the kallikrein–kinin system in the airway mucosa by hydrolysing bradykinin. Indeed, we demonstrated that cultured human bronchial epithelial cells degraded bradykinin to bradykinin-(1–5) and that this enzymatic activity was inhibited by angiotensin-converting enzyme selective inhibitors. The EIA for both bradykinin and bradykinin-(1–5) shows less than 0.1% cross-reactivity between bradykinin and bradykinin-(1–5), and further, less than 0.2% cross-reactivity with Des-Arg^9-bradykinin and bradykinin-(1–7). Therefore, our data suggest that angiotensin-converting enzyme is one of the key enzymes that limits the effects of bradykinin in the respiratory system.

To examine the hydrolysis of bradykinin added to the conditioned medium of bronchial epithelial cells, we used nanomolar concentrations of bradykinin in order to more closely imitate pathophysiological conditions. Consequently, the inhibition of bradykinin hydrolysis by angiotensin-converting enzyme inhibitors seemed weaker than the inhibition of hippuric acid production. This discrepancy may be caused by differences in the concentrations of the substrates, bradykinin and HHL. The inhibition of hippuric acid production was examined in the presence of an excess amount of HHL. In contrast, the inhibition of bradykinin degradation was examined in the presence of a small amount of the substrate (bradykinin). Therefore, the IC_{50} values of the angiotensin-converting enzyme inhibitors were different in this study because the relative concentrations of the enzyme and substrates were different in the assays.

Although we did not test the effect of bradykinin on bronchial epithelial cells, bradykinin receptors have been localised in airway epithelial cells (Denning and Welsh, 1991; Mak and Barnes, 1991; Ohkubo et al., 1994). Stimulation of the bradykinin receptor may induce the release of prostanoids such as prostaglandin E_2, which counteract the bronchoconstrictor effect of bradykinin (Bramley et al., 1990; Hulsmann et al., 1994). The peptide has been shown to regulate airway epithelial cell functions, such as airway ciliary motility (Tamaoki et al., 1989), release of neutrophil and monocyte chemotactic activity (Koyama et al., 1995), and chloride transport (Denning and Welsh, 1991). Thus, angiotensin-converting enzyme may play an autocrine role in modulating airway epithelial cell function by regulating the local concentration of bradykinin.

The contractile effect of bradykinin on guinea pig airway smooth muscle may be inhibited by the protective function of the epithelium, which releases relaxant prostanoids (Frossard et al., 1990). The bronchoconstrictor effect of bradykinin in ferrets in vitro and in guinea pigs in vivo is enhanced by the inhibition of neutral endopeptidase by phosphoramidon and the inhibition of angiotensin-converting enzyme by captopril (Dusser et al., 1988; Ichinose and Barnes, 1990). These studies also suggest that kininase I is not involved in the metabolism of bradykinin. The relative importance of the three main carboxypeptidases (angiotensin-converting enzyme, neutral endopeptidase, kininase I) involved in the metabolism of kinins in serum is different in rats, dogs, rabbits, and humans (Décarie et al., 1996). In rats, angiotensin-converting enzyme and the kidney are important to the metabolism of circulating kinins, while carboxypeptidase N, neutral endopeptidase, and aminopeptidase B are not (Ishida et al., 1989). Together, these studies suggest that the metabolic pathway of bradykinin differs depending on the animal species and/or tissue investigated. However, our data suggest that the participation of angiotensin-converting enzyme is of importance in human airway epithelium.

Not only bradykinin but also angiotensin II has a potential role in the respiratory system. Angiotensin II causes bronchoconstriction in vivo in humans and plasma levels of angiotensin II have been shown to be elevated in patients with acute severe asthma (Millar et al., 1994). Angiotensin II has been shown to be a weak mitogen and a potent hypertrophic stimulus for human airway smooth muscle cells in vitro (McKay et al., 1998). Thus, angiotensin II has been implicated in bronchoconstriction and in airway smooth muscle growth during airway remodelling in asthma. Although the physiological and pathophysiological role of angiotensin-converting enzyme in airway epithelium in vivo remains to be clarified, we speculate that bronchial epithelial angiotensin-converting enzyme is involved in both angiotensin II formation and bradykinin degradation in the airway epithelium.

Gene expression of angiotensin-converting enzyme in vessels and epithelia is increased by angiotensin-converting enzyme inhibition but not by angiotensin II suppression in normal rodents in vivo (Costerousse et al., 1998). Our in vitro study showed that the angiotensin II receptor antagonist did not change the level of angiotensin-converting enzyme in the conditioned medium of bronchial epithelial cells (Fig. 4). However, comparison of Figs. 3 and 4 shows that the enzyme inhibitors do not have a pronounced effect on the expression of angiotensin-converting enzyme, although it is necessary to determine the concen-

tration of angiotensin-converting enzyme after removal of the inhibitors by dialysis. It remains to be established whether long-term treatment with the enzyme inhibitors alters the concentration or gene expression of angiotensin-converting enzyme in human bronchial epithelial cells.

Dry cough is the most common adverse effect of angiotensin-converting enzyme inhibitors. The mechanism for this dry cough is considered to be linked to the suppression of the enzyme activity, which may be followed by the accumulation of kinins. The newest class of antihypertensive agents, angiotensin II receptor antagonists such as valsartan, act directly on the renin–angiotensin cascade by blocking specifically the angiotensin II receptor site. They have a different mechanism of action from that of angiotensin-converting enzyme inhibitors, which prevent the formation of angiotensin II via inhibition of the enzyme (Criscione et al., 1993; Foote and Halstenson, 1993). Therefore, they would be assumed to be less problematic in terms of unwanted side effects like cough (Holwerda et al., 1996). The mechanism underlying angiotensin-converting enzyme inhibitor-induced cough is still unclear, although the involvement of bradykinin has been postulated. The effect of bradykinin has been assessed not only on the direct action of smooth muscle but also on the indirect and alternative activity of sensory nerves in airways (Fox et al., 1996). In airways, if bradykinin is mainly degraded by bronchial epithelial angiotensin-converting enzyme and if the accumulation of bradykinin really causes dry cough, our present data are compatible with the mechanism presented in the studies cited above.

In summary, the present results indicate that cultured human bronchial epithelial cells possess angiotensin-converting enzyme and have the potential to secrete the enzyme. We also demonstrated that the degradation of bradykinin by bronchial epithelial cells was inhibited by angiotensin-converting enzyme inhibitors. Bronchial epithelial angiotensin-converting enzyme might play an important role in regulating local concentrations of bradykinin in the airways.

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