Endothelial Renin-Angiotensin Pathway: Evidence for Intracellular Synthesis and Secretion of Angiotensins

Imre Kifor and Victor J. Dzau

Cultured bovine aortic endothelial cells (BAEC) contain renin and angiotensinogen. To examine whether angiotensins are synthesized intracellularly and secreted by these cells, we assayed cell extracts as well as serum-free media of intact confluent BAEC. Angiotensins were identified by their retention time on reverse phase high performance liquid chromatography and direct radioimmunoassay. BAEC and their media contain angiotensin II and angiotensin III. The rate of angiotensin accumulation in the media was a nonlinear function of time; the highest rate occurred in the first 15 minutes. The amount of angiotensin II accumulated in 30 minutes exceeded 200% of the intracellular concentration and that of angiotensin III exceeded 500% of the initial intracellular content. Neither renin nor angiotensinogen could be detected in the media. The viability of these cells was supported by low lactic dehydrogenase activity in the media (<0.5% of cellular level). These data suggest that BAEC is capable of synthesizing and secreting angiotensins. We postulate that this endothelial-derived angiotensin system may play an important paracrine or autocrine role in influencing local vascular tone. (Circulation Research 1987;60:422-428)

The classic concept of the renin-angiotensin system (RAS) is that it is a blood-borne hormonal system whose principal components, enzyme (renin) and substrate (angiotensinogen), are secreted into the circulation by the kidney and liver, respectively. Angiotensin I, the product of renin angiotensinogen reaction, is cleaved by the angiotensin-converting enzyme (ACE) in blood and on endothelial cells to the vasoactive peptide, angiotensin II. This peptide, produced and transported in the circulating blood, acts on its major target organs, i.e., blood vessels, adrenal cortex, kidney, and possibly brain.1-3

Recent evidence has demonstrated that the major target organs of angiotensin II may have independent endogenous renin-angiotensin systems. The essential components of RAS have been revealed in the brain4-6 and the vascular wall.6-10 In addition, immunoreactive renin was also demonstrated in many other tissues, including adrenal glands, anterior pituitary, the Leydig cells of testis, the follicular epithelial cells of thyroid gland, the uterus, and the prostate.11-17 The physiologic implication of tissue RAS is that angiotensin-mediated functions of these target organs may be locally regulated, independent of circulating RAS.

In recent years, the role of the vascular endothelium in determining vascular responses to vasoactive agents has been elucidated. The endothelium acts as a transducer of signals to the underlying smooth muscle. One of its mechanisms of influencing smooth muscle function is through the synthesis of vasoactive substances, e.g., endothelium-derived relaxant factor18 and autacoids such as prostacyclin.19 Recently, it was reported that cultured bovine aortic endothelial cells contain the immunoreactive renin and angiotensins.20 This observation led us to postulate that the endothelium-derived renin may play a role in regulating local angiotensin production. For this hypothesis to be valid, these cells should contain renin substrate and have the capability of producing angiotensins. The purposes of this study are to examine whether cultured bovine aortic endothelial cells contain renin substrate and angiotensins, to identify and characterize endothelial angiotensins, and to examine which components of the renin-angiotensin system are secreted by these cells.

Materials and Methods

Cell Culture

Bovine aortic endothelial cells, line SC 11 (a kind gift of Dr. M. Gimbrone), were grown in 10-cm plastic culture dishes (Falcon, Becton Dickinson Labware, Oxnard, Calif.) at 37°C in room air and 5% carbon dioxide in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% calf serum and 100,000 units/l penicillin, 100 mg/l streptomycin, and 3 mg/l fungizone (Gibco Labs, Grand Island, N.Y.). The cell cultures were characterized by morphological and biochemical criteria. The presence of factor VIII, a characteristic feature of vascular endothelial cells, was demonstrated with immunofluorescent techniques, using fluorescein-tagged factor VIII antibody.20
**Experimental Protocol**

All experiments were carried out with cell cultures obtained after 20–36 passages. Lilly et al demonstrated that cells of this late passage contained comparable levels of renin and maintained their usual phenotypic expression (e.g., factor VIII staining) as compared with the cells from earlier passages. The release of angiotensin as well as renin and angiotensinogen was followed in confluent cell cultures, employing a DMEM in which the calf serum was replaced with 0.5% crystalline bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.). This will be referred to as "assay medium." Both calf serum and BSA were heat inactivated so that they contained no detectable renin or renin substrate activities (Kifor and Dzau, unpublished observations). With each culture dish of endothelial cells, 3 successive experiments were performed. First, the cells were incubated at 37°C in room air and 5% carbon dioxide for two consecutive 24-hour periods (Periods I and II), each in 10 ml of fresh assay medium per culture dish in a CO₂ incubator. This was followed by a short-term experiment (Period III), during which the cells were incubated up to 60 minutes at 37°C in a water bath in 95% oxygen and 5% carbon dioxide with 5 ml of fresh assay medium per culture dish. After each study period, the medium was collected for analysis and replaced with fresh medium. At the end of the short-term experiment, the cells were extracted for further analysis. To obtain a sufficient amount of material, in each experiment 18–20 culture dishes were used, and the media, as well as the cell extracts, were pooled.

**Angiotensin Assays**

The pooled media were immediately mixed with trifluoroacetic acid (final concentration 4%) and heated to boiling. The protein precipitate was removed by centrifugation and the supernatant was extracted with RP 18 SEP PAK cartridges (Waters Associates, Milford, Mass.). After washing with 0.01 M trifluoroacetic acid for two successive 24-hour periods (Periods I and II), each in 10 ml of fresh assay medium per culture dish, the cartridge was eluted with 0.5 ml of 0.02 M acetic acid. The eluate was lyophilized, then redissolved in 0.02 M acetic acid and subjected to high performance liquid chromatography (HPLC). As a control, 100 ml of fresh assay medium not previously exposed to cells was processed in the same manner. The intracellular angiotensins were extracted by the following technique: Immediately after removal of the medium, 0.2 ml of concentrated trifluoroacetic acid was added to each dish. The dishes were covered with a lid for 5 minutes, then washed twice with 1 ml of 0.02 M acetic acid. The wash fluids containing detached, denatured cells were collected and the suspension sonicated for 2 x 10 seconds using a Sonicator Cell Disrupter (Heat Systems-Ultrasonics Inc., Farmingdale, N.Y.). The sonicate was then heated to boiling and further processed in the same manner as the assay medium.

Reverse-phase HPLC was performed employing a Varian 5000 solvent delivery system combined with a Spectroflow 757 variable UV monitor tuned to 216 nm. The data handling and processing was performed using an Apple IIe PC, loaded with Chromatograph software (Interactive Microwave, Inc., State College, Penn.). The angiotensins were separated on a 5-cm Supelco 518 column (Supelco Inc., Bellafonte, Penn.). The solvent system consisted of 20% methanol in Na acetate 10 mM, pH 5.6 (A) and 80% methanol in Na acetate 10 mM, pH 5.6 (B). The gradient at 0–5 minutes was B = 0%; at 50 minutes, B = 100%. The flowrate was 1 ml/min. The fractions were collected in polypropylene test tubes; the collection time for a fraction was 30 seconds.

After lyophilization, the samples were redissolved in 0.4 ml of 0.02 M acetic acid and mixed with 0.65 mL radioimmunoassay (RIA) buffer consisting of KH₂PO₄ 0.001M, pH 7.4, ethylenediaminetetraacetic acid (EDTA) 3 mM, 0.15 mM 8-hydroxy-quinoline, 0.02% neomycin sulphate, and 2.5 mg/ml crystalline BSA (Sigma Chemicals Co., St. Louis, Mo.). To inactivate the peptidase contaminating the BSA, the buffer was first heated at 56°C for 30 minutes. The fractions corresponding to the elution times of synthetic angiotensin II and angiotensin III (Peninsula Labs, Belmont, Calif.) were assayed with an angiotensin II antibody, which has 100% crossreactivity with angiotensin III (a kind gift of Dr. Arthur Freedlander). Since the RIA was always performed after HPLC, in fractions corresponding only to the elution times of angiotensins II and III, the crossreactivity of antibody with nonangiotensin peptides was not a major issue.

**Renin and Angiotensinogen Assays**

To examine whether renin and/or angiotensinogen were secreted, 600 ml of assay medium collected from 60 tissue culture dishes from Period II was examined (n = 3). The medium was concentrated by ultrafiltration using an Amicon-stirred cell ultrafiltration system loaded with Amicon Diaflo PM 10 ultrafiltration membrane (Amicon Co., Lexington, Mass.). The hundredfold concentrated medium was assayed for renin activity using excess undiluted sheep anephric plasma (containing 8-hydroxy-quinoline, dimercaptopropanol, phenylmethylsulfonyl fluoride, EDTA, captopril) as source of substrate at 37°C, as previously described. Renin activity was expressed as generation of angiotensin I/hr at pH 7.4.

Renin substrate was assayed in the hundredfold concentrated medium using excess purified human renin (R-18, Ciba-Geigy, Basel, Switzerland) and semipurified bovine renal renin, and the reaction was driven to exhaustion of substrate in the presence of multiple protease inhibitors according to protocol, as previously described. Intracellular angiotensinogen was also determined in cell homogenates by the above method (n = 4). For these experiments, the cells were sonicated, as described earlier, in the presence of the following protease inhibitors: 5 mM EDTA, 5 mM sodium tetrathionate, 0.1 mm phenylmethylsulfonyl fluoride, 0.1 mM captopril, 1 mM 8-hydroxy-quinoline, and 5 mM 2,3-dimercaptopropanol, as described by Lilly et
al. The authenticity of endothelial angiotensinogen was examined using anti-rat angiotensinogen antibody (a kind gift of Dr. Hilgenfeldt). This antiserum cross-reacts with bovine angiotensinogen (authors’ unpublished observation). In these experiments, cell homogenates were preincubated with antiangiotensinogen antiserum (1:10) or preimmune serum (1:10) at 37°C for 1 hour prior to the addition of excess renin. Angiotensinogen concentration was expressed as total angiotensin I generation at pH 7.4. The stability of angiotensin I in the incubation mixture with protease inhibitors was always verified by examining the recovery of a standard amount of exogenously-added angiotensin I in control experiments. Only greater than 80% recovery was acceptable.

Lactic Dehydrogenase Assay

To follow the nonspecific release of cell components as a result of cell damage and leakage, cells and media were assayed for lactic dehydrogenase (LDH) activity using Sigma test kits. The LDH activity was measured in the assay media during Period II and Period III, intracellularly and at different time points.

Statistical Analysis

For any statistical analysis, the logarithms of data were used. The analysis of variance was performed using a Basic Business software package (Basic Business Software, Inc., Las Vegas, Nev.).

Results

The separation of angiotensin in the endothelial cells and in their media was accomplished with reverse-phase HPLC. The baseline separation of angiotensin II and angiotensin III by 92 ± 12 seconds allowed separate collections (Figure 1). These peptides were then measured by radioimmunoassay using angiotensin II antibody, which crossreacts 100% with angiotensin III. The data indicate that the cultured bovine aortic endothelial cells, as well as their assay media, contain angiotensin II and III (Table 1). Lilly et al. have previously shown that angiotensin I is present in these cells. Considering the fact that angiotensin II and angiotensin III are the major bioactive components of the renin-angiotensin system, we focused on these peptides. The amount of angiotensin II and angiotensin III in the assay medium, collected after the second 24-hour incubation, is twofold higher than in the medium collected after the first 24-hour period (Figure 2). The time course of angiotensin accumulation in the culture media in a short-term experiment (Period III) was also studied. In this experiment, the endothelial cells were incubated in fresh assay medium, and the time course of the appearance of angiotensins in the medium was followed. The accumulation of angiotensins in the medium is a nonlinear function of time (Figure 3). The rate of accumulation is highest in the first 15 minutes of incubation; thereafter, the rate declines (Figure 4). If the amount of angiotensin appearance in the medium is expressed as a percent of intracellular content, it becomes evident that the angiotensin II accumulated is more than twofold and the angiotensin III is more than fivefold their respective initial intracellular amounts (Figure 5). In the same period, less than 0.5% of the intracellular lactic dehydrogenase activity is accumulated in the assay medium (Figure 5). This feature suggests angiotensin is actively produced and secreted by these cells. The angiotensin levels in the endothelial cells decrease during the short-term experiment (Figure 6). The exact angiotensin form secreted by the cells cannot be determined in our experiment. It is also

<table>
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<tr>
<th>Renin Activity (ngAl/h/10^6 cells)</th>
<th>Concentration</th>
<th>% Inhibition</th>
<th>n</th>
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<tbody>
<tr>
<td>1.2 ± 0.2*</td>
<td>40-80†</td>
<td>8</td>
<td></td>
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<tr>
<td>Renin substrate (ngAl equiv/10^6 cells)</td>
<td>0.9 ± 1.4</td>
<td>90-100‡</td>
<td>4</td>
</tr>
<tr>
<td>Angiotensin II (pg/10^6 cells)</td>
<td>17.9 ± 1.6</td>
<td>. . .</td>
<td>3</td>
</tr>
<tr>
<td>Angiotensin III (pg/10^6 cells)</td>
<td>12.5 ± 1.2</td>
<td>. . .</td>
<td>3</td>
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</tbody>
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* = Reported by Lilly et al. † = percent inhibition by antirenin antibody as described by Lilly et al. ‡ = % inhibition by angiotensinogen antibody (i.e., to 0.06 ± 0.01 ngAl equiv/10^6 cells); n = number of experiments.
The amount of angiotensin II and III accumulated in the assay media during the short term incubation (Period III), as function of time of incubation.

It is possible that angiotensin I and/or des-ASP angiotensin I rather than angiotensin II or III are secreted and subsequently processed to angiotensin II and III.

Cultured endothelial cells contain renin activity and renin substrate as previously described. Endothelial renin substrate is immunoreactive angiotensinogen, since preincubation with an antibody (1:10 dilution), which crossreacts with bovine angiotensinogen, completely neutralized the ability of the cell homogenate to generate angiotensin I when incubated to exogenous renin (Table 1). Neither renin nor angiotensinogen could be detected in the medium despite hundredfold concentration (n = 5).

Discussion

The results presented here demonstrate that in addition to immunoreactive renin, angiotensinogen, and angiotensins are present in cultured bovine aortic endothelial cells. It has previously been shown that angiotensin I is present in these cells. In this study, we demonstrate that angiotensins II and III are present intracellularly and also appear in the culture medium. The higher increment of angiotensin II and III accumulation in the media during the second 24-hour period after removal of calf serum, as well as the observation during the short-term secretion experiment (Period III) that the amount of angiotensins II and III accumulated exceeds severalfold the initial amount of intracellular levels, are evidences against cellular release of preformed, endocytized, and stored angiotensin II or III. They suggest, rather, a production of angiotensins in the cells followed by an active secretion. Our data do not establish definitively that angiotensin II or III are the peptide(s) secreted by the endothelial cells. It is also possible that angiotensin I and/or des-ASP angiotensin are released and subsequently converted to angiotensin II and/or III in the medium.

It is not probable that the release of angiotensins is due to cell leakage since less than 0.5% of intracellular lactic dehydrogenase activity escaped during the ex-
periment. In contrast, over the same 60-minute period more than 200% of intracellular angiotensin II and more than 500% of angiotensin III were accumulated. The time course of angiotensin and LDH accumulation is also different. Angiotensin accumulates rapidly in the medium, peaking in the first 15 minutes. In contrast, LDH accumulation is linear with time and is highest at 60 minutes.

The increase of angiotensin II and angiotensin III concentrations in the assay media after removal of calf serum resembles the increase of intracellular renin and angiotensin in cultured rat juxtaglomerular cells grown without serum supplementation. A similar phenomenon of serum-mediated decrease of plasminogen activator production in cultured bovine aortic endothelial cells has also been reported. These data suggest that the removal of calf serum from the medium of confluent cell culture may cause a switch in cellular biology favoring increases in synthetic and secretory functions.

The rate of accumulation of angiotensin II and angiotensin III in the medium followed by the short-term secretion experiment does not appear to be a linear function of time. The highest rate was observed during the first 15 minutes of Period III. Thereafter, the rate of accumulation declined. Possible explanations for this phenomenon include feedback regulation of secretion by angiotensins or a rapid degradation of angiotensin II as well as the exhaustion of preformed substrate or product pools. The fast decline in angiotensin accumulation rate in the culture medium renders it difficult to estimate the kinetics of angiotensin production and release by the endothelial cells. To assess the angiotensin output rate of endothelial cells, very short secretion periods or a superfusion model should be used. Under these conditions, the feedback inhibition and the degradation of angiotensins may be substantially reduced.

The high level of angiotensin III in the endothelial cells and in their media indicate an intensive ASP-aminopeptidase activity. The removal of amino terminal aspartyl residue may be a rate-limiting reaction in angiotensin II degradation. The product, angiotensin III, possesses only a fraction of the pressor activity of parent peptide and is more susceptible to further non-specific degradation. However, angiotensin III has also been shown to possess other biological activities and can exert actions in a number of target tissues (e.g., adrenal, renal vasculature, blood vessels, central nervous system, etc.). Angiotensin II and angiotensin III appear to be equipotent in potentiating aortic contractility.

The inability to detect renin could indicate that the renin is membrane-bound and not secreted. This form of renin has been previously reported. Alternatively, the amount of renin secreted by these cells is too low for detection. In this regard, the culture medium was concentrated a hundredfold to enhance the detection of the enzyme. A third possibility is that secreted renin is rapidly and completely degraded. This possibility should have been circumvented with the addition of large amounts of protease inhibitors to the media prior to assay.

The absence of detectable angiotensinogen in the culture medium suggests that either angiotensinogen is present in nonsecretory cellular compartments but can be readily converted into angiotensins and packaged into vesicles for secretion or that endothelial angiotensinogen is completely converted into angiotensins intracellularly prior to secretion. The high intracellular angiotensinogen concentration reported in this study tends to support the former possibility. It is unlikely that the inability to detect angiotensinogen in the culture medium is due to rapid conversion and degradation of secreted angiotensinogen, since the medium is free of renin-like enzymes.

The observations presented here (that the endothelial cells secrete the active products of the renin-angiotensin systems), may have important physiological, pathophysiological, and pharmacological implications. Endothelial-derived angiotensins may influence vascular tone in a variety of settings. Angiotensin II can influence endothelial prostaglandin biosynthesis (autocrine effect). Angiotensin II can induce vascular smooth muscle cells (paracrine effect) and by enhancing norepinephrine release from noradrenergic nerve endings in the blood vessel wall (paracrine effect). The potential importance of vascular renin is supported by the observations that vascular renin-angiotensin activities are increased in several experimental models of hypertension. In some patients with essential hypertension associated with low plasma renin levels, inhibitors of the RAS can elicit depressor responses despite low plasma renin activity. One possible explanation of the depressor response is that these agents exert their actions by inhibiting vascular renin-angiotensin activity.

Finally, there is increasing evidence that renin is colocalized with angiotensin in a number of cells. This phenomenon was described in the juxtaglomerular cell, the neuroblastoctumor cell, the Leydig cell of rat testis, and the Leydig cell of rat testis. There is also increasing evidence that angiotensins are secreted from these cells. Sometimes they are cosecreted with renin; sometimes they are secreted alone. In human renovascular hypertension, the concentration of angiotensin II is 3.6 times higher in the vein of the renin-secreting kidney than in the arterial blood. All data presented here support the possibility that the direct
secretion of angiotensins is a general phenomenon rather than an exception. The observation that the endothelial cells secrete angiotensins is in keeping with the emerging concept that angiotensins are produced in peripheral tissues and only a portion of plasma concentration is produced by the "classic" circulating renin-angiotensin system.46,54

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