Properties of Angiotensin-Converting Enzyme in Intact Cerebral Microvessels

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SUMMARY Angiotensin-converting enzyme (ACE) was studied in preparations of microvessels isolated from rabbit cerebral cortex. Activity was determined by measuring the degradation of hippuryl-histidyl-leucine (Hip-His-Leu) by the intact microvessels in a physiological salt solution at pH 7.4. ACE activity was dependent on both substrate and chloride ion concentration and was inhibited by captopril in a manner similar to that observed previously with tissue homogenates. Angiotensin I was rapidly degraded by the intact microvessels, even in the presence of 10^-6 M captopril. An advantage of the methodology employed was the ability to pretreat the microvessels and then assess the effect of pretreatment by transfer to a postincubation assay system. Pretreatment with a hyperosmolar urea solution did not change ACE activity or cause release of ACE from the microvessels, although lactic dehydrogenase and lysosomal enzymes were released. Pretreatment with captopril caused a lag in the subsequent degradation of Hip-His-Leu, presumably reflecting dissociation of inhibitor from the cell-associated enzyme. ACE activity was unaffected by hypoxic or anoxic incubation conditions. The ability to measure ACE activity of the microvessels in vitro provides a unique opportunity to study the properties of the enzyme in intact cerebrovascular endothelial cells. (Hypertension 3: 198-204, 1981)

KEY WORDS • angiotensin converting enzyme • cerebral microvessel • endothelial cells

ANGIOTENSIN-converting enzyme (peptidyl-dipeptide hydrolase, EC 3.4.15.1) is known to have an important physiological role in the metabolism of circulating angiotensin I and bradykinin.1-4 ACE activity has been demonstrated in many tissues and has been localized on the vascular endothelium.5, 4 Techniques for the isolation and culture of vascular endothelial cells have led to several reports documenting ACE activity in isolated or cultured cells from pulmonary artery, aorta, and umbilical vein of several species.5, 4 The enzyme has served as a convenient cellular marker for cultured endothelial cells, and tissue culture systems have been used to examine several properties of the enzyme including subcellular localization and synthesis,6 release into culture medium,6 and inhibition by hypoxic conditions.6

We have recently described the presence of ACE in homogenates of isolated microvessels prepared from rabbit cerebral cortex.10 Such microvessel preparations consist primarily of arterioles, venules, and capillaries, and are both morphologically intact and metabolically active.11, 12 Activity also was reported in microvessels obtained from bovine retina.13 Because these microvessel preparations are enriched in endothelial cells, they offer a unique opportunity to study the in vitro properties of ACE using freshly prepared endothelial cells not previously exposed to the somewhat artificial conditions inherent in tissue culture systems. In the present study we have developed an in vitro assay system for measuring ACE using intact cerebral microvessels, and have used this methodology to examine the effect of captopril and the influence of hypoxia and hyperosmolarity on this enzyme.

Experimental Materials and Methods

Materials

Hippuryl-histidyl-leucine (Hip-His-Leu) was purchased from Peninsula Laboratories, Inc. Labeled Hip-His-Leu containing [1-14C] glycine (specific activity 3.8 mCi/m mole) was obtained from New England Nuclear Corporation. Angiotensin I, angiotensin II, and bradykinin were purchased from Peninsula Laboratories Inc., and D-3-mercapto-2-methylpropanoyl-L-proline (captopril) was obtained from Squibb Pharmaceutical Company.
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Preparation of Microvessels

All studies were performed using male New Zealand White rabbits weighing between 1-1.5 kg. Animals were killed by decapitation; the brains were removed and placed in a buffered salt solution containing 27 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.4; 118 mM NaCl; 5.4 mM KCl; 1.8 mM CaCl2; 1.0 mM MgSO4; 1.0 mM Na2HPO4 and 5.5 mM glucose. This solution was used throughout the preparation procedure and also as the incubation solution for most enzymatic studies with the intact microvessels.

The microvessels were prepared using a modification of previously described procedures.

The microvessels were prepared using a modification of previously described procedures. Routinely, brains from two to three animals were cleaned and the cerebral cortices, free of white matter and pial membrane, were pooled, minced, and homogenized in 15 ml buffer in a loose-fitting, hand-held Dounce homogenizer using six vertical passages. The homogenate was filtered through a 149 µm nylon mesh contained in a 49 mm diameter Swinnex holder. The material collected on this mesh was homogenized again with the Dounce homogenizer using two strokes, and refiltered through a 149 µm mesh. The material collected on this second 149 µm mesh was discarded, and the filtrate was added to that of the first passage. The combined filtrates were passed successively through a 105 and 74 µm mesh in the 49 mm Swinnex holder and then through a 74 µm mesh in a 25 mm diameter holder. The microvessels contained on the three meshes were collected, pooled, and gently homogenized in a Dounce homogenization apparatus with a clearance of 0.25 mm. This homogenate was filtered serially through a 105 µm and two 74 µm meshes contained within 25 mm Swinnex holders, and the material collected on these meshes was pooled, and finally collected onto one or two 53 µm mesh. Segments of the mesh were used for subsequent studies.

Angiotensin-Converting Enzyme Assay

Under standardized conditions, the microvessels, entrapped on a segment of the final mesh, were placed in 1 ml of incubation solution (defined above) which also contained 5 mM Hip-His-Leu as substrate. The reaction mixture was incubated at 37°C for 60 minutes, and a 0.25 ml aliquot of the reaction mixture was removed, mixed with an equal volume of 1 N HCl, and extracted with 1.5 ml ethyl acetate. A 1 ml aliquot of the ethyl acetate extract was evaporated under a stream of nitrogen and the residue dissolved in 1 ml of 1.0 M NaCl. The amount of hippuric acid formed as a result of hydrolysis was determined spectrophotometrically, essentially as described by Cushman and Cheung. The protein content of the microvessels was determined by the method of Lowry et al. as described previously. ACE activity was expressed as nmole of hippuric acid formed per minute per milligram of microvessel protein.

In several experiments, ACE activity also was determined by a radioassay using 14C-labeled Hip-His-Leu, labeled in the glycine 1-14C position. In these studies, the labeled substrate was used at a final specific activity of 2.0 µCi/mnmole in the standard assay solution. Following incubation, the reaction was stopped with an equal volume of 1 N HCl. Free hippuric acid was extracted into ethyl acetate as described above, and an aliquot of this organic phase added directly to 10 ml of scintillation cocktail (Liquisint, National Diagnostics) for scintillation counting. Blanks, obtained by omitting tissue from the standard assay solution, contained in the organic phase 1.1% of the total radioactivity originally added. When expressed as nmole hippuric acid formed, values obtained with the radioassay were identical to those obtained spectrophotometrically, using conditions where direct comparisons were made on aliquots of the same ethyl acetate extract.

For the experiments performed under hypoxic or anoxic conditions, microvessels entrapped on a segment of the nylon mesh were placed into Dispo beakers containing 1 ml of the incubation solution. This reaction vessel was connected to the appropriate gas supply using an apparatus described previously, and the gas flowed through continuously at the rate of 0.2 R³/hr/tube. Aliquots of the reaction mixtures were removed and assayed for released hippuric acid at designated times.

For the experiments involving pretreatment of microvessels with urea, segments of nylon mesh containing entrapped microvessels were placed into 1 ml of the standard incubation solution containing 2 M urea for 15 seconds at 25°C. Then the mesh was removed with forceps, gently rinsed in a beaker containing 100 ml of the standard incubation solution, and finally placed in 1 ml of the standard solution for postincubation at 37°C for 60 minutes. Aliquots of the incubation mixture were assayed spectrophotometrically for the following enzymes: lactate dehydrogenase was determined by measuring the rate of NADH disappearance; N-acetyl β-glucosaminidase (NAGA) was determined in the presence of 0.1% Triton X-100 as described previously by us, and acid phosphatase was assayed using p-nitro-phenyl phosphate as substrate at pH 4.2. The microvessels remaining on the mesh were homogenized in 0.01 M Tris, pH 7.4, and aliquots of the homogenates assayed for lactate dehydrogenase, NAGA, and acid phosphatase as described above. To determine the effect of urea pretreatment on ACE activity, microvessels were preincubated with urea as described above, transferred to the standard incubation solution for postincubation at 37°C for 60 minutes, and the supernatant assayed for ACE. The remaining microvessels were then assayed for ACE by a second postincubation in the presence of Hip-His-Leu.

Radioimmunoassay for Angiotensin I

The procedure used was a minor modification of the method employed for routine clinical determination of plasma renin activity. Aliquots of the incubation mixture containing angiotensin I were diluted serially,
and 20 µl aliquots were incubated overnight at 4°C with antibody and 125I-labeled angiotensin I. Bound and free peptides were separated by charcoal adsorption, and the amount of unlabeled angiotensin I present was calculated based on a standard curve derived from parallel incubations containing known amounts of angiotensin I.

Results

Properties of the Assay for ACE in Microvessels

Several characteristics of the assay system for measuring ACE activity in intact microvessels are shown in figure 1. All experiments were performed at 37°C in a physiological salt solution at pH 7.4 using Hip-His-Leu as substrate. Studies on the effect of substrate concentration showed that maximal activity was obtained at a substrate concentration of approximately 3 mM. To ensure zero order kinetics, all subsequent experiments were performed using 5 mM Hip-His-Leu. Activity was completely abolished in the presence of 10^-6 M captopril, a substance shown previously to inhibit ACE in cell-free systems.10' The reaction rate was directly proportional to incubation time for up to 180 minutes and was linear with respect to the amount of microvessel protein added between 0-250 µg. With intact microvessels, pH had only a slight effect on the hydrolysis of Hip-His-Leu between pH values of 7.0 and 8.8.

ACE activity in the intact microvessel preparation was dependent on the presence of chloride ion in the reaction mixture (fig. 2). Incubations were performed using a modified standard buffer where the NaCl was replaced by sucrose in osmolar equivalents. At concentrations below 5 mM, KCl also was replaced by sucrose. Almost complete loss of activity was observed when the chloride concentration was reduced below 5 mM. In separate experiments, it was established that addition of 0.25 M sucrose to the standard assay system did not diminish enzymatic activity.

The effect of several peptides on ACE activity is summarized in figure 3. When the intact microvessels were incubated in 1 ml of the standard incubation solution containing both 5 mM Hip-His-Leu and the designated concentrations of captopril, inhibition was observed even at 10^-6 M captopril, and complete inhibition occurred at about 10^-5 M. Bradykinin, angiotensin I, or angiotensin II also inhibited activity toward Hip-His-Leu, but only at concentrations between 10^-4 to 10^-3 M.

Experiments were performed to determine the metabolism of a physiological substrate, angiotensin
by the intact microvessel preparations (fig. 4). Angiotensin I was added at a concentration of $3 \times 10^{-4}$ M, and peptide present in the incubation mixture was estimated by radiimmunossay. As shown in figure 4, angiotensin I was rapidly degraded by the microvessels when incubated at 37°C with almost complete loss of immunoreactive peptide by 30 minutes. Interestingly, the presence of $10^{-4}$ M captopril had only a slight effect on angiotensin I metabolism, indicating that peptidases other than those inhibited by captopril were present in the system. This observation clearly emphasizes the advantage of using the synthetic substrate Hip-His-Leu, which is not readily degraded by proteolytic enzymes other than ACE.

Effect of Preincubation with Captopril

The marked inhibition of ACE activity toward Hip-His-Leu by captopril (fig. 3) indicated a strong interaction between the drug and the microvessel-associated enzyme. To assess the nature of this interaction in the intact cerebral microvessels, experiments were performed where the microvessels were preincubated for 10 minutes at 37°C in 1 ml of the standard incubation mixture which lacked substrate but contained the designated amount of captopril. Following preincubation, the microvessels were briefly washed in 10 ml of buffer and transferred into a reaction tube containing 5 mM Hip-His-Leu. Incubations were then performed to measure the extent and duration of the inhibition produced by preincubation with captopril. As shown in figure 5, microvessels preincubated in the absence of captopril were able to hydrolyze Hip-His-Leu at a linear rate for up to 90 minutes. When preincubated in the presence of $10^{-4}$ M captopril, hydrolysis of substrate was not observed for the initial 20 minutes of postincubation, and subsequently occurred at a reduced rate relative to control for the remaining incubation period. When microvessels were preincubated with a lower concentration of captopril, subsequent hydrolysis also was delayed relative to control, and the effect was proportional to the concentration of captopril contained in the preincubation mixture.

Effect of Hyperosmolarity and Freeze-Thawing

Since hyperosmolar solutions were reported to have deleterious effects on the cerebral microvasculature in vivo, it was of interest to determine if preincubation of the isolated microvessels with hyperosmolar solutions would affect the activity of ACE, or other enzymes with different subcellular localization.

![Figure 2. Effect of chloride concentration on angiotensin-converting enzyme activity in intact microvessels.](image)

![Figure 3. Effects of different peptides on angiotensin-converting enzyme activity in intact microvessels. All experiments were performed under standard assay conditions in the presence of the designated concentration of added peptide.](image)

![Figure 4. Degradation of angiotensin I by intact microvessels. Microvessels were incubated with angiotensin I ($3 \times 10^{-4}$ M) in the absence or presence of $10^{-4}$ M captopril. Aliquots of the incubation medium were removed at the designated time and assayed for immunoreactive angiotensin I.](image)
Microvessels were preincubated in 2 M urea for 15 seconds at 37°C, then quickly rinsed and transferred to the standard incubation solution lacking urea but containing Hip-His-Leu, and incubated for up to 60 minutes. The effect of the brief urea preincubation was assessed by measuring the release into the incubation medium of several enzymes known to be associated with the microvessels. As shown in table 1, when aliquots of incubation mixture were assayed for individual enzymes, the brief urea preincubation caused the subsequent release of about 25% of the cytoplasmic enzyme lactic dehydrogenase and increased significantly the release of the lysosomal enzymes NAGA and acid phosphatase, suggesting that cellular damage had occurred. However, ACE activity was not found in the incubation medium of either control or urea-pretreated microvessel preparations, and the activity remaining in the microvessels was unchanged by exposure to urea. Furthermore, when the microvessels were incubated with Hip-His-Leu under standardized conditions but in the presence of 2 M urea for up to 60 minutes, ACE activity was unchanged (data not shown), demonstrating that even prolonged exposure to urea did not alter ACE.

Comparative studies where microvessels were damaged by a freeze-thaw procedure also were performed. Microvessels were rapidly frozen by placing the nylon mesh onto dry ice for 15 seconds, then thawed by immersion into the incubation buffer. Following a 60-minute postincubation period, about 80% of the lactic dehydrogenase and 70% of NAGA were released, suggesting considerable damage. However, ACE activity remained associated with the microvessels with no apparent inactivation or release into the medium.

In separate experiments, microvessels were incubated at 37°C for up to 3 hours in the absence of Hip-His-Leu, and then aliquots of the incubation solution were assayed for ACE. No activity was released from the microvessels into the medium for up to 3 hours.

Effect of Hypoxia

Several experiments were performed to determine if hypoxic conditions would influence ACE activity in cerebral microvessels. The microvessels were incubated with Hip-His-Leu in incubation vials where the gaseous phase could be controlled. Figure 6 shows results of representative experiments using either hypoxic (97% N₂, 3% oxygen) or anoxic (100% N₂) conditions. In all cases, ACE activity was unchanged for at least 1 hour of incubation. Similar results were obtained when the gas phase was changed during the incubation (results not shown). These results are in marked contrast to those obtained with cultured vascular endothelial cells where hypoxia caused a rapid, but reversible, decrease in ACE activity.

**Discussion**

An assay for ACE in cerebral microvessels was developed permitting enzymatic activity to be determined in a tissue preparation containing freshly isolated and intact endothelial cells. The substrate used, Hip-His-Leu, has been shown repeatedly to be a useful synthetic substrate for this enzyme, particularly when whole tissue preparations have been assayed, since it is not readily attacked by extraneous peptidases. Thus, the hippuric acid formed gives a reasonable estimate of ACE activity.

**Table 1. Effect of Urea Pretreatment or Freeze-Thawing on the Release of Microvessel-Associated Enzymes**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Total activity in microvessels (nmole min⁻¹ mg⁻¹)</th>
<th>Activity released (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 7)</td>
<td>Control (n = 4)</td>
</tr>
<tr>
<td>Lactic dehydrogenase</td>
<td>225 ± 14</td>
<td>7.6 ± 2.1</td>
</tr>
<tr>
<td>NAGA</td>
<td>45 ± 5</td>
<td>5.4 ± 1.2</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>14 ± 3</td>
<td>4.0 ± 1.1</td>
</tr>
<tr>
<td>ACE</td>
<td>12 ± 3</td>
<td>&lt; 2</td>
</tr>
</tbody>
</table>
ACE is thought to be localized on the plasma membrane of endothelial cells, and since previous studies have shown that the endothelial cells in the microvessel preparations are morphologically and metabolically intact, it is likely that the substrate, Hip-His-Leu, was degraded at the luminal surface during the assay. Other studies have indicated that the lumen of the microvessels remain patent, and we have shown that another enzyme thought to be associated with the plasma membrane of the endothelial cell, lipoprotein lipase, was released from intact microvessels by heparin, reflecting an interaction known to occur in vivo when heparin interacts with the luminal surface of the microvasculature.

The rapid degradation of angiotensin I by the intact microvessel preparation, even when captopril was present, implicates the presence of peptidases other than ACE. These enzymes may be associated with the cerebrovascular endothelial or smooth muscle cells, or with membrane fragments or other contaminating cell types present in the preparation. We previously have characterized the microvessel preparation by electron microscopy and noted the presence of small amounts of lymphocytes, heterophilic leukocytes, plasma cells, and membranous cell debris.

The degradation of Hip-His-Leu by the intact microvessel preparation was dependent on both substrate and chloride ion and inhibited by captopril in a manner similar to that reported for ACE activity of tissue homogenates. One difference was the effect of pH on activity, which remained relatively unchanged at between pH 7 to 8.5 in the intact microvessels, whereas in a previous study using microvessel homogenate preparations, the pH optimum for Hip-His-Leu was above pH 8, with almost no activity in the physiological range. This difference could be due to the use of HEPES buffer in the present study and phosphate buffer for homogenate assays, since phosphate was shown to inhibit lung ACE activity at pH values below 8, whereas HEPES did not have an inhibitory effect.

A particular advantage of the methodology employed was the ability to transfer the intact microvessels from a pre- to postincubation solution. Thus, when the microvessels were pretreated with captopril and subsequently assayed for ACE in a solution lacking the inhibitor, a noticeable lag in the degradation of Hip-His-Leu was observed, suggesting that captopril dissociated slowly from its site of inhibition.

We examined the effect of acute exposure of the microvessels to a hyperosmolar urea solution because of recent reports showing that brief infusion of hyperosmolar solutions throughout the cerebral microcirculation caused definite, but reversible, changes in the blood-brain barrier in vivo. Since these changes may be attributable to either morphological or biochemical changes in cerebral endothelial cells, it was of interest to determine whether ACE, a potential marker enzyme for plasma membrane, was affected by urea pretreatment. Our data showed that cell "damage" did occur, as reflected by the leakage of cytoplasmic and lysosomal enzymes, but ACE activity remained unchanged. Furthermore, the activity was not altered even when the assay was performed in the presence of 2 M urea for up to 60 minutes, again emphasizing the stability of the enzyme in cerebral endothelial cells.

ACE was associated tightly with the microvessels, and release of enzyme into the incubation solution did not occur even after prolonged incubation for up to 3 hours. Furthermore, the ACE activity in microvessels was stable during several procedures deleterious to the preservation of cellular integrity, including urea pretreatment or freeze-thawing. Hayes et al. in studies on ACE activity in cultured hog aortic endothelial cells, showed that the enzyme was released into the culture and suggested that release of ACE activity by endothelial cells in vivo was the source of soluble ACE found in plasma. Appropriate organ culture conditions would be required to maintain microvessels for periods up to 3 days, and such studies were not performed here. It is possible that species differences may exist in the stability of ACE. However, the present studies would suggest that ACE in rabbit cerebral endothelial cells is remarkably stable, an observation that may be related to the stability of cerebral endothelial cell membranes which are joined together by tight junctions and function to maintain what is often called the blood-brain barrier.

Another major difference between the microvessels and cultured endothelial cells was the effect of hypoxia which was shown by Stalcup et al. to markedly inhibit ACE activity in cultured cells. In our studies, hypoxia had no effect on intact microvessels. Several factors could contribute to these contrasting results. Stalcup et al. measured the degradation of immunoreactive bradykinin or angiotensin I whereas we measured the hydrolysis of Hip-His-Leu. The former study did not test the effects of hypoxia on the activity toward synthetic substrates, and in our studies the presence of peptidases insensitive to captopril in the microvessels made it impractical to use angiotensin I as substrate. Furthermore, Stalcup et al. used cells obtained from

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Figure 6 Angiotensin-converting enzyme activity in intact microvessels incubated under hypoxic or anoxic conditions.
sources other than the cerebral vasculature, and it is possible that hypoxia may exert specific effects on endothelial cells from some vascular sources but not from cerebral microvessels. A recent study by Arregui and Barer showed that chronic hypoxia in rats produced reduced ACE activity in the nigra and striatum but had no effect on seven other regions of the brain, including the cortex.

Although there are obvious experimental problems in obtaining comparative data on the properties of ACE in different endothelial cell types, the ability to measure the activity of the enzyme in intact cerebrovascular endothelial cells provides a unique experimental system for assessing the potential physiological role of the renin-angiotensin system in the brain.

References


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