Production of Angiotensin-(1–7) by Human Vascular Endothelium

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The heptapeptide angiotensin-(1–7) is a circulating biologically active product of the renin-angiotensin system. In this study, we evaluated the role of the vascular endothelium in the formation of angiotensin-(1–7). Metabolism of ^{125}I-angiotensin I was investigated using confluent cultured bovine and human aortic and umbilical vein endothelial cells. The fetal calf serum–supplemented medium was replaced by serum-free medium containing 0.2% bovine serum albumin. One hour later, this medium was replaced by serum-free medium containing ^{125}I-angiotensin I. After incubation of ^{125}I-angiotensin I for various intervals at 37°C, the medium was collected and analyzed for formed products by high-performance liquid chromatography. Products of angiotensin I metabolism were identified by comparison of their retention times with those of radiolabeled standards. The contribution of proteases released into the medium was evaluated by incubation of ^{125}I-angiotensin I with medium previously incubated for 1 hour with endothelial cells. Incubation of ^{125}I-angiotensin I with bovine and human endothelial cells produced a time-dependent generation of ^{125}I-angiotensin-(1–7) > ^{125}I-angiotensin II > ^{125}I-angiotensin-(1–4). Generation of angiotensin peptides was not due to the presence of proteases in the medium. When human umbilical endothelial cells were incubated in the presence of the angiotensin converting enzyme inhibitor enalaprilat (1 μM), generation of angiotensin II was undetectable. In contrast, angiotensin-(1–7) production increased by an average of 30%. Experiments in which the metabolism of ^{125}I-angiotensin I was determined in the presence of the prolyl endopeptidase inhibitor Z-pro-prolinal are consistent with the presence of a membrane-bound form of this endopeptidase being in part responsible for the production of angiotensin-(1–7) from labeled angiotensin I. The data showed that generation of angiotensin-(1–7) by an angiotensin converting enzyme–independent route is a major metabolic pathway for the processing of angiotensin I by human endothelium. (*Hypertension* 1992;19[suppl II]:II-56–II-61)

It is now recognized that the endothelium is a layer of metabolically active cells playing a critical role in the modulation of vascular responses to constrictor hormones and growth factors.[1] In addition, the endothelium acts as a local autocrine–paracrine system in the processing of vasoactive hormones.[1] Endothelial cells play a key role in the processing of angiotensin I (Ang I) into angiotensin II (Ang II) because of the presence of high concentrations of angiotensin converting enzyme (ACE) in their plasma membrane.[2,3] Cultured bovine aortic endothelial cells contain immunoreactive renin, angiotensinogen, and angiotensins.[4] Thus, in addition to their role in the formation of circulating Ang II, endothelial cells may possess an independent endogenous renin-angiotensin system.

Recent studies in this laboratory have shown that the amino terminal heptapeptide Asp-Arg-Val-Tyr-Ile-His-Pro [angiotensin-(1–7)] [Ang-(1–7)] is a principal product of the hydrolysis of Ang I in cell systems,[5] tissue homogenates,[6,7] and the circulatory system of the dog.[8,9] Ang-(1–7) is a potent vasopressin secretagogue in vitro[10] and also stimulates the release of prostaglandins.[11,12] Other studies have shown that Ang-(1–7) exerts cardiovascular effects comparable to those obtained with Ang II when microinjected in nuclei of the rat dorsomedial medulla.[13] In this study, we investigated the presence of an ACE-independent pathway for the generation of Ang-(1–7) by determining the metabolism of Ang I in cultured vascular endothelial cells.

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Methods

Cell Cultures

Bovine and porcine aortic endothelial cells were isolated by a modification of the method of Schwartz[14] as described by DiCorleto and Bowen-Pope.[15] Human umbilical vein and aortic endothelial cells were isolated as reported by Lewis et al.[16] Cells were plated onto fibronectin-coated plates (5 μg/ml rabbit fibronectin) and grown in Dulbecco's modified Eagle's (DME)/Ham's F-12 medium (1:1) (Irvine Scientific Co., Irvine, Calif.). The medium was supplemented with 0.24% sodium bicarbonate, 1× minimum essential medium with nonessential amino acids, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). Porcine and bovine cells grown in DME/F-12 medium were supplemented with 5% fetal calf serum (GIBCO, Grand Island, N.Y.), and human cells were supplemented with a mixture of 15% fetal calf serum, heparin sulfate (90 μg/ml), and endothelial cell growth supplement (150 μg/ml). Cells were maintained in an incubator at 37°C in a humidified atmosphere of 95% air–5% CO_2. At confluence, endothelial cells were collected with 0.06% trypsin/0.2% EDTA and plated at ratios of 1:3 to 1:5 in 12-well cell culture cluster dishes. Bovine and porcine endothelial cells were used between the third and seventh passages, and human endothelial cells were used at the third subculture.

Angiotensin Metabolism

Iodine-125–labeled Ang I (2,200 Ci/mmol) was initially purified by high-performance liquid chromatography to remove the peptidase inhibitor trasylol present in the radiolabeled peptide preparations supplied by New England Nuclear (Waltham, Mass.).[6] Processing of ^{125}I-Ang I into angiotensin peptides was studied in confluent cells plated at a density of 5×10^5 cells per well. The fetal calf serum–supplemented medium was replaced by 1 ml serum-free medium containing 0.2% bovine serum albumin (Pentex, Miles Scientific, Naperville, Ill.). One hour later, endothelial cells were incubated with a fresh solution of serum-free medium containing 50 pM ^{125}I-Ang I, and aliquots of the medium were collected at 15, 30, and 60 minutes by rapid chilling to 0–2°C. The solution was mixed immediately on ice with 1 ml 0.26% heptafluorobutyric acid (HFBA) containing 5 mM EDTA and 5 mM *o*-phenanthroline. Time zero controls were nonincubated mixtures of 0.5 ml serum-free medium, 0.26% HFBA, and 50 pM of the labeled peptide. Metabolism of Ang I by culture medium preincubated with endothelial cells for 1 hour was investigated to determine the potential influence of proteases released into the medium. In these experiments, 50 pM ^{125}I-Ang I was incubated with the cell-free medium for 45 minutes.

Samples were extracted by adsorption onto Bond-Elut phenylsilica cartridges. Columns were activated by sequential washes with 10 ml 80% acetonitrile/0.13% HFBA and 10 ml 0.13% HFBA containing 0.01% bovine serum albumin. After sample application, columns were washed with 20 ml 0.13% HFBA, and the peptides were eluted with 5 ml 80% acetonitrile/0.13% HFBA into polypropylene tubes rinsed with 0.1% bovine serum albumin. We have consistently observed that recovery of radiolabeled peptides using this procedure is greater than 95%.[17] After evaporation, samples were analyzed using a Model 400 chromatograph (Kratos Analytical, Inc., Ramsey, N.J.) equipped with a Brownlee Aquapore C_8 column (2×220 mm) and a Brownlee C_4 guard column (3.2×1.5 mm) (Applied Biosystems, Inc., Foster City, Calif.) using 0.13% HFBA (mobile phase A) and 80% acetonitrile/0.13% HFBA (mobile phase B).[17] The column was developed with a linear gradient from 20% to 50% B over 20 minutes and then run isocratically at 50% B for 10 minutes at a flow rate of 0.5 ml/min at room temperature. Fractions were collected every 20 seconds and counted in a gamma counter. Products of the metabolism of ^{125}I-Ang I were identified by comparison of their retention times with those of radiolabeled standards as described previously.[6,17] It was further confirmed that both synthetic Ang-(1–7) and that generated from incubation of Ang I with endothelial cells yielded no differences in the displacement curve obtained in the Ang-(1–7) radioimmunoassay. We also verified that the recovery of ^{125}I-Ang I as either ^{125}I-Tyr or ^{125}I-labeled peptides was consistently greater than 90% after high-performance liquid chromatographic separation.

Angiotensin Converting Enzyme Assay

Measurements of ACE activity in control and enalaprilat-treated cells were done as described elsewhere.[18] The cells were washed twice with phosphate-buffered saline (pH 7.4). A buffer solution made of 0.25 ml 0.1% Triton X-100 in 0.4 M sodium borate/0.9 M NaCl (pH 8.3) was added to each well. The reaction was initiated by addition of 0.25 ml Hip-His-Leu (8 mM) placed into the well. After a 20-minute period of incubation at 37°C, 0.4 ml of the assay solution was removed, mixed with 1.2 ml 0.34 M NaOH, and the product His-Leu measured as described previously.[18] Blanks were prepared by addition of 10 μM enalaprilat into the assay mixture. Substrate hydrolysis was found to be linear for up to 1 hour of incubation. No significant hydrolysis of the product His-Leu was found under the assay conditions described above. Moreover, the slope of the standard curve was not changed by extracts obtained from endothelial cells, showing that endothelial cells did not quench the fluorescence of the product.

Results

The addition of ^{125}I-Ang I to cultured endothelial cells obtained from blood vessels of either animals or humans was followed by the generation of Ang-(1–7), Ang II, and angiotensin-(1–4) [Ang-(1–4)] as major products of Ang I metabolism. Both the rate and amount of Ang I metabolism differed among the various screened cultures. Human and bovine aortic endothelial cells hydrolyzed approximately 30–40%

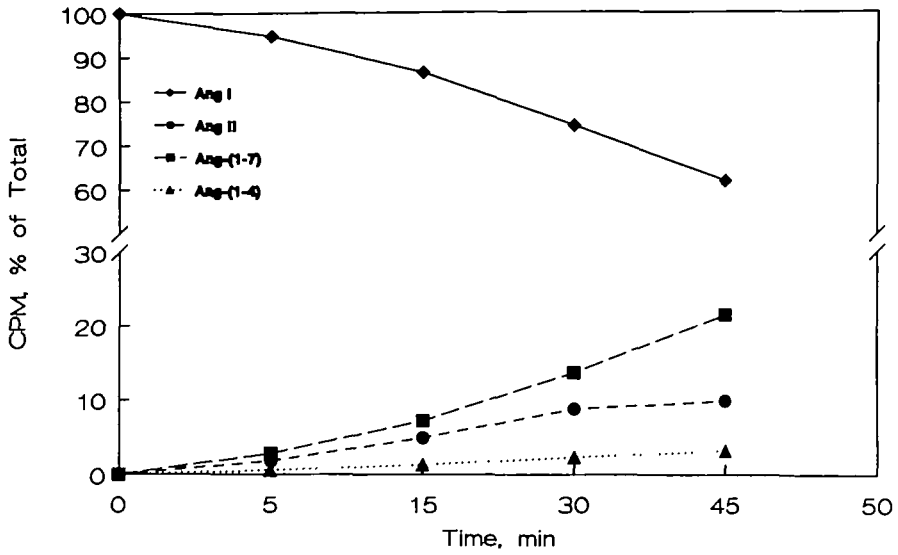

FIGURE 1. *Line graph shows time course of the metabolism of ^{125}I-angiotensin I (Ang I) (50 pM) by human umbilical endothelial cells at third passage. Data are expressed as percent total radioactive counts per minute (CPM) recovered after separation of peptides by high-performance liquid chromatography. Samples were obtained from a pool of cells. Experiments were replicated three times. Ang II, angiotensin II; Ang-(1–7), angiotensin-(1–7); Ang-(1–4), angiotensin-(1–4).*

of ^{125}I-Ang I into angiotensin peptides. Ang-(1–7) was the predominant peptide formed. In contrast, ^{125}I-Ang I hydrolysis over a 45-minute period of incubation with endothelial cells obtained from porcine aorta amounted to less than 5%. In these subcultures, fractional metabolism of the labeled peptide was associated only with the production of trace amounts of ^{125}I-Ang-(1–7) as the major peptide. Further comparisons of the rate of ^{125}I-Ang I metabolism among the various preparations used in these experiments showed that Ang I hydrolysis was consistently greater in endothelial cells obtained from human umbilical vein. Therefore, these cells were used for all additional experiments.

Figure 1 shows the time course of the metabolism of ^{125}I-Ang I by human umbilical vein endothelial cells. We found that ^{125}I-Ang-(1–7) production averaged 21.5% of the total ^{125}I-Ang I metabolism 45 minutes after addition of labeled Ang I. At this time, corresponding levels of ^{125}I-Ang II and ^{125}I-Ang-(1–4) were 9.9% and 3.2%, respectively. Peptidases released into the medium did not contribute to the generation of either ^{125}I-Ang-(1–7) or other Ang I congeners. Incubation of ^{125}I-Ang I with serum-free medium, previously incubated for 1 hour with human umbilical vein endothelial cells, showed less than 1% conversion into ^{125}I-Ang-(1–7).

Measurements of the ratio between prohormone level and formed products provided a reliable estimate of hormone processing.[8] Therefore, we calculated radiolabeled Ang-(1–7)/Ang I, Ang II/Ang I, and Ang-(1–4) Ang I ratios from the data obtained in three separate metabolism experiments. Figure 2 shows that the ^{125}I-Ang-(1–7)/^{125}I-Ang I ratio as a function of the corresponding concentration of remaining ^{125}I-Ang I was always higher than that obtained for either ^{125}I-Ang II/^{125}I-Ang I or ^{125}I-Ang-(1–4)/^{125}I-Ang I ratios. Forty-five minutes after addition of ^{125}I-Ang I, the levels of ^{125}I-Ang-(1–7) constituted 53% of the total ^{125}I-Ang I. Thus, these data confirmed that ^{125}I-Ang I is metabolized primarily into ^{125}I-Ang-(1–7). The data shown in Figure 2 also suggest that Ang-(1–7) generation occurred at a rate greater than that of Ang II. The slope of the relation between the ^{125}I-Ang-(1–7)/^{125}I-Ang I ratio and Ang I averaged -0.71 ± 0.12. This value is 2.5-fold greater than that measured for the ratio of ^{125}I-Ang II/ ^{125}I-Ang I (-0.28 ± 0.04). In other words, progressive exhaustion of ^{125}I-Ang I is not accompanied by a diminished rate of ^{125}I-Ang-(1–7) production.

We also investigated the dependence of Ang I metabolism on the activity of ACE by pretreatment of human umbilical vein endothelial cells with enalaprilat (1 μM final concentration). In extracts of cultured endothelial cells, baseline ACE activity averaged 172 ± 7 nmol His-Leu per 10^6 cells per hour. In endothelial cells exposed to enalaprilat, ACE activity fell to values below the detectable level of the assay. Figure 3 shows that the metabolism of radiolabeled Ang I in the presence of ACE inhibition was characterized by blockade of ^{125}I-Ang II production, whereas the formation of ^{125}I-Ang-(1–7) increased by an average of 30% when compared with values obtained in nontreated cells run in parallel experiments. Therefore, the hydrolysis rate of ^{125}I-Ang I was not affected by blockade of ACE. In addition, small amounts (<1%) of Ang-(1–4) were produced in enalaprilat-treated cells. These results suggest that production of Ang-(1–7) represents a major parallel pathway of the metabolism of Ang I in human umbilical endothelial cells. In additional experiments, endothelial cells were incubated in the presence of 1 μM *N*-benzyloxycarbonyl-prolyl-prolinal (Z-pro-prolinal), a competitive inhibitor of prolyl endopeptidase (EC 3.4.21.26).[19] In these experiments, the production of ^{125}I-Ang-(1–7) was reduced between 25% and 40% of the amounts generated in nontreated cells. These data agree with findings obtained in studies of Ang I metabolism in neuroblastoma×glioma cell lines.[5]

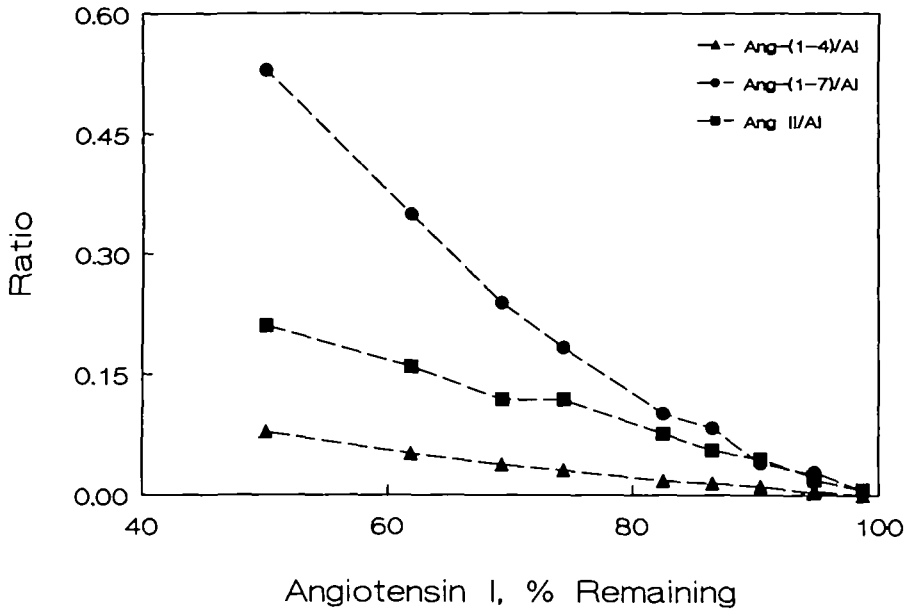

FIGURE 2. *Line graph shows relation between angiotensin peptide ratios and the extent of angiotensin (Ang) I hydrolysis. Data are means from three separate experiments. Values for the regression line* ($y=a+bx$) *determined by the method of least-squares of peptide ratios vs. Ang I substrate are: [Ang-(1–7)/Ang I]=70.6±0.71 Ang I,* $r=-0.90$; *(Ang II/Ang I)=30.16±0.28 Ang I,* $r=-0.91$. *AI, angiotensin I.*

Discussion

Experiments with cultured endothelial cells showed a systematic processing of Ang I into Ang-(1–7), the newly established biologically active product of the renin-angiotensin system.[20] Conversion of the labeled Ang I precursor into Ang-(1–7) as the major product followed similar patterns of processing in both human and bovine vascular endothelial cells. Only porcine subcultures of aortic endothelial cells had limited hydrolysis of Ang I, but even in this situation, the slow metabolism of the precursor yielded trace amounts of Ang-(1–7). These data extend previous conclusions derived from similar metabolism studies in neuroblastoma×glioma cell lines[5] and in homogenates of canine hypothalamus.[6] In the current experiments, we further demonstrated that the catalytic activity of vascular endothelial cells of different origins also leads to the preferential formation of Ang-(1–7) from Ang I.

In agreement with other studies, cells in culture cleaved Ang I into Ang II at a rate lower than that found in isolated organs or the intact circulation.[21,22] Recently, Dezsö et al[23] reached a similar conclusion from the analysis of Ang I metabolism by rat alveolar macrophages. However, at all time points of a 60-minute incubation, we found that the relative concentrations of Ang-(1–7) always surpassed those of Ang II. Ang-(1–4) was the only other angiotensin fragment produced in concentrations above the detectable level of the high-performance liquid chromatographic procedure used in these experiments. Examination of the rate of formation of the products produced during incubation with labeled Ang I in human umbilical vein endothelial cells showed that peak levels of Ang II occurred within 30–45 minutes. These data agree with those obtained by others.[24] In contrast, the rate of Ang-(1–7) production, whether expressed either in terms of the level of the product or as the ratio of Ang-(1–7) to Ang I, continued to increase throughout the period of precursor hydrolysis. These data suggest that Ang-(1–7) may be an immediate product of the metabolism of Ang I. A similar conclusion was derived from studies in NG108-15 neuroblastoma×glioma hybrid cell lines, in which the peak of Ang-(1–7) generation preceded the production of Ang II, and Ang-(1–7) generation increased when Ang II production was blocked.[5]

ACE inhibition prevented the production of Ang II but further increased the rate of Ang-(1–7) production. Similar findings were obtained in studies of angiotensin metabolism in intact dogs,[8,9] brain homogenates,[7] and NG108-15 neuroblastoma cell lines.[5] These data demonstrate that Ang-(1–7) may be formed directly from Ang I by an enzymatic pathway distinct from that involved with the generation of Ang II. In intact endothelial cells, production of Ang-(1–7) was partially blocked by the addition of Z-pro-prolinal, an inhibitor of prolyl endopeptidase. Although earlier studies suggested that prolyl endopeptidase is a cytosolic enzyme,[25] Checler et al[26] found an ectocellular form of this enzyme in neuronal cells. In our experiments, we verified that the catalytic activity associated with the production of both Ang-(1–7) and Ang II was not due to the presence of enzymes released into the medium. Only trace amounts of angiotensin peptides were produced in the 1-hour incubation of ^{125}I-Ang I with medium that was collected after previously being incubated in the presence of endothelial cells. In addition, we found that extracts from endothelial cells, previously incubated with ^{125}I-Ang I for 60 minutes, contained between 0.5% and 1% of the total radioactivity. These data suggest that the catalytic activity of the enzymes responsible for the production of either Ang II or Ang-(1–7) exists on the surface of these cells. Although further evidence

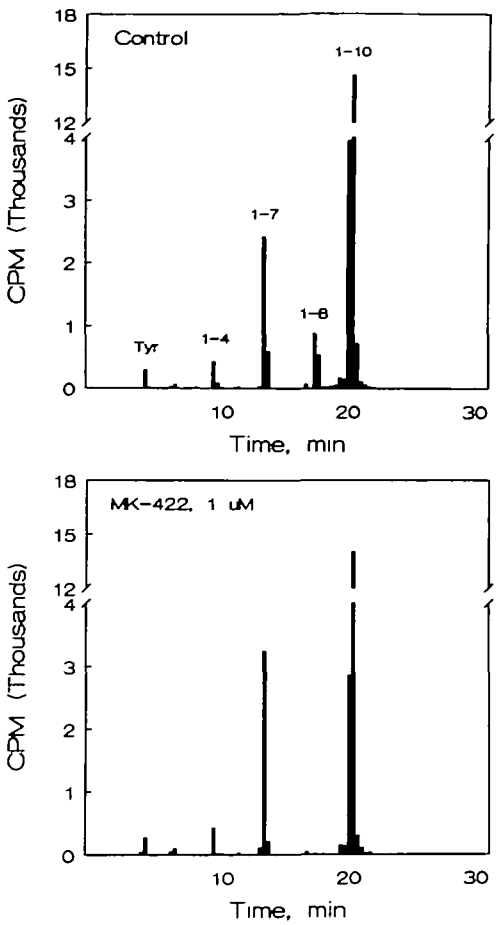

FIGURE 3. *Histograms show profile of angiotensin peptides after pretreatment of human umbilical vein endothelial cells with the angiotensin converting enzyme inhibitor enalaprilat (MK-422).* Top panel *denotes cells incubated for 5 minutes with vehicle (10 μl of 0.9% sodium chloride).* Bottom panel *illustrates effects of preincubation of endothelial cells with 1 μM MK-422. ^{125}I-Angiotensin I was added to the cultures at a concentration of 50 pM, and high-performance liquid chromatographic data are findings after incubation for 45 minutes at 37°C.*

is provided for a role of prolyl endopeptidase in the production of Ang-(1–7), we again note that inhibition of this enzyme does not block production of Ang-(1–7) in its entirety. The nature of the other peptidases able to cleave the postproline bond of Ang I is not known. In NG108-15 neuroblastoma cells, complete inhibition of ^{125}I-Ang I metabolism into Ang-(1–7) was obtained by the addition of *p*-chloromercuriphenylsulfonate, suggesting the contribution of a sulfhydryl-containing peptidase other than prolyl endopeptidase.[5] In addition, other peptidases that may contribute to the production of Ang-(1–7) include neutral endopeptidase 24.11 (EC 3.4.24.11).[2] However, further studies are needed to determine its role in the production of Ang-(1–7).

In summary, the principal finding of our study is that the biologically active Ang-(1–7) can be generated from Ang I by cultured vascular endothelial cells via an ACE-independent pathway. In addition, we further confirmed that prolyl endopeptidase is partially involved in the generation of Ang-(1–7).

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KEY WORDS • angiotensins • angiotensin I • blood pressure • endothelium • angiotensin converting enzyme • endopeptidase

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