Angiotensin-converting enzyme activity in ovine bronchial vasculature

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Moldobaeva, Aigul, and Elizabeth M. Wagner. Angiotensin-converting enzyme activity in ovine bronchial vasculature. J Appl Physiol 95: 2278–2284, 2003. First published August 1, 2003; 10.1152/japplphysiol.00266.2003.—Angiotensin-converting enzyme (ACE) plays a major role in the metabolism of bradykinin, angiotensin, and neuropeptides, which are all implicated in inflammatory airway diseases. The activity of ACE, which is localized on the luminal surface of endothelial cells (EC), has been well documented in pulmonary EC; however, few data exist regarding the relative activity of ACE in the airway vasculature. Therefore, we measured ACE activity in cultured EC from the sheep bronchial artery and bronchial mucosa (microvascular) and compared it with pulmonary artery EC. The baseline level of total ACE activity (cellular plus secreted) was significantly greater in bronchial microvascular EC (1.24 ± 0.24 mU/10⁶ cells) compared with bronchial artery EC (0.59 ± 0.15 mU/10⁶ cells; P < 0.05) and comparable to pulmonary artery EC (1.12 ± 0.14 mU/10⁶ cells; P > 0.05). Measured ACE activity secreted into culture medium for each cell type was 64–74% of total activity and did not differ among the three EC types (P = 0.17). Hydrocortisone (10 μg/ml; 48–72 h) treatment resulted in a significant increase in ACE activity in bronchial EC. Likewise, TNF-α (0.1 ng/ml) treatment markedly increased ACE activity in all cell lysates (P < 0.05). We confirmed the importance of ACE activity in vivo since, at the highest dose of bradykinin studied (10⁻⁸ M), bronchial artery pressure at constant flow showed a greater decrease after captopril treatment (36% before vs. 60% after; P = 0.05). These results demonstrate high ACE expression of the bronchial microvasculature and suggest an important regulatory role for ACE in the metabolism of kinin peptides known to contribute to airway pathology.

brachial endothelial cells; captopril; hydrocortisone; tumor necrosis factor-α

ANGIOTENSIN-CONVERTING ENZYME (ACE), although known primarily for the conversion of angiotensin I into the potent vasoconstrictor angiotensin II, also inactivates bradykinin and tachykinins that have been implicated in the pathogenesis of asthma (4, 11). These kinin peptides are produced in both plasma and airway tissue during an inflammatory response and elicit vasodilation (41), vascular leak (24, 41), and bronchospasm (8). Thus ACE may serve a crucial regulatory role within the airway for the inactivation of these inflammatory peptides. ACE has been shown to be expressed in high levels in the lung (12). Although localization of ACE specifically on the luminal surface of pulmonary endothelial cells (EC) has been reported (33), little is known regarding ACE activity in the airway circulation, which is the more relevant vasculature in inflammatory airway disease. Roisman and colleagues (32), using immunohistochemical techniques on human airway biopsy material, confirmed that ACE is expressed by EC of the bronchial mucosa as well as submucosal vessels surrounding airway smooth muscle. Furthermore, ACE activity in bronchoalveolar lavage fluid was shown to be increased in subjects with asthma compared with normal subjects (30). Although the increase in ACE activity could serve a compensatory function and enhance the metabolism of bradykinin and tachykinins, asthmatic subjects have been shown also to have increased levels of bradykinin and tachykinins in bronchoalveolar lavage fluid (10, 39) and in plasma (1). Collectively, these observations suggest the inability of normal or enhanced metabolic activity to deal with an excessive inflammatory burden and thereby contribute to sustained levels of kinin peptides and the pathogenesis of airway disease.

Increased ACE activity could also result in increased levels of angiotensin II. Indeed, the plasma angiotensin II level has been shown to be elevated in acute severe asthma (27, 31). Furthermore, infused angiotensin II induces bronchoconstriction (27) and potentiates the effects of other bronchoconstrictive agents in subjects with mild asthma (28). Angiotensin II also has been shown to promote the hypertrophy of human airway smooth muscle cells in vitro, and it is thereby suggested to play a role in the airway remodeling of asthma (16, 25).

Although ACE inhibition is known to play a pivotal role in the regulation of vascular disease (15), the control of ACE is incompletely understood. ACE activity has been shown to be modulated by several factors implicated in the pathogenesis of asthma such as platelet-activating factor (21), endothelin-1 (22), reactive oxygen species (9), and TNF-α (34). Additionally, growth factors such as VEGF (35) and glucocorticoids (20) have been shown to increase ACE activity. However, an overriding concern about these published re...
sults relates to the observation that EC from different vascular beds show differential ACE expression (6). Most studies documenting ACE activity have used EC from human umbilical vein, pulmonary artery, or other organs. Consequently, the present study was undertaken to determine ACE activity in bronchial vascular EC, where its regulation has not been characterized. We measured ACE activity in sheep bronchial conduit EC and bronchial microvascular EC at baseline and after treatment with corticosteroids and the inflammatory mediator TNF-α.

METHODS

Materials. Dulbecco’s PBS (DPBS), DMEM, MEM nonessential amino acids solution 10 mM (100×), 10,000 U/ml of penicillin and streptomycin, 25 μg/ml of amphotericin B, 0.05% trypsin, 0.53 mM EDTA, and lamb serum were purchased from Life Technologies (Gaithersburg, MD). 1,1-Dioctadecyl-1,3,3,3′-tetramethylindocarbocyanine perchlorate–labeled low-density lipoproteins were obtained from Molecular Probes (Eugene, OR). Collagenase, gelatin, bradykinin, O-phthalaldehyde, captopril, and hydrocortisone were obtained from Sigma Chemical (St. Louis, MO). EC growth supplement was purchased from Upstate Biotechnology (Lake Placid, NY). Cloning disks were from Scienceware (Fisher, Pittsburgh, PA). Z-Phe-His-Leu was from Bachem Bioscience (King of Prussia, PA). TNF-α was obtained from R&D Systems (Minneapolis, MN).

In vivo experiments. To determine the role of ACE activity in the regulation of vascular tone in the bronchial circulation, bradykinin dose-response relationships were studied in sheep before and after treatment with an ACE inhibitor. The study protocol was approved by the Johns Hopkins Animal Care and Use Committee. Anesthesia was induced in sheep (20–35 kg) with intramuscular ketamine (30 mg/kg) and intravenous pentobarbital sodium (0.1–0.2 mg/ml) and a subcutaneous injection of atropine (0.1 mg/kg). The sheep was intubated, and the lungs were mechanically ventilated (10–12 breaths/min). After thoracotomy, the bronchial branch of bronchosophageal artery was isolated, cannulated, and perfused (0.6 ml/min 12–15 mm Hg) through a normal-speed roller pump (42). Sheep were administered heparin (20,000 U iv), and bronchial artery pressure was measured continuously at a side arm of the inflow cannula. Bradykinin (10−10–10−6 M) was infused (1 ml/min for 2 min) directly into the bronchial artery perfusion circuit. After the highest dose of bradykinin was infused and bronchial artery pressure returned to baseline, the ACE inhibitor captopril (1 mg/kg; 10 ml) was delivered through the perfusion circuit and the bradykinin dose response was repeated. Control reproducibility studies in two sheep showed no alterations in the dose-response relationship with repeat bradykinin administration.

Isolation of bronchial artery, bronchial microvascular, and pulmonary artery EC. Bronchial artery and bronchial microvascular EC were isolated and cultured as previously described (29). After cannulating the bronchial artery (as above) and dissecting it free, we gently flushed the vessel with DPBS to remove residual blood and filled it with collagenase. After a 10-min incubation period at room temperature, the vessel was perfused with DMEM, and the perfusate was collected in a tube and centrifuged at 500 g for 7 min. The cell pellet was resuspended in 2 ml of culture medium (DMEM with 20% of lamb serum, 150 μg/ml EC growth supplement, 100 U/ml penicillin/streptomycin, 0.25 μg/ml amphotericin B, 0.1 mM MEM nonessential amino acids) and placed in a 0.2% gelatin-coated 35-mm tissue culture dish. The medium was changed every 2 days thereafter. Bronchial artery EC were identified by their cobblestone morphology. When cells were grown to confluence, they were transferred to T-25 flasks and subcultured.

Bronchial microvascular EC were isolated from sheep mainstem bronchi and subtended airways. The epithelial cell layer was removed, and the underlying tissue with microvessels to the level of cartilage was dissected free. These tissue pieces were placed in 1 ml of collagenase and were incubated for 10 min at room temperature. After incubation, 15 ml of DMEM were added and cells were filtered through a nylon mesh. The remaining procedures were the same as those applied in the isolation of bronchial artery EC.

Pulmonary artery EC were isolated from the main pulmonary artery in sheep. The vessel was separated from connective tissue, cut longitudinally, washed in DMEM, and incubated for 10 min in collagenase. EC were pelleted at 500 g for 7 min. The remaining procedures were the same as those applied in the isolation of bronchial artery EC. EC were studied between passages 5 and 8, and the passage number was matched among studies of the three EC types.

Measurement of ACE activity. ACE activity was measured by a modification of the fluorometric method as previously described (2). EC were grown to confluence in 35 mm petri dishes, and ACE activity was determined 2 days after cells reached confluence. Cells were washed twice with DMEM and incubated with DMEM for 24 h with or without agonists. Because serum has been shown to exhibit significant levels of ACE activity that is derived from EC (17), all measurements were performed after 24 h incubation in serum free medium. Doses of agonists were selected based on the published results of others and included hydrocortisone (0.1–10 μg/ml) (20) and TNF-α (0.001–20 ng/ml) (34). The culture medium was collected, centrifuged to remove cell debris, frozen and stored at −20°C. For cell lysis, EC were washed twice with PBS and then 50 mM Tris-HCl, 0.15 M NaCl, and 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfo nate (Calbiochem, La Jolla, CA) were added. Lysed cells were scraped, homogenized by sonication at 4°C, and frozen. To determine ACE activity, frozen samples were thawed, and culture medium (10 μl) and cell lysate (10 μl) were added to substrate (Z-Phe-His-Leu; 2 mM, 200 μl) and incubated for 3 h at 37°C. The reaction was terminated with NaOH (0.28 N; 1.5 ml) and the His-Leu product was revealed by incubation (10 min at 37°C) after addition of O-phthalaldehyde in methanol (100 μl of 10 mg/ml). After 10 min, HCl (2 N; 200 μl) was added and the fluorescence of samples was measured spectrophotometrically (363 nm excitation wavelength; 500 nm emission wavelength). In initial studies, absolute baseline ACE activity (nM/min = mU) of each endothelial subtype was determined for 106 cells, which had been counted (mU/106 cells). In subsequent experiments, cell lysate and culture medium ACE activities were expressed in milliunits per milliliter and compared with vehicle control activity (% control).

Cytotoxicity assay. Toxicity of agonists was evaluated by measuring lactate dehydrogenase (LDH) activity released into the medium 24 h after exposure to test agonists using CytoTox 96 nonradioactive cytotoxicity assay (Promega; Madison, WI) and quantified by measuring absorbance at 490 nm. Data are normalized to the amount of LDH activity from cell lysates (100%) and corrected for baseline LDH release from vehicle-treated cells.
Statistical analysis. All data are presented as the means ± SE. A one-way ANOVA followed by Newman-Keuls multiple-comparisons test was used to compare responses of the three EC types as well as dose-response data within a cell type. Student’s t-test was used to compare ACE activity in cell lysates relative to activity in culture medium. A P value of 0.05 was accepted as significant.

RESULTS

In vivo infusion of bradykinin into the bronchial artery in sheep (n = 5) caused a dose-dependent vasodilation in the bronchial circulation as evidenced by the decrease in bronchial artery pressure at constant inflow (16 ± 2 ml/min). Baseline bronchial artery pressure was 121 ± 22 mmHg. The changes in bronchial artery pressure observed with increasing concentrations of bradykinin (10–10 M–10–8 M) are presented in Fig. 1. Bradykinin caused a dose-dependent vasodilator effect (∗P < 0.05). These results demonstrate the in vivo importance of ACE activity in the metabolism of infused bradykinin.

The baseline level of total ACE activity (cellular plus secreted) was significantly greater in bronchial microvascular EC (1.24 ± 0.24 mU/106 cells) compared with bronchial artery EC (0.59 ± 0.15 mU/106 cells; P < 0.05) and comparable to pulmonary artery EC (1.12 ± 0.14 mU/106 cells; P > 0.05). Basal ACE activity in EC lysates and in culture medium of pulmonary artery EC cultures, bronchial artery EC cultures, and bronchial microvascular EC cultures are presented in Fig. 2 (n = 4/cell type). As can be seen, ACE activity measured in EC lysates was significantly less than the secreted ACE activity measured in culture medium for pulmonary artery EC and bronchial artery EC (∗P ≤ 0.05). Although not statistically significant, the average activity showed a similar trend in bronchial microvascular EC (∗P = 0.15). ACE activity measured in the culture medium for each cell type averaged 2.0- to 2.8-fold greater than the activity expressed in the paired EC lysates. ACE activity secreted into the culture medium did not differ among the three EC types (∗P = 0.17).

However, baseline levels of ACE activity were significantly lower in bronchial artery EC lysates (0.15 ± 0.02 mU/106 cells) compared with bronchial microvascular EC lysates (0.45 ± 0.10 mU/106 cells) and pulmonary artery EC ACE activity (0.38 ± 0.05 mU/106 cells; ∗P < 0.05).

To confirm the inhibitory effects of captopril on ACE activity, EC were treated with captopril (10 µg/ml) for 1 h before ACE activity was measured in pulmonary artery EC (n = 5), bronchial artery EC (n = 3), and bronchial microvascular EC (n = 3). A substantial reduction in ACE activity was observed in EC lysates (42–49% of control activity) and ACE activity secreted into culture medium (52–58% of control activity). No difference in response to captopril was seen among the cell types in ACE activity in either cell lysates (∗P = 0.93) or culture medium (∗P = 0.83). The reduction in ACE activity after captopril treatment in EC lysates as a group was not different from culture medium (∗P = 0.14).

To determine whether any of the selected agonist concentrations were cytotoxic to any of the three EC subtypes, we performed an initial cytotoxicity screening assay (n = 2 experiments/cell type). The highest concentration of hydrocortisone and the complete dose range for TNF-α were studied. Only high concentrations of TNF-α (10–20 ng/ml) showed substantial cytotoxicity as reflected by the amount of LDH released compared with vehicle-treated cells (Fig. 3).

In initial studies (n = 6/EC type), no change in ACE activity was observed in either bronchial EC subtype within the experimental hydrocortisone dose range (0.1–10 µg/ml) after 24-h incubation. Therefore, the highest concentration of hydrocortisone (10 µg/ml) studied, which resulted in a significant increase (81%; ∗P = 0.04) in pulmonary artery EC lysate ACE activity, was selected for additional experiments in bronchial EC. ACE activity in EC lysates after short treatment times (4, 8, 18, and 24 h) and treatment times longer (48 and 72 h) than the original series (24 h) were grouped for each bronchial EC subtype. Results are
shown in Fig. 4 and demonstrate that for both bronchial artery EC (n = 11 short, 6 long) and bronchial microvascular EC (n = 11 short; 6 long) significant increases in ACE activity were observed after longer hydrocortisone treatment (P < 0.01 and P = 0.02, respectively). ACE activity secreted into the culture medium ranged from 98 to 132% of vehicle controls (P > 0.05).

Because TNF-α concentrations >10 ng/ml caused substantial cytotoxicity (Fig. 3) that could affect ACE activity, we measured ACE activity after incubation with a TNF-α concentration (0.1 ng/ml) that caused negligible cytotoxicity. This low concentration of TNF-α caused substantial increases in ACE activity in all EC lysates (Fig. 5). ACE activity increased in pulmonary artery EC (n = 7; P = 0.01), bronchial artery EC (n = 9; P = 0.02), and bronchial microvascular EC (n = 9; P = 0.02). However, only in culture medium from bronchial microvascular EC was a significant increase in ACE activity observed (n = 9; P = 0.01).

**DISCUSSION**

ACE plays a major role in the metabolism of bradykinin, angiotensin, and several neuropeptides, all of which have been implicated in the pathogenesis of inflammatory airway diseases. However, most work defining ACE activity in the lung has focused on its regulation in pulmonary vascular endothelium. Given EC heterogeneity within the lung, including the airway tree supplied by the systemic vasculature, we questioned whether ACE activity in cultured EC from sheep bronchial artery and bronchial microvascular EC is similar to activity in pulmonary artery endothelium. Our results document differences in basal ACE activity as well as responsiveness of the endothelial subtypes to growth factors. Additionally, we confirmed a significant in vivo role for ACE within the conducting airways. These results provide further support for the importance of ACE in the metabolism of peptides implicated in airway disease.

As previously shown by several investigators, bradykinin exerts a profound vasodilatory effect on the bronchial vasculature (Fig. 1). In vivo treatment by intrabronchial artery delivery of the ACE inhibitor captopril caused significant enhancement of bronchial vasodilation. These observations indicate that normally bradykinin is rapidly inactivated by ACE within the bronchial vasculature. With ACE inhibition, bradykinin metabolism was attenuated and greater vasodilatory effects of this peptide were observed. Additionally, these results are suggestive of ACE activity in bronchial conduit EC because the changes in bronchial artery pressure are related to changes in larger vessel resistance. Previous work by Grantham and colleagues (18) in an in situ perfused bronchial artery sheep preparation showed that the ACE inhibitor SQ 20881 significantly depressed metabolism of a synthetic peptide substrate for ACE. These authors concluded that the bronchial circulation is pharmacokinetically and metabolically active with respect to bradykinin and that the enzymes responsible for this metabolic activity line the vascular lumen. The results of the present in vivo experiments indicate also that ACE exerts a substantive functional influence on the bronchial vasculature by modulating vascular smooth muscle tone. Insofar as bronchial blood flow has been shown to modulate agonist-induced airway smooth muscle constriction, the bronchial circulation is permissive in allowing leukocyte recruitment to the bronchial air-
ways from the level of the carina to the terminal bronchioles, and airway edema that contributes to airways narrowing is due to fluid and protein exudation from the bronchial circulation, ACE activity may play an important regulatory role during airway inflammation.

This study is the first to investigate the distribution of ACE activity in bronchial artery, bronchial microvascular, and pulmonary artery EC. Previous studies of ACE activity have shown differential ACE expression in EC from different vascular beds (6), decreased ACE activity with EC subcultivation (2), and increased ACE activity in the presence of serum and growth factors (17, 35). Therefore to assess accurately EC heterogeneity requires standardization of conditions across cell types. To make relevant comparisons we studied pulmonary artery, bronchial artery, and bronchial microvascular EC of the same passage number, using the same culture medium and EC isolated from the same species. We evaluated ACE activity within EC lysates as well as that secreted into the EC culture medium. During basal conditions when all EC were treated similarly, all three cell types showed greater ACE activity secreted into culture medium than in cell lysates (Fig. 2). This result is consistent with observations in human umbilical vein EC and pulmonary vascular EC cultures (2, 37). Interestingly, however, increased ACE activity with application of hydrocortisone and TNF-α was observed predominantly in cell lysates. The mechanism responsible for this differential regulation is unclear but may relate to the timing of exposure of agonists. We can only speculate that other rate-limiting factors exist for secretion, and further experimentation is required to understand more fully the kinetics of this enzyme.

Bronchial microvascular EC lysates demonstrated significantly greater ACE activity than conduit bronchial artery EC. Previous work by Tamaru and colleagues (37) showed that ACE activity in cell lysates and culture medium of pulmonary microvascular EC was greater than in pulmonary artery EC cultered from large central pulmonary arteries. Although we did not study ACE activity of pulmonary microvascular EC, this previous study and our present work together are suggestive of microvascular EC demonstrating greater ACE activity than EC from their respective conduit vessels. Furthermore, pulmonary endothelium has been shown to express considerably more ACE activity than other peripheral vascular beds (3, 6). Thus the fact that bronchial microvascular endothelium expresses levels of ACE activity equivalent to that of the pulmonary vasculature suggests an important role for this enzyme in airway vascular homeostasis. Interestingly, conduit bronchial EC show less ACE activity despite the in vivo observation that blockade of ACE has a major impact on vasodilation. Bronchial microvascular EC that demonstrated greater ACE activity are unlikely to be involved in regulation of vasmotion based on location; however, they may participate in other regulatory control of kinins such as modulation of permeability or inflammatory cell recruitment.

Although the control of ACE activity in vascular endothelium is not completely understood, several studies have demonstrated that glucocorticoid treatment increases ACE activity. ACE activity has been shown to be increased after glucocorticoid treatment in bovine aortic EC in culture and in rat lung in vivo (13, 20, 26). However, glucocorticoid treatment has also been shown to inhibit the secretion of ACE (5, 26). Given the potential importance of ACE in asthma and inflammatory airway disease in which steroid treatment is common, we evaluated the effects of glucocorticoid treatment on bronchial and pulmonary EC. Using a treatment time (24 h) and dose range previously shown to cause an increase in ACE activity in bovine aortic EC (20), we saw no effect on ACE activity in any of the lung EC or culture medium. We performed additional experiments both increasing and decreasing the incubation time with the highest hydrocortisone concentration and found that both bronchial artery and bronchial microvascular EC lysates showed increased ACE activity after longer incubation times (48–72 h) with hydrocortisone. Secreted ACE of all cell types remained unchanged. We conclude that sheep EC are relatively resistant to the effects of glucocorticosteroids with regard to ACE activity induction. However, prolonged treatment can significantly increase ACE activity within airway endothelium. Extrapolation of this observation to airway disease in human subjects suggests that corticosteroid treatment may increase the metabolism of peptides released in the inflamed airway.

We also studied the effects of TNF-α on ACE activity in lung EC. TNF-α is a potent proinflammatory cytokine that plays a role in the initiation of allergic airway inflammation and the generation of airway hyperreactivity (19, 38). Furthermore, an interaction between TNF-α and ACE has been suggested by studies of the genetic basis for hyperactive airway disease (7, 43). TNF-α also has been shown to have regulatory effects on EC barrier function, adhesion molecule expression, and vasoregulation (23). Saijonmaa and colleagues (34) previously reported that TNF-α downregulated ACE activity in cultured human umbilical vein EC and human aortic EC. Thus we studied the same concentration range as these investigators, expecting to find a decrease in ACE activity. However, an initial cytotoxicity screening revealed that the higher TNF-α concentrations resulted in significant cytotoxicity of all the lung EC subtypes. Cytotoxicity to TNF-α has been likewise observed in bovine capillary EC (36). Furthermore, Diez and colleagues (14) showed a direct correlation between increased ACE activity and cell apoptosis in cardiomyocytes. We chose to use a lower TNF-α concentration that did not result in significant cytotoxicity. ACE activity was markedly enhanced in each of the EC types studied. As observed after steroid treatment, no change was observed in the secreted ACE measured in culture medium. Overall these results are opposite to the observations of Saijonmaa and col-

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leagues. We can only speculate that the difference in response is related to differences between lung EC and human umbilical vein EC or the effects of agonist concentration.

Additionally, we have previously shown that intravascular infusion of TNF-α caused vasoconstriction in the bronchial circulation (40). Although much of this response was attributed to secondary release of endothelin-1, a portion of the vasoconstriction was not explained by this mechanism. Because in the present study TNF-α caused an increase in ACE activity of the bronchial vascular endothelium, this increase could contribute to vasoconstriction in vivo by enhanced metabolism of circulating vasodilator peptides. Additional in vivo studies with ACE and endothelin-1 antagonist treatment before TNF-α challenge will provide further information regarding the mechanism of vasoconstriction.

Although we have previously shown bronchial EC heterogeneity with regard to barrier properties of confluent monolayers (29), results observed in the present study showed qualitatively similar responses. We have shown that bronchial artery and bronchial microvascular EC in vitro demonstrate similar responsiveness to hydrocortisone and TNF. Furthermore, ACE activity of the bronchial vascular endothelium was equivalent to that expressed in pulmonary artery endothelium. Thus we confirm that the bronchial vascular endothelium exhibits high ACE activity and has the potential to regulate metabolic activity in response to an inflammatory stimulus. Control of this enzyme within the airway may have a major impact on peptide metabolism in inflammatory airway disease. Furthermore, conjugation of therapeutic agents to antibodies targeted to ACE in the airway vascular endothelium might provide selective airway treatment options.

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