Metabolism of Vasoactive Peptides by Human Endothelial Cells in Culture

ANGIOTENSIN I CONVERTING ENZYME (KININASE II) AND ANGIOTENSINASE

ALICE R. JOHNSON and ERVIN G. ERDÖS

From the Departments of Pharmacology and Internal Medicine, University of Texas Health Science Center, Dallas, Texas 75235

ABSTRACT Cultured endothelial cells provide a model for the study of interactions of vasoactive peptides with endothelium. Endothelial cells cultured from veins of human umbilical cords contain both angiotensin I converting enzyme (kininase II) and angiotensinase activities. Intact monolayers of cells can both activate angiotensin I and inactivate bradykinin when the peptides are added to culture flasks in protein-free medium. Intact suspended cells or lysed cells convert angiotensin I to angiotensin II, inactivate bradykinin, and hydrolyze hippuryl-diglycine to hippuric acid and diglycine. These actions are inhibited by SQ 20881, the specific inhibitor of converting enzyme. The kininase activity of endothelial cells was partially inhibited by antibody to human lung converting enzyme. Endothelial cells also inactivate longer analogs of bradykinin, such as kallidin, methionyl-lysyl bradykinin, and bradykinin coupled covalently to 500,000 mol wt dextran. The endothelial cells retained converting enzyme activity through four successive subcultures, indicating that the enzyme is synthesized by the cells. The converting enzyme is localized on the cell surface, and it is apparently a marker for endothelial cells, since cultured human fibroblasts, smooth muscle cells, and baby hamster kidney cells do not have it. Endothelial cells also contain an aminopeptidase which hydrolyzes both angiotensin II and the synthetic substrate, α-L-aspartyl β-naphthylamide. The angiotensinase activity increased when the cells were lysed, which suggests that the enzyme is localized within the cells. Hydrolysis of both α-L-aspartyl β-naphthylamide and angiotensin II was inhibited by *o*-phenanthroline, indicating that the enzyme is an A-type angiotensinase.

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INTRODUCTION

The vascular endothelium occupies a unique position in metabolism and transport of substances from both blood and tissues. The endothelium functions as more than a simple diffusion barrier between these compartments, because by metabolizing blood-borne peptides it can determine the fate of these materials in circulation.

The metabolism of vasoactive peptides by the lung has been studied extensively (1–3). An enzyme, presumably on the surface of the endothelial cells (4, 5), cleaves angiotensin I and bradykinin during passage through the pulmonary circulation. This angiotensin I converting enzyme, or kininase II, acts as peptidyldipeptidase (EC 3.4.15.1) since, by the release of a carboxyl-terminal dipeptide, it both generates angiotensin II from angiotensin I and inactivates bradykinin (6–8).

Although the pulmonary circulation was first recognized as a major site for metabolism of bradykinin and angiotensin I, these peptides are hydrolyzed in other vascular beds, presumably by contact with the converting enzyme (9–14). Angiotensin II also may be cleaved during contact with vascular endothelium by an angiotensinase, since it is inactivated in the peripheral circulation and in isolated, perfused vascular beds (1). Some investigators (15) report that angiotensin II activity is lost after a single passage through coronary or renal vascular beds, but the peptide can pass through the

pulmonary circulation without loss of biologic activity (1, 9, 10, 16). Thus, it appears that enzymes that both activate and inactivate angiotensins are associated with endothelium, but they have a different distribution in the various vascular beds.

The availability of human endothelial cells in culture provided new impetus to studies of endothelial cell function. Cells derived from the veins of umbilical cords have been cultured successfully by several laboratories (17–19). These cultures provide homogeneous cell populations that exhibit the morphologic and biochemical characteristics of endothelium in situ. We utilized cultured human endothelial cells to study the angiotensin I converting enzyme and angiotensinase activities associated with endothelium (20).

METHODS

Materials. Culture medium, enzyme solutions and additives for the culture media were purchased from Grand Island Biological Co. (Grand Island, N. Y.). Peptides were purchased from Schwarz/Mann Div., Becton, Dickinson & Co., (Orangeburg, N. Y.) and from Calbiochem (San Diego, Calif.). SQ 20881 was obtained from Dr. Zola Horovitz of the Squibb Institute. The substrate, 1 dimethylaminonaphthalene-5-sulfonyl-triglycine (dansyl-triglycine) was synthesized as described (8). Hippurylglycyl-glycine (hippuryl-diglycine) was obtained from Bachem (Marina del Rey, Calif.) and α-L-aspartyl β-naphthylamide and β-naphthylamine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Antibodies to human lung converting enzyme (21) were elicited in goats and the IgG fraction of immune serum was isolated by precipitation with 33% ammonium sulfate and chromatography on a DEAE-cellulose column. Bradykinin was coupled to soluble dextran of 500,000 mol wt that was activated with sodium meta-periodate (22). Swine kidney converting enzyme (23) and human serum albumin were labeled with ^{125}I by the chloramine-T method described by Freedlender et al. (24).

Cell cultures. Endothelial cells from veins of human umbilical cords were collected according to the methods described by others (17–19). The cords were obtained within minutes after normal vaginal delivery or caesarean sections and were placed in sterile bottles containing phosphate-buffered saline and antibiotics. The specimens were processed with sterile technique in a laminar flow hood. The vein was cannulated with an 18-gauge needle fitted with polyethylene tubing. Perfusion with 5–10 ml of phosphate-buffered saline removed the remaining cord blood. Areas of injury or distension were cut off and discarded. Intact veins were filled with a solution of 0.25% trypsin in phosphate-buffered saline and both ends were clamped with hemostats. After 5 min at room temperature the trypsin solution was flushed into a polyethylene tube containing medium 199 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 20% fetal calf serum, glutamine (2 mM), and antibiotics. The vein was flushed with an additional 5–10 ml of trypsin solution and the washings were collected with the initial perfusate. The cells were centrifuged to remove the trypsin-containing medium, resuspended in fresh culture medium, and plated in 250-ml Falcon culture flasks or culture plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The cultures were maintained at 37°C in an atmosphere of 5% CO_2. After 12–24 h, unattached cells were removed by suction and the adhering cells were covered with fresh medium 199 that contained 10% human serum (type O+) as well as the other additives.[1] A confluent monolayer of cells was obtained within 1–2 wk. The cells were usually transferred or used for experiments when they were 70–80% confluent.

Cells from other sources were screened for angiotensin I converting enzyme. Human smooth muscle cells were cultured from umbilical veins by the technique of Gimbrone and Cotran (25). Smooth muscle cells were distinguished from endothelial cells by examining the cultures with light and electron microscopy. While both types of cells grew slowly in culture, the smooth muscle cells could be easily distinguished from endothelial cells by their larger size, irregular shape, and the presence of bundles of myofilaments in the cytoplasm. After several weeks in culture, smooth muscle cells grew into multilayered patches interspersed with bands of monolayered cells. In contrast, endothelial cells remained in monolayers even after many weeks of culture.

Human skin fibroblasts were cultured from skin biopsy material by the method of Taylor et al. (26). Once the cultures grew into monolayers, the fibroblasts could be easily distinguished from smooth muscle cells or endothelial cells by their rate of growth and their morphology. Fibroblasts appeared as long spindle-shaped cells that organized into parallel arrays in the cultures. They grew very rapidly after the initial passage and reached confluency within 3 days. Smooth muscle cells and endothelial cells required at least 1 wk to form confluent monolayers.

Baby hamster kidney cells (BHK 21 13s) were provided by Dr. Fred Grinnell of the Department of Cell Biology, University of Texas Health Science Center. All cells were grown in medium 199 supplemented with 10–20% fetal calf serum and antibiotics. The human cell cultures were also supplemented with 10% human serum. Cells were used or transferred at approximately 80% confluency.

Electron microscopy. For examination by transmission electron microscopy, washed cell monolayers were fixed with 8% glutaraldehyde for 4 min at room temperature. The glutaraldehyde was removed, and 10 ml of medium 199 with 20% fetal calf serum was added to the flask. The cells were scraped from the flask with a rubber spatula and centrifuged to a pellet. The supernatant fluid was discarded and the pellet was fixed with a second addition of 8% glutaraldehyde. The pellet was washed once with phosphate-buffered saline and post-fixed with 1% OsO_4 for 30 min. The material was dehydrated by passage through a series of ethanol solutions and propylene oxide and embedded in Epon (Shell Chemical Co., New York). Thin sections were cut on a Porter-Blum Mt-2 microtome (DuPont Instruments, Sorvall Operations, Newton, Conn.) and examined with a JEOL 100 C electron microscope (JEOL, Ltd., Tokyo, Japan).

Enzyme assays. A variety of techniques was applied to detect and measure the activity of enzymes associated with endothelial cells. The angiotensin I converting enzyme (kininase II) was detected in cultured cells by a thin-layer chromatography method (8). Cell extracts were incubated with dansyl-triglycine in converting enzyme buffer (0.2 M Tris, pH 7.4, plus 0.2 M NaCl) containing 2 mM $CoCl_2$ for 3 and 24 h. The enzyme was identified by showing cleavage of diglycine from the substrate. 20 × 20-cm sheets of ChromAR 1,000 (Mallinckrodt Inc., St. Louis,

[1] Neither fetal calf serum nor human serum was heat inactivated before use in the culture media.

Mo.) were spotted with the reaction mixture and were then developed with $CHCl_3$-benzyl alcohol-acetic acid (150:45:7.5) as the solvent system. The fluorescent spots were visualized under ultraviolet light and the product was identified as dansyl-glycine by comparison of the Rf values with standards.

The activity of the converting enzyme was determined quantitatively by measuring the rate of cleavage of peptide substrates. The specific activity of the converting enzyme was defined as the amount of substrate hydrolyzed per minute per million cells that was inhibited by SQ 20881 (0.1 mM), the specific peptide inhibitor of converting enzyme (14). The cleavage of hippuryl-diglycine to hippuric acid and diglycine was determined by measuring the amount of diglycine released. Cells or cell extracts (0.05 ml) were added to 0.95 ml of converting enzyme buffer containing hippuryl-diglycine in a final concentration of 1 mM. Selected samples of cells were treated with $CoCl_2$ (1 mM) before incubation with the substrate. The samples were incubated at 37°C for 1–24 h and the reaction was stopped by addition of 1 ml of 3% sulfosalicylic acid. The precipitated protein was sedimented by centrifugation and the supernatant was applied to the short column of a Beckman 121 amino acid analyzer (Beckman Instruments, inc., Fullerton, Calif.) in 0.2 N citrate buffer (pH 3.3).

Angiotensinase A activity was measured by two methods: a chemical assay and a bioassay. Aspartyl-aminopeptidase activity was measured by a modification of the method of Nagatsu et al. (27). Cells were disrupted by sonication and incubated with α-L-aspartyl β-naphthylamide (0.7 mM) in 0.2 M Tris-maleate buffer (pH 7.0) with $CaCl_2$ (0.3 mM). After incubation for 30 min at 37°C the reaction was stopped by addition of alkaline EDTA to make a final concentration of 5 mM EDTA and bring the reaction mixture to pH 11. Fluorescence of the liberated β-naphthylamine was measured at 410 nm in an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) and compared with standards of β-naphthylamine.

The activation of angiotensin I to II and the inactivation of kinins and angiotensin II were determined by bioassays. The bioassay technique proved to be the most satisfactory means of rapidly identifying kininase or angiotensinase activities in cell cultures and for quantitation of the enzyme activity in a small amount of material, such as the cells from a single culture flask. The estrus rat uterus was used to assay inactivation of kinins or angiotensin II or activation of angiotensin I (28). One horn from an animal pretreated with diethylstilbesterol (0.4 mg/kg) was suspended in a smooth muscle bath in DeJalon's solution[2] containing atropine sulfate (1 mg/ml). Either standard peptides or test samples were injected every 5 min, and the amount of peptide in an incubation mixture was determined by comparison of the contractions with those produced by standards.

The rate of inactivation or generation was measured by incubating the cells or cell lysates with peptide substrates at 37°C in converting enzyme buffer. Aliquots were withdrawn at various times, diluted with 0.2–1 ml of saline and tested on the smooth muscle. The reaction rates were linear with time over a period of 0–30 min. Since angiotensinase in lysed cell preparations (see below) inactivated both angiotensin I and II, inactivation of bradykinin was the most reliable means of measuring converting enzyme activity. The hydrolysis of bradykinin by the cells was 80–95% inhibited by the specific inhibitor, SQ 20881 (0.1 mM).

Angiotensinase activity and its inhibition by *o*-phenanthroline (1 mM) was measured in cells and cell lysates by incubating them at 37°C with angiotensin II in converting enzyme buffer. Aliquots were withdrawn at various times and injected into the muscle bath as described. A decrease in the height of contraction with time of incubation was interpreted as degradation of the peptide in the reaction mixture.

Lactic dehydrogenase activity of cell fractions was measured with a Sigma assay kit (Sigma Chemical Co.). Sodium pyruvate (1.6 mM) was the substrate. The decrease in optical density at 254 nm that resulted from oxidation of DPNH (10 μM) was measured in a Cary model 118 spectrophotometer (Cary Instruments, Monrovia, Calif.).

Treatment of cells. Enzymatic activities were measured in monolayers of cells, suspended intact cells, cell lysates, and fractions of lysed cells that were separated by differential centrifugation.

The cell monolayers were tested for enzymatic activities after the cell surfaces were washed at least three times with 10 ml of protein-free culture medium. In some experiments SQ 20881 was added to the final wash and the monolayers were incubated with the inhibitor for 15 min at 37°C. The peptide substrates (angiotensin I and II and bradykinin) were added directly to the flasks in 10 ml of protein-free culture medium. An aliquot of 0.5 ml was immediately removed and the flasks were placed on a rocker platform in the CO_2 incubator at 37°C. Aliquots were withdrawn at various times during the incubation and were kept on ice until assayed. At the end of the experiment the cells were suspended by brief treatment with 0.25% trypsin, resuspended in 5–10 ml of fresh medium containing 10% fetal calf serum to stop the action of the trypsin, and the cells were counted in a hemocytometer. Trypan blue was added to the sample taken for counting to determine the number of viable cells. The remaining cell suspension was centrifuged at low speed (1,000–2,000 rpm) for 5 min in an International model HN table top centrifuge (International Equipment Co., Needham Heights, Mass.). The cell pellet was washed three times with 10 ml of protein-free culture medium and was resuspended to 0.5–1.5 ml in phosphate-buffered saline or culture medium. Aliquots of this final suspension were then assayed for enzyme activity and DNA and protein concentrations (see below).

To measure enzyme activities in cell suspensions, several methods for suspending the cells were compared. Cells were detached from monolayers by treatment with either 0.25% trypsin or collagenase in protein-free medium. The action of trypsin was generally more rapid than that of collagenase and resulted in more complete detachment of the cells. Cells were also suspended by repeated washing with calcium- and magnesium-free phosphate-buffered saline, but this technique frequently failed to detach all of the cells. Alternatively, the cells could be completely removed by scraping monolayers gently with a rubber spatula. While this latter method did not expose the cells to proteolytic enzymes, many of the cells were damaged, as indicated by their staining with trypan blue. The activity of the converting enzyme, as measured by hydrolysis of hippuryl-diglycine, was similar in all of these preparations (Table I). Since the trypsin-suspended cells were consistently viable as indicated by exclusion of trypan blue and retention of lactic dehydrogenase ac-

[2] DeJalon's solution contained 154 mM NaCl, 5.4 mM KCl, 0.5 mM $CaCl_2$, 6 mM $NaHCO_3$, and 5.5 mM glucose.

tivity, this method was used for most of the experiments with suspended cells.

For experiments in which the enzyme activity was inhibited by SQ 20881, *o*-phenanthroline, or antibody to converting enzyme, cells were suspended by 0.25% trypsin and then washed as described. The cell suspensions were incubated at 37°C with inhibitors for 15 min before the addition of substrate.

Cell lysates were prepared from suspended cells by either freezing and thawing several times or by sonication with an Artek sonic dismembrator (Artek Systems Corp., Farmingdale, N. Y.) for 15 s. The crude lysates were used directly for enzyme assays and for protein and DNA determinations.

For fractionation experiments endothelial cells from 5–10 confluent cultures were suspended by treatment with 0.25% trypsin, washed four times with 10 ml of protein-free medium, resuspended in homogenization buffer (5 mM Tris, 0.25 M sucrose, 0.2 mM $MgSO_4$) and then disrupted by the nitrogen cavitation method of Wallach and Kamat (29). The cells were placed in an Artesan nitrogen bomb (Artesan Industries, Inc., Waltham, Mass.) under 600 psi pressure for 20 min. After a sudden release of the pressure, the disrupted cells were collected in a thick-walled glass centrifuge tube and the bomb was rinsed with 0.5 ml of homogenization buffer which was added to the homogenate. The cell homogenate was sampled for determination of enzyme activities and DNA and protein content. The remainder of the homogenate was centrifuged at 5°C in a Sorvall RC-2 refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) at a maximum force of 30,000 *g* for 10 min. The first pellet was resuspended in 1 ml of phosphate-buffered saline and used for assay of enzymes, DNA and protein. The supernate was placed in a screw top polycarbonate tube and centrifuged in a Beckman model L5-65 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 1 h at 100,000 *g*. The second pellet was resuspended in 0.5 ml of phosphate-buffered saline and saved for assay of enzyme activity, DNA, and protein. In one experiment the high-speed supernate was centrifuged at 400,000 *g* for 3 h but there was no further sedimentation. The high speed (100,000-*g*) supernate was assayed without dilution for enzyme activity and protein.

Uptake of proteins from media. To determine whether the endothelial cells took up converting enzyme from serum added to the culture medium, some cultures were grown in media that contained either ^{125}I-labeled human serum albumin or ^{125}I-labeled converting enzyme (24). After 72 h of contact with the labeled materials (2,000,000 cpm/ml) the cells were detached with 0.25% trypsin and washed with protein-free medium as described above. The final cell pellet was counted in a Packard 5120 autogamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.)

Relation of enzyme activity to cell number. Initially, an aliquot of suspended cells from each experiment was counted in a hemocytometer and the cell counts were compared with DNA measurements. In five experiments the DNA content of the cultured cells was 6.1±0.3 μg per million cells. This figure is in close agreement with the DNA content of mammalian cells given by Lehninger (30). Thus, to avoid errors caused by clumping of the cells during suspension and handling, the cell number was determined by the DNA content of each cell suspension.

DNA and protein determinations. An aliquot of suspended cells or a cell pellet was first treated with 0.25 ml of cold 0.5 N perchloric acid and allowed to stand in ice for 10 min. The sample was centrifuged to sediment the precipitated material and the pellet was washed once with cold perchloric acid. The pellet was then resuspended in 0.25 ml of perchloric acid and DNA was extracted by heating to 70°C for 20 min. The amount of DNA in the extracts was determined by the diphenylamine method of Burton (31). Calf thymus DNA was used as a standard.

Protein in cell samples and fractions was measured by the method of Lowry et al. (32). Values were compared with those of standard solutions of bovine serum albumin.

TABLE I
Comparison of Methods for Suspending Endothelial Cells

Treatment	Converting enzyme activity		Trypan blue staining	LDH release
	nmol/h/10⁶ cells		%	%
Trypsin (0.25%)	19.4±1.3	(5)	3	10
Collagenase	21.3±5.0	(3)	2	5
Phosphate-buffered saline	18.8±3.1	(3)	43	33
Scraping	17.7±3.9	(4)	38	—

Converting enzyme activity was measured with hippuryl-diglycine as substrate. Values are means±SEM. Numbers in parentheses are the number of experiments.

RESULTS

Identification of endothelial cells. The cells cultured from human umbilical cords were examined by light and electron microscopy. Their morphology was similar to that described by others for cultured endothelium (17–19, 33). The starting cultures were compact colonies of hexagonal cells that spread into monolayers at a relatively slow rate. Typically, 1 wk or more was required to obtain a confluent monolayer when the cells of a single cord were divided into two 250-ml flasks. Examination of the cultured cells with transmission electron microscopy revealed fibrils, pinocytotic vesicles, and the specific endothelial granules, or Weibel-Palade bodies (34) in the cytoplasm. These latter inclusions occur in endothelial cells but not in cultured smooth muscle cells or in fibroblasts which might be collected as contaminants of the endothelial cells from umbilical veins (17, 19, 25). Fig. 1 shows the Weibel-Palade bodies and pinocytotic vesicles in cultured endothelial cells.

Endothelial cell cultures secrete a protein that is apparently specific for endothelial cells, Factor VIII antigen (35, 36). As another means of identifying our cultures as authentic endothelial cells, this marker was measured by the immunoelectrophoretic method of Zimmerman et al. (37). Culture fluids were collected after 24–48 h in contact with the cells. Fluids from both primary and subcultures of endothelial cells contained the antigen, but fluid from the fibroblasts cultures did not.

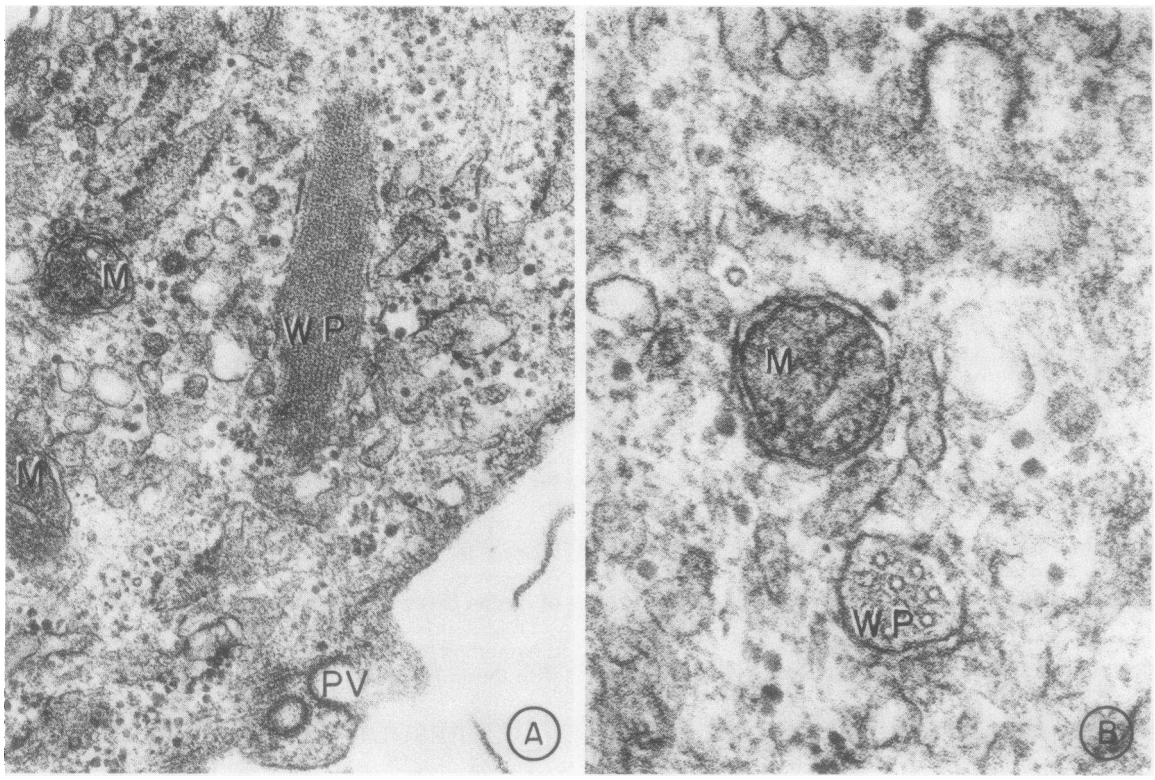

FIGURE 1 Transmission electron micrograph of Weibel-Palade bodies in human endothelial cells. The cells were fixed with 8% glutaraldehyde in cacodylate buffer and post-fixed with OsO_4. WP = Weibel-Palade body, M = mitochondria, and PV = pinocytotic vesicle. (A) Longitudinal section through a Weibel-Palade body and pinocytotic vesicle at cell surface. Magnification ×40,000. (B) Cross section through Weibel-Palade body and mitochondrion. Magnification ×82,000.

Measurement of enzymatic activities. Initially, a thin-layer chromatography method was used to detect converting enzyme activity in cultured cells. Four out of five different primary cultures selectively cleaved a dipeptide from the dansylated triglycine substrate, indicating the presence of angiotensin I converting enzyme in the cells. The reaction was completely inhibited by addition of the peptide inhibitor SQ 20881 (0.1 mM) to the incubation mixture. Three cultures of baby hamster kidney cells were tested but they did not cleave the substrate.

TABLE II
Aspartyl Aminopeptides (Angiotensinase) Activity in Endothelial Cells

Cell preparation	Treatment	Number of experiments	Activity
			pmol/h/10⁶
Trypsin (0.25%)	None	8	361±32
	o-phenanthroline	4	94±12
Scraped	None	10	467±74
	o-phenanthroline	4	134±23

Values are mean±SEM.

Endothelial cells contained aminopeptidase activity as determined by the hydrolysis of α-L-aspartyl β-naphthylamide. Cells that were removed from monolayers by scraping were as active as cells detached by trypsin. Hydrolysis was inhibited approximately 70% when the cells were incubated with 1 mM *o*-phenanthroline before contact with the substrate. These data are given in Table II.

The conversion of angiotensin I to II, inactivation of bradykinin and inactivation of angiotensin II were measured by bioassay (Table III). The enzyme activity depended upon the type of cell preparation. In six separate experiments the rate of inactivation of bradykinin in the monolayer cultures was 1.6±0.2 nmol/h per million cells. The rate of angiotensin II generation from angiotensin I in three experiments was 1.2±0.1 nmol/h per million cells. SQ 20881 (0.1 mM) inhibited both reactions. An example of the bioassay of these peptides is given in Fig. 2. When

the cells were suspended by treatment with trypsin or collagenase or by scraping, the kininase activity increased 10-fold but the rate of angiotensin I conversion increased only 3- to 4-fold. Disruption of the cells by sonication or by nitrogen cavitation did not increase the activity further. Freezing and thawing of the cells decreased the activity of the kininase by nearly 70% as compared with intact cell preparations or cells lysed by sonication or nitrogen cavitation (Table III).

Angiotensinase was not detected in intact cell monolayers during a 60–90-min incubation period. However, angiotensin II was hydrolyzed by suspended cells at a rate of approximately 4 nmol/h per million cells. When the cells were lysed the activity of angiotensinase doubled. The inactivation of angiotensin could be partially (66–87%) inhibited by addition of *o*-phenanthroline (1 mM) to the reaction mixture.

The synthetic substrate hippuryl-diglycine was also used to assay the activity of converting enzyme in endothelial cells and the amount of glycyl-glycine released was measured with an amino acid analyzer. The concentration of the dipeptide product increased with the time of incubation as shown in Fig. 3. In untreated suspended cells that were incubated with the substrate (1 mM) for 18–24 h the rate of cleavage was 17.7 nmol/h per million cells (Table IV). While this method was not as sensitive as the bioassay methods, it confirmed that the enzyme released a dipeptide from the carboxyl terminal end of the substrate. This reaction also was inhibited by the specific inhibitor, SQ 20881, and all data are given as specific activity of the converting enzyme, i.e., the difference in activity between uninhibited and inhibited cell preparations. Suspended cells and sonicated cells had comparable enzyme activities when measured by this method. However, as indicated earlier (Table III), cells that were lysed by freezing and thawing several times had only a third of the activity of unfrozen cells. Although $CoCl_2$ has been shown to enhance the activity of converting enzyme from plasma (7), addition of $CoCl_2$ (1 mM) did not affect the activity in endothelial cells. EDTA, which inhibits the enzyme in both plasma and tissues (14), almost completely blocked the hydrolysis of hippuryl-diglycine by endothelial cells (Table IV).

Converting enzyme activity in other cultured cells. Baby hamster kidney cells, human skin fibroblasts, and smooth muscle cells were tested for converting enzyme activity with both bradykinin and hippuryl-diglycine as substrates. Although there was kininase activity in baby hamster kidney cells and in both human fibroblasts and smooth muscle cells, the activity was not inhibited by SQ 20881 and these cells did not cleave hippuryl-diglycine. Thus, the major kininase activity was not attributed to the angiotensin I converting enzyme.

Several cultures of human endothelial cells that appeared fibroblastic after several weeks in culture were also tested. These cells were a mixture of epi-

TABLE III
Converting Enzyme and Angiotensinase Activities in Endothelial Cells

Cell preparation	Inhibitor and concentration	Activity: Converting enzyme: Bradykinin	Activity: Converting enzyme: Angiotensin I	Activity: Angiotensinase (Angiotensin II)
	mM	*nmol/h/10⁶* cells		
Monolayer	None	1.6±0.2	1.2±0.1	0
	SQ 20881 (0.1)	0.3±0.1	0	—
Suspended cells	None	16.7±1.7	6.4±0.8	3.5±0.5
	SQ 20881 (0.1)	2.1±1.1	0.2±0.1	—
	o-phenanthroline (1.0)	—	—	1.2±0.2
Scraped cells	None	17.8±2.5	—	4.4±0.5
	SQ 20881 (0.1)	2.9±0.3	—	0.2±0
Sonicated cells	None	22.1±3.2	—	7.9±1.5
	SQ 20881	4.9±1.2	—	—
	o-phenanthroline (1.0)	—	—	1.1±0.1
Frozen cells	None	4.9±1.3	—	—
	SQ 20881 (0.1)	0.9±0.2	—	—

Values are means±SEM of three or more experiments. Substrate concentrations were: bradykinin, 1 μM; angiotensin I, 20 μM; and angiotensin II, 20 μM.

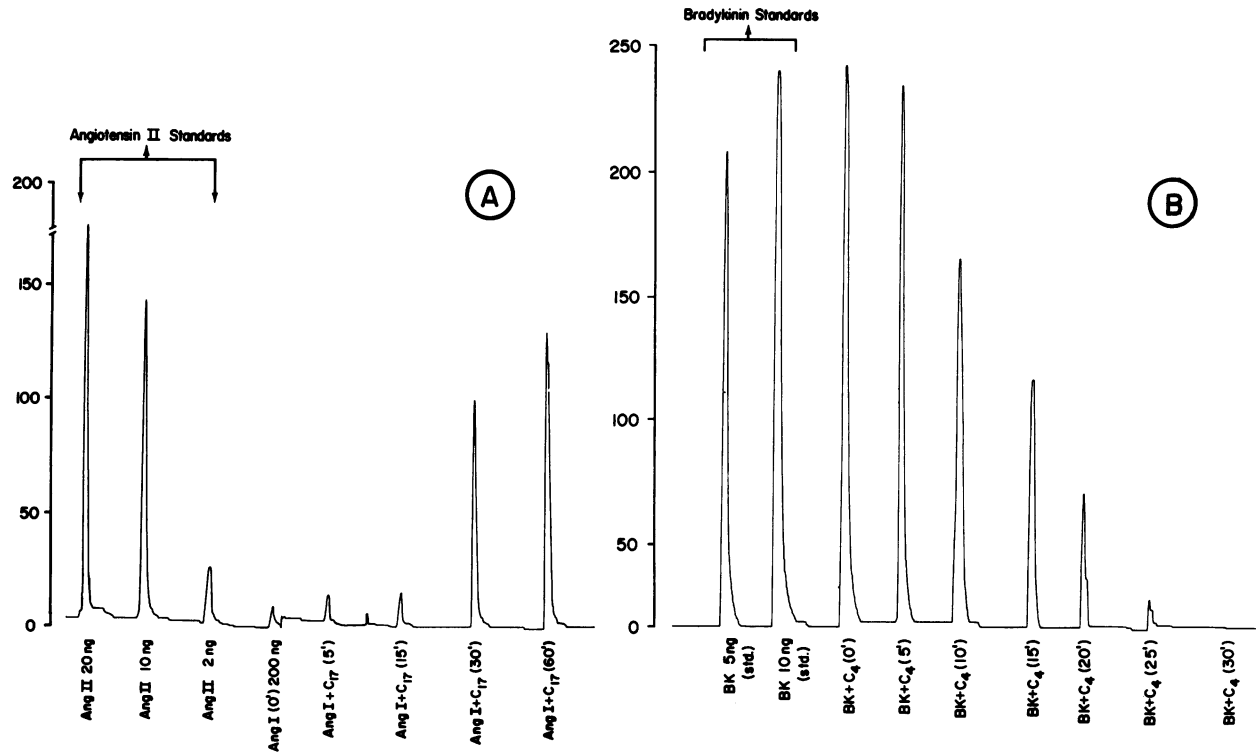

FIGURE 2 Conversion of angiotensin (Ang I) (A) and inactivation of bradykinin (BK) (B) by converting enzyme of cell monolayers as measured by bioassay on the rat uterus. The peptides substrates were added to washed cell monolayers (C_4, C_{17}) in protein-free culture medium. Samples were withdrawn at the times indicated on the abscissa and injected into the muscle bath. The height of contraction in millimeters is given on the ordinate. Incubation of peptides alone (without cells) did not alter their activity on smooth muscle.

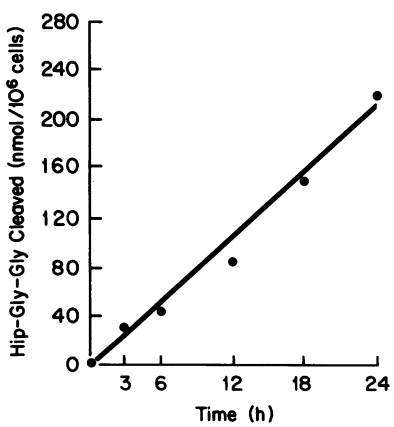

FIGURE 3 Release of diglycine from hippuryl-diglycine by cultured endothelial cells. Cells were suspended with trypsin, washed, disrupted by sonication, and then incubated with the substrate at pH 7.4. Samples were withdrawn at the times indicated on the abscissa. The amount of diglycine released was determined in an amino acid analyzer after correction for hydrolysis to glycine. The amount of substrate cleaved per million cells is given on the ordinate.

thelioid and spindle-shaped cells, and the rate of growth was more rapid than that of typical endothelial cell cultures. These fibroblastic cultures cleaved only 3–9 nmol/h per million cells as measured with bradykinin as the substrate. SQ 20881 (0.1 mM) decreased the activity in these mixed cultures by approximately 50%. The mixed endothelial-fibroblast cultures cleaved hippuryl-diglycine but at a lower rate than true endothelial cells (3.3 compared with 21.6 nmol/h per million cells).

Uptake of proteins from culture medium. Both fetal calf serum and human serum used in the growth medium for endothelial cells had converting enzyme activity, as determined by hydrolysis of hippuryl-diglycine. Human serum albumin and purified converting enzyme (23) were labeled with ^{125}I and added to the culture media. When cell monolayers were incubated with either of the labeled proteins for 72 h and then detached with trypsin there was no significant retention of the label by the cells.

Converting enzyme activity in transferred endothelial cells. Activity of the converting enzyme as measured by cleavage of hippuryl-diglycine was re-

TABLE IV

Activity of Converting Enzyme in Endothelial Cells. Effect of $CoCl_2$ and EDTA Measured with Hippuryl-Diglycine as Substrate

Cell preparation	No. expts.	Treatment	Activity
			nmol gly-gly/h/10^6 cells
Suspended cells	7	None	17.7±3.2
Sonicated cells	7	None	20.8±4.0
Frozen cells	4	None	6.7±1.4
Sonicated cells	7	$CoCl_2$ (1 mM)	22.2±5.1
Sonicated cells	5	EDTA (1 mM)	3.1±0.8

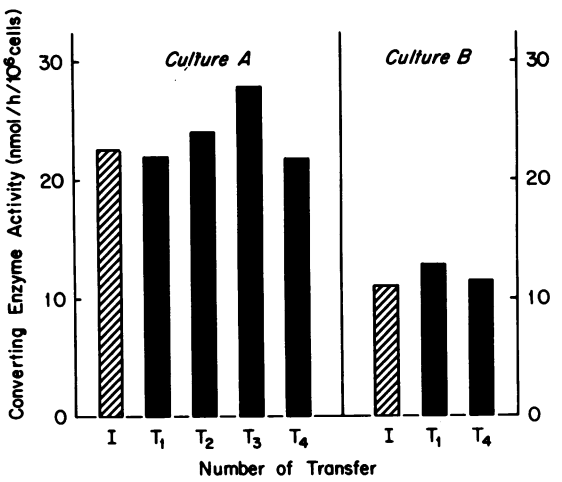

FIGURE 4 Activity of converting enzyme in endothelial cells grown in subcultures. Two separate cell lines were tested for enzyme activity as measured by cleavage of hippuryl-diglycine. The activity in the initial (I), or primary cultures, and in each transferred culture (T_1–T_4) is given on the ordinate.

tained by cultured endothelial cells through several successive subcultures. Fig. 4 shows the enzymatic activity in two different cell lines that were tested as primary cultures and at the time of each transfer. Although there was approximately a twofold difference in the basal level of activity, each culture retained the original activity, even after the fourth transfer. Furthermore, the converting enzyme in subcultured cells was inhibited by SQ 20881. Since such subculture starts with only one-third or one-fourth of the original culture, retention of the enzyme activity through four separate passages indicates that the cells synthesize the enzyme during growth of the cell monolayer.

Other substrates. The converting enzyme from human endothelial cells hydrolyzed both kallidin and methionyl-lysyl-bradykinin but at slower rates than bradykinin (Table V). Hydrolysis of all three kinins was inhibited by SQ 20881 at a concentration of 0.1 mM (not shown). The enzyme was also active on bradykinin that was coupled at the amino terminal end to a soluble dextran of 500,000 mol wt. (This complex retained about 50% of the activity of unbound bradykinin when tested on the rat uterus.) The inactivation of the macromolecular complex was also inhibited by SQ 20881. However, the coupled peptide was inactivated more slowly than bradykinin. Cleavage of this high molecular complex suggests that the kininase is localized on the surface of the endothelial cells (Table V).

Effect of antibody to converting enzyme. The kininase activity in endothelial cells was partially inhibited by antibody to human lung converting enzyme. When cells were incubated with the IgG fraction of goat antiserum to the human lung enzyme for 15 min before addition of bradykinin, enzyme activity decreased by approximately 45%. Incubation with antibody for 60 min did not result in any greater inhibition. In five separate experiments the concentration of antibody required for this maximal inhibition was between 45 and 100 $\mu g/10^6$ cells (mean = 65.8). Fig. 5 shows an experiment where the cells were incubated with varying concentrations of the partially purified antibody for 15 min before measurement of the kininase activity by bioassay.

Fractionation of endothelial cells. Cultured endothelial cells were disrupted by N_2 cavitation and fractionated by differential centrifugation at 30,000 and 100,000 *g* (Table VI). All of the fractions (nuclear pellet, microsomal pellet, and high-speed supernatant) had some kininase activity, but most of the activity (80%) was found in the final, high-speed

TABLE V

Inactivation of Kinins by Converting Enzyme of Endothelial Cells

Substrate	Concentration	Activity	No. expts.
	μM	*nmol/h/10^6 cells*	
Bradykinin	2	16.0±2.7	7
Kallidin	2	4.2±0.8	5
Methionyl-lysyl-bradykinin	2	3.1±1.2	3
Bradykinin coupled to dextran*	1.1	2.0, 2.6	2
	2	6.0, 2.0	2

* Concentration of bradykinin in the complex was determined by amino acid analysis. Data are given for two separate preparations of the complex.

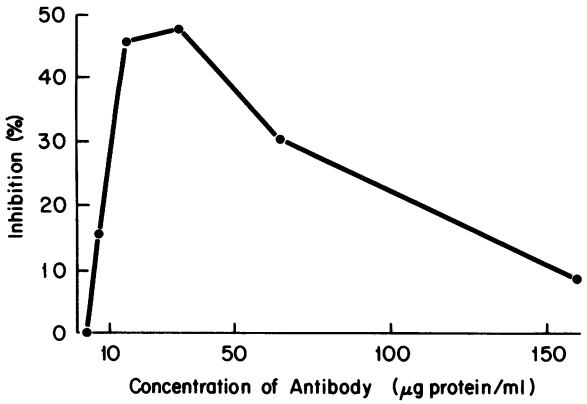

FIGURE 5 Inhibition of kininase activity of cultured human endothelial cells by antibody to human lung converting enzyme. Endothelial cells in suspension (0.5×10^6/ml) were incubated with antibody in the concentrations given on the abscissa. The kininase activity was determined with bradykinin (1 μM) substrate on the rat uterus. The percent inhibition of bradykinin inactivation is indicated on the ordinate. The enzyme in cells treated with normal goat serum (not shown) was not inhibited.

supernatant fraction. Approximately 17% of the total activity remained in the 30,000-*g* pellet, presumably in association with unbroken cells. Only trace amounts of activity were recovered in the high-speed (membrane) pellet. The total amount of activity recovered relative to the crude homogenate varied between 39 and 80% in four experiments. The specific activity for both the starting material (cell homogenate) and the final supernatant fraction was approximately 20 nmol/h per mg protein.

DISCUSSION

We identified two enzymes that metabolize vasoactive peptides in the endothelial cells cultured from human veins: angiotensin I converting enzyme (kininase II) and an aminopeptidase which cleaves angiotensin II (angiotensinase A). However, our primary interests were the properties and localization of the angiotensin converting enzyme associated with endothelial cells.

The angiotensin converting enzyme occurs in both soluble and membrane-bound forms. For example, human or guinea pig plasma are sources of soluble enzyme while lung or kidney of man and experimental animals contain membrane-bound enzyme (14). The converting enzyme in rabbit (5), rat, and hog (4, 38) lungs was identified in vascular endothelium by a combination of immunofluorescence and other immunochemical methods. When the converting enzyme was purified from homogenates of lung, however, a substantial amount of activity was in the soluble fraction (21). In the kidney, the converting enzyme is concentrated on the brush border of epithelial cells in the proximal tubules (5, 39, 40).

The converting enzyme in human endothelial cells was identified by measuring inactivation of bradykinin and activation of angiotensin I in vitro. Cleavage of the carboxyl terminal dipeptide from shorter peptide substrates was shown qualitatively by thin-layer chromatography and was measured quantitatively in an amino acid analyzer. Inhibition of these activities by the specific inhibitor of converting enzyme SQ 20881 (8, 14) identified the enzyme as angiotensin I converting enzyme.

The converting enzyme of endothelial cells appears to be localized on or very near the cell surface. It is readily accessible to peptide substrates, since intact cell monolayers metabolize both bradykinin and angiotensin I and the activities are inhibited by SQ 20881. Furthermore, the enzyme on intact, suspended cells is accessible to high molecular weight substances, such as the complex of bradykinin and dextran or the antibody to human lung converting enzyme. Although the suspended cells inactivate bradykinin bound to dextran more slowly than free bradykinin, the fact that the reaction is inhibited by SQ 20881 indicates that inactivation is by the converting enzyme. The inhibition of the kininase activity by antibody to human lung converting enzyme shows that the enzyme on suspended cells is available for combination with the antibody. Since lysis of the cells by sonication does not increase the activity beyond that in intact, suspended cells (Tables III and IV), the converting enzyme of cultured

TABLE VI
Distribution of Kininase Activity in Fractions of Endothelial Cells

Fraction	Total activity	Specific activity	Percent of total* activity recovered
	nmol/h	*nmol/h/mg protein*	%
Cell lysate‡	170.7±25.0	20.4±6.3	—
First pellet (30,000 *g*, 10 min)	9.3±4.1	8.3±4.2	17
Second pellet (100,000 *g*, 60 min)	3.1±0.6	6.4±1.9	3
Final supernatant (100,000 *g*, 60 min)	67.2±14.2	20.2±6.5	80

* Sum of the activities recovered in the three fractions = 100%.

‡ Cells were lysed by N_2 cavitation. Data are means of four separate experiments.

human endothelial cells must be associated with the surface of intact cell membranes.

Endothelial cells contain an enzyme that cleaves both aspartyl β-naphthylamide and angiotensin II. Nagatsu and co-workers (27) described an enzyme in human serum that released aspartic acid from both angiotensin II and α-L-aspartyl β-naphthylamide. They classified this enzyme as angiotensinase A (41) on the basis of its dependence upon calcium and inhibition by EDTA. The enzyme in endothelial cells is inhibited by another metal sequestering agent, *o*-phenanthroline, and it is probably angiotensinase A.

Angiotensin II was not inactivated during incubation with cell monolayers (Table III), which indicates either that the angiotensinase was not active on the surface of intact cells or the level of activity was below the sensitivity of our assay. Angiotensinase activity could be detected, however, in suspended cells and it increased in lysed cells, indicating that it is probably localized within the cells.

When the cells were suspended by trypsin or collagenase treatment, kininase activity increased approximately 10-fold. Endothelial cells that were suspended by scraping contained as much converting enzyme activity as did those detached by trypsin or collagenase. Thus, it is not likely that the enzyme treatment causes the increase in activity. Since the enzyme is localized on the cell surface, this increase in enzyme activity may be due, in part, to an increase in the cell surface area. When the monolayer is adherent to the surface of the culture flask, only one surface of the cell is in contact with the bathing medium. When cells are suspended, they pull apart and assume a spherical shape. This change in shape could also change infoldings of the cell surface, but we do not have evidence to support this. Another factor that may accelerate the rate of substrate cleavage is the frequency of contact between enzyme and substrate. In experiments with cell monolayers, the medium containing the peptide is slowly moved past only one surface of the cell by agitation of the flasks on a rocker platform. When the cells are suspended, they contact the substrate on all sides during constant agitation in a metabolic shaker.

It was reported that converting enzyme in tissues such as lung or kidney is localized in a microsomal or membrane fraction (6, 14, 39). However, the distribution of enzyme activity in fractions of homogenized tissues varies according to the organ of origin (8, 21, 23). We found approximately 80% of the converting enzyme activity from endothelial cells in the high-speed supernatant, or soluble, fraction. This indicates that the enzyme is loosely bound to the cell surface and that it dissociates under the conditions used for disruption and fractionation of the cells. The converting enzyme of endothelial cells may be easily denatured, since repeated freezing and thawing of the cells results in loss of activity.

The converting enzyme is presumably synthesized by cultured endothelial cells since the activity is retained by successive subcultures. Several pieces of evidence suggest that the converting enzyme is synthesized by endothelial cells rather than taken up from the serum added to the culture media. First, endothelial cells did not accumulate ^{125}I-labeled converting enzyme when it was included in the culture medium. Second, several cell lines, including human fibroblasts, smooth muscle cells and baby hamster kidney cells had no significant converting enzyme activity, although they were grown in media containing serum. Finally, the converting enzyme activity was retained by successive subcultures of endothelial cells. When a particular cell line was followed from the initial (primary) culture through several transfers, no activity was lost as long as the cells retained their typical endothelial cell morphology and slow rate of growth. In several cultures, however, there was a distinct change in morphology and rate of cell growth, presumably as a result of overgrowth with fibroblastic cells. These cultures had much less converting enzyme activity than did the authentic endothelial cell cultures, which could be explained by the finding that human skin fibroblasts have little or no converting enzyme activity.

The converting enzyme in plasma and tissues is inhibited by metal sequestering agents such as EDTA or *o*-phenanthroline, and the activity of the enzyme from plasma is enhanced by the addition of $CoCl_2$ to the reaction mixture (7). The enzyme from cultured endothelial cells is inhibited by EDTA and thus appears to have a similar requirement for a metal ion, but its activity is not affected by addition of $CoCl_2$.

The homology of the converting enzyme from endothelial cells with the enzyme from human lung was not established in these experiments. However, since an antibody prepared against purified human lung converting enzyme partially inhibited kininase activity of the cultured cells, the two enzymes presumably contain some of the same antigenic determinants. It was shown previously that antibody to purified converting enzyme is not organ, but species specific (23).

The specificity of the endothelial cell converting enzyme was studied with several kinin peptides. The deca- and undecapeptides, kallidin and methionyl-lysyl-bradykinin, are less potent than bradykinin in stimulation of smooth muscle. However, these longer analogs have a more potent vasodilator action than bradykinin when they are injected in vivo (42). We found that bradykinin was hydrolyzed by endothelial cells at a faster rate than

the longer peptides. Previously Dorer and associates (43) noted that a preparation of converting enzyme purified from hog lung cleaved kallidin and methionyl-lysyl-bradykinin more slowly than bradykinin in vitro. The prolonged in vivo actions of kallidin and methionyl-lysyl-bradykinin may be due, in part, to slower inactivation by kininase II in endothelium.

The localization of angiotensin I converting enzyme in endothelial cells of blood vessels may explain some of the actions and fate of vasoactive peptides in the systemic circulation. Although the endothelial cells of the lung can hydrolyze angiotensin I that is released by renin into venous blood, the peptide in arterial blood could be hydrolyzed by contact with endothelium in the systemic circulation or after glomerular filtration on the brush border of renal tubular cells (39). The converting enzyme in the vessels of the peripheral circulation may be important for the regulation of flow in selected vascular beds. In this regard, Collier and Robinson found that angiotensin I injected into the forearms of human volunteers caused significant changes in blood flow of that area (12).

Liberated angiotensin II is also metabolized in the peripheral circulation (1). The angiotensinase in endothelial cells cultured from human veins is inhibited by *o*-phenanthroline, and it may be angiotensinase A (41). Angiotensinase A is an aminopeptidase that could release angiotensin III (des-Asp^1-angiotensin II) by cleavage of the amino terminal aspartic acid from angiotensin II. The resulting heptapeptide is less active than angiotensin II on smooth muscles but it stimulates aldosterone secretion and thus affects salt and water retention (44, 45). The presence of enzymes that metabolize angiotensin I and II to angiotensin III (46) in cultured endothelial cells suggests that endothelium may be a site of angiotensin II generation in vivo.

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