Renin Expression by Vascular Endothelial Cells in Culture

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SUMMARY. Cultured bovine aortic endothelial cells were examined for renin activity by biochemical, immunological, and immunohistochemical techniques. When cell sonicates were incubated with renin substrate, linear generation of angiotensin I was observed (1.12 ± 0.2 ng angiotensin I/10⁶ cells per hr). The effect of pH on this activity was similar to that of bovine renal renin, and renin antibodies inhibited a large portion of the enzymatic activity. Furthermore, immunofluorescence microscopy with antirenin antisera confirmed the presence of renin within these cells. Biosynthetic radiolabeling, followed by immunoprecipitation, demonstrated de novo synthesis of a renin precursor in the endothelial cells, which was processed to a more mature protein. Thus, bovine aortic endothelial cells in culture contain and biosynthesize renin, a key component of the renin-angiotensin system. The expression of renin activity by endothelium may contribute to the local regulation of vascular tone. (Circ Res 57: 312-318, 1985)

THE vascular endothelium actively participates in the control of vascular tone through the synthesis and metabolism of a number of vasoactive substances. Angiotensin-converting enzyme (ACE), for example, has been localized to the endothelial cell surface (Ryan et al., 1976; Johnson et al., 1977; Hial et al., 1979), where it acts to convert angiotensin I (AI) to angiotensin II (All), thus serving to increase vascular smooth muscle tone. Although circulating renin has been considered to be the major rate-limiting determinant of All production, recent data suggest that renin residing within the vascular wall, or at the blood/vessel interface, may also play an important role (Gould et al., 1964; Swales and Thurston, 1973; Basso and Taguini, 1974; Thurston and Swales, 1977; Barrett et al., 1978; Thurston et al., 1979; Aguilera et al., 1981). However, the exact cellular localization of renin in blood vessels in vivo and its site(s) of synthesis remain incompletely understood. In the current study, we have examined the expression of renin by cultured vascular endothelial cells. Using biochemical, immunological, and immunohistochemical techniques, we have obtained evidence for the biosynthesis and intracellular processing of renin in cultured bovine aortic endothelial cells.

Methods

Cell Cultures

Bovine aortic endothelial cells (11-BAEC strain) were isolated and characterized, as previously described (Gimbrone, 1976), and studied at the 17th-26th subpassages. Replicate-plated cultures in plastic petri dishes, 100 mm in diameter, were grown to confluence in Dulbecco's modified Eagle's medium (DME) containing 10% calf serum (CS), 100 U/ml penicillin, and 100 g/ml streptomycin. Confluent monolayers were routinely incubated for the final 24 hours before assay in DME containing 0.5% bovine serum albumin (BSA). The cultures were washed twice with ice-cold Dulbecco's phosphate-buffered saline (DPBS) and scrape-harvested in DPBS containing 0.5% BSA on ice. Cell counts were performed by hemacytometry, and cellular viability was assessed by trypan-blue exclusion. Cell sonicates were prepared (Ultrasoundics microtip probe ultrasonicator, position 3, 15 seconds, 4°C) in the presence of the following mixture of protease inhibitors: 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium tetrathionate (NATT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM captopril, 1 mM 8-hydroxy-quinoline (8-OH-Q), and 5 mM 2,3-dimercaptopropanol (BAL).

Characterization and Identification of Cellular Renin-like Activities

Cellular homogenates were immediately assayed for renin-like activity as measured by AI generation. Aliquots of sonicate were incubated with nephrectomized sheep (containing 810 ng of angiotensinogen/ml) at 37°C, pH 6.1, for 0–3 hours. Incubates were boiled, then spun in an Eppendorf centrifuge (13,000 g for 5 minutes), and the supernatants were assayed for AI generation by the RIA method of Haber et al. (1969). The effect of pH on AI-generating activity was studied over the pH range 3.5–9.0. The pH of the sonicates was adjusted before the assay by 0.2 M solutions of sodium citrate, sodium acetate, or potassium phosphate. The resultant pH profile was compared with that of bovine renal renin (specific activity of 6.7 ng Al/hr per mg protein), which was derived from a three-step purification procedure (Dzau et al., 1982b).

To determine whether cellular Al generation was dependent on the activity of intracellular aspartyl proteases, we evaluated the effect of pepstatin, the specific inhibitor.
for these enzymes (Gross et al., 1972). Cellular sonicates were incubated at 37°C with 0.1–1 mM pepstatin at pH 5.5 for 1 hour. Incubates then were reacted with renin-free nephrectomized sheep plasma at 37°C for 1 hour, and residual AI generation was measured at pH 6.1 by the method described above. Inactive renin was examined by means of trypsin activation. Ninety microliters of cell sonicate (approximately 10^6 cells) were incubated with trypsin (10^−6 to 1 mg/ml) for 20–60 minutes at 4°C. The reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/ml) and aprotinin (0.44 TIU) at 25°C for 10 minutes. Renin activity then was assayed as described above.

We evaluated the immunoreactivity of cellular renin-like activity, using goat anticanine renin (GLN 816) antisera derived and characterized as reported previously (Dzau et al., 1982a, 1982b, 1984). This antisera is specific for renin and does not bind or inactivate cathepsin D, cathepsin B, trypsin, kallikrein, converting enzyme, or a large number of other proteins tested. Cross-reactivity between the anticanine antiserum and bovine renal renin has been shown (Dzau et al., 1982b). Additional data on the immunoreactivity of cellular renin-like activity were obtained using rabbit antihuman renin (R1723) antisera. This antisera is raised in rabbits immunized with pure human renal renin, as described (Ishue et al., 1983). This antisera forms a single precipitin arc with pure renin and crude kidney homogenate on immunodiffusion and immunoelectrophoresis. Immunoradiometric assay indicated that R1723 did not bind cathepsin D, trypsin, albumin, or angiotensin-converting enzyme. Furthermore, this antisera inhibited human renin at a titer of 1:30,000, but did not inhibit cathepsin D enzymatic activity. Cross-reactivity also exists between antihuman antiserum and bovine renal renin (see Results). Cellular sonicates were preincubated with serial dilutions of these antisera for 1 hour at 37°C. Residual AI-generating activity then was determined after the addition of renin-free nephrectomized sheep plasma and incubation at 37°C for 3 hours.

Further evidence for immunoreactive renin within these cells was assessed by immunofluorescence chromatography. Canine renin-specific immunoglobulin G (GLN 816) was isolated (Dzau et al., 1982b) and coupled to cyanogen bromide-activated sepharose 4B (Pharmacia Fine Chemicals) by the method of Cuatrecasas (1970). One hundred microliters of the antibody-sepharose complex (binding capacity for canine renin of 10^−6 ng Al/hr per ml gel) were equilibrated with DPBS containing 0.25 mM EDTA, 0.25 mM NaTT, and 0.1 mM PMSF. Five hundred microliter aliquots of endothelial sonicate (containing 0.20 ± 0.04 ng Al/hr) were reacted with the antibody-sepharose over-night at 10°C. In control experiments, bovine renal renin was reacted with the immunofluorescence column, and cell sonicates were reacted with uncoupled sepharose. The renin-antibody-sepharose complex was separated from the unbound supernatant by centrifugation and was washed extensively with 0.025 M sodium phosphate containing 0.5 mM sodium chloride. Renin bound to the immunofluorescence column then was eluted with 2.0 M LiBR in 0.1 M Tris (pH 7.4) containing 1 mg/ml BSA and the protease inhibitors listed above.

**Intracellular Angiotensin I and Angiotensinogen**

The concentration of pre-formed intracellular AI was measured by boiling aliquots of cellular sonicates for 5 minutes, followed by Eppendorf centrifugation (13,000 g for 5 minutes) and immediate radioimmunoassay of the supernatant for AI. The concentration of cell-associated angiotensinogen was estimated by the method described by Herrmann and Dzau (1982): aliquots of cellular sonicate, containing the protease inhibitors described above, were incubated with excess partially purified human renin (4 × 10^7 ng AI/ml per hr) to exhaust all renin-substrate. Angiotensinogen concentration was then estimated from total AI concentration. Under these experimental conditions, we have documented that the generated AI is stable and does not undergo degradation.

**Immunofluorescence Microscopy**

Bovine aortic endothelial cells were examined for the presence of immunoreactive renin by indirect immunofluorescence microscopy. To reduce the possibility of carry-over of renin from the serum in the culture medium, monolayers were incubated for the final 24 hours in serum-free DPBS supplemented with 0.5% BSA. Subconfluent cultures on glass coverslips were fixed and permeabilized with 10% formalin/0.1% NP-40 (Sigma). In preliminary studies, other fixation/permeabilization protocols tested (methanol, formalin alone) yielded poorer results. The fixed cells were incubated with a 1:60 dilution of goat anticanine renin (GLN 816), or nonimmune goat serum, for 30 minutes at 37°C and were washed with PBS. The cells then were incubated with fluorescein-conjugated rabbit antigoat IgG (1:100 dilution; Miles-Yeda Ltd.) for 30 minutes at 37°C, followed by washes with PBS and deionized water. The stained coverslips were mounted on slides with glycerol/PBS and examined with a Leitz Orthoplan microscope under epifluorescent illumination (100-W mercury lamp, Leitz L2 filter block, Zeiss Planapo 63× oil immersion phase objective (NA 1.4)).

In addition to the standard methodological controls for autofluorescence and nonspecific antibody binding (e.g., omitting primary or secondary antibodies; use of nonimmune sera), the specificity of staining was also tested by preincubation of the antirenin antiserum with an excess of purified canine renin adsorbed to nitrocellulose. Similar experiments were performed with rabbit antihuman-renin antiserum (R1723).

**Radiolabeled Biosynthesis Experiments**

De novo synthesis of renin was evaluated by immunoselection of extracts of radiolabeled cells. Cultured BAEC were grown to confluence (approximately 3 × 10^6 cells/60 mm plate) in DME + 10% CS and then were incubated for 24 hours at 37°C in DME + 0.5% BSA. BAEC were radiolabeled with 1 mCi [35S]methionine (>1000 Ci/mmol, New England Nuclear) in 2 ml of methionine-deficient DME ± 0.5% BSA for 3 hours. In additional experiments, the 3-hour labeling period was followed by 20 hours of incubation in DME ± 0.5% BSA with excess unlabeled methionine. After the incubation, each culture was scraped harvested in 2 ml of 20 mM sodium phosphate, 0.1% Triton X-100, and 1 mg/ml BSA containing the following protease inhibitors: 5 mM EDTA, 5 mM NaTT, 1 mM PMSF, 5 mM 8-OH-Q, 1.6 mM BAL, 4 × 10^−8 U/ml aprotonin (Apro), 1.0 ng/ml soybean trypsin inhibitor (SBTI), and 1 mM diisopropyl fluorophosphate. The cells were sonically disrupted, cellular debris was removed by centrifugation (Eppendorf microfuge, 3 minutes at 13,000 g) and the supernatant extracts were frozen (−70°C) until analyzed.

Before immune selection of the 35S-labeled renin and...
TABLE 1
Renin-Angiotensin Components in Cultured Bovine Aortic Endothelial Cells

<table>
<thead>
<tr>
<th>Component</th>
<th>Activity</th>
<th>(Mean ± SD)</th>
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<tbody>
<tr>
<td>Renin activity</td>
<td>1.2 ± 0.2 ng AI/10^6 cells per hr (n = 8)</td>
<td></td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>90 ± 25 pg/10^6 cells (n = 6)</td>
<td></td>
</tr>
<tr>
<td>Renin substrate</td>
<td>14.8 ± 1.18 ng/10^6 cells (n = 8)</td>
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precursors, 150-microliter aliquots of the thawed cellular sonicate (total acid precipitation, counts/min = 5 × 10^6) was precleared by incubation for 1 hour with control rabbit serum (21 microliters). After the addition of protein A-sepharose 4B (20 microliters packed volume, Pharmacia Fine Chemicals), the mixture was incubated for an additional 2–4 hours at 4°C, followed by centrifugation (13,000 g for 3 minutes) to remove the protein A-sepharose and bound constituents. This preclearing step was essential in reducing nonspecific adsorption (data not shown). The precleared supernatant was then incubated for 24 hours at 4°C with 12 microliters of rabbit antihuman renin antiserum, and the immune complex was isolated with protein A-sepharose, as described above. The protein A-sepharose immune complexes was washed three times in PBS containing 0.05% SDS and 0.1% TX-100 and the protease inhibitors EDTA, NaTT, PMSF, aprotinin, and SBTI at the concentrations listed above. After being boiled in 2% SDS and 5% mercaptoethanol, the immune complexes were analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography using Enhance (New England Nuclear) to increase the sensitivity of autoradiography. Molecular weight standards in the gels were detected by Coomassie blue staining. Similar experiments were performed with goat anticanine-renin antiserum (GLN 816).

Results
Characterization and Identification of Cellular Renin-like Activity

Angiotensin I generation was demonstrated in sonicates of cell suspensions prepared from confluent cultures of bovine aortic endothelial cells. This generation was linear with incubation time, with a mean value (n = 8) of 1.12 ± 0.2 ng AI/10^6 cells per hour (Table 1). Nephrectomized sheep plasma and conditioned growth media assayed in the absence of cell sonicate demonstrated no renin-like activity. The pH profile of this activity (Fig. 1A) showed optimal AI generation at pH 6.5, which is similar to that of partially purified bovine renal renin, shown in the figure.

Pepstatin (0.1–1 mm) inhibited greater than 90% of this endothelial-associated AI-generation, confirming that the enzymatic activity belonged to the acid protease class. To define further the nature of this activity, antirenin antisera were employed. Preincubation with renin-specific anticanine and antihuman antisera at various dilutions resulted in similar suppressions of endothelial cell angiotensin I-generating activities with comparable inhibitory curves for bovine renal renin (Fig. 1B). In control experiments, preincubation with nonimmune serum did not inhibit renal renin or endothelial cell AI-generating activity.

The immunological relationship between endothelial renin and renal renin was studied further by immunoaffinity chromatography using anticanine-renin antibody (Table 2). A substantial quantity of the endothelial renin-like activity bound to and was eluted from the antibody column, confirming the results of inhibitory assay using the same antiserum. In contrast, no activity bound to or could be eluted from sepharose alone. In parallel experiments, the capacity of this immunoaffinity column for bovine renal renin was 150 ng AI/hr per ml gel. Thus, under these conditions, a significant proportion of endothelial renin-like activity is immunologically similar to renal renin.

It is unlikely that this cellular Al-generating activity was derived from the calf serum in which the cells were grown, since complete growth medium (not incubated with cells) did not contain active renin or trypsin-activated renin. Active renin, or trypsin-activated renin, also was not detectable in 50% calf serum (five times the concentration in growth medium). Furthermore, when cells were maintained in the absence of calf serum for 48 hours, there was a 4-fold increase in renin activity per cell (data not shown).
Angiotensinogen and Angiotensin I

As shown in Table 1, angiotensinogen (renin substrate) and immunoreactive AI also were detected within the endothelial cell sonicates. However, neither angiotensin nor angiotensinogen was detectable in unconcentrated growth medium (DME-10% CS) before incubation with cells.

Immunofluorescence Microscopy

Examination of bovine aortic endothelial cells by indirect immunofluorescence microscopy using the goat anticanine renin antiserum and fluoresceinated rabbit antigoat IgG revealed a coarse, granular cytoplasmic staining, as well as more diffuse perinuclear staining (Fig. 2B). Parallel controls using non-immune goat serum showed only a faint background fluorescence. In a more critical control, when the antirenin antiserum was preabsorbed with partially purified canine renin, the immunofluorescent staining pattern shown in Figure 2B was abolished (Fig. 2C), thus confirming the specificity of this staining. Similar results were obtained with antihuman antiserum.

Biosynthetic Studies

The presence of renin in these cultured endothelial cells results from de novo synthesis. Figure 3A (lane 2) shows that within 3 hours, these cells synthesize a precursor with an apparent molecular weight of 64 ± 2 kD which was immunoprecipitated by rabbit antihuman renin antiserum. The specificity of this precipitated band is supported by the observation that the monospecific antibody to dog renin (GLN 816, Dzau et al., 1982b) also precipitated this band (data not shown). In addition, this protein band was not precipitated with non-immune serum (Fig. 3A, lane 1). The precursor nature of this protein is suggested by its disappearance, followed by the appearance of a duplex with a molecular weight of 45 Kd after an additional 20 hours of incubation (Fig. 3B, lane 2). The upper band of this duplex may represent nonspecific adsorption to the antiserum and/or the protein A sepharose, since a single band at this molecular weight also appears in the nonimmune control (Fig. 3B, lane 1). The lower band probably represents authentic renin, as it is not present in the nonimmune control. Similar results were observed with anticanine-renin antiserum. Thus, the evidence strongly suggests that endothelial cells synthesize a renin precursor which is processed intracellularly to the mature form.

Discussion

This study provides evidence that cultured bovine aortic endothelial cells synthesize and process renin. The pH optimum of endothelial renin activity indicates that the enzyme involved is active at near neutral pH, thus minimizing the possible contribution of other acid proteases such as cathepsin D under our assay conditions. Inhibition of this renin-like activity by the aspartyl protease inhibitor pepstatin, and by antirenin antiserum provides further evidence for the presence of renin in these cells. The fact that antisera raised against dog or human renins are unable to neutralize bovine endothelial cell AI generation completely is probably due to cross-species structural differences in renin (Dzau et al., 1982b). Indeed, the inhibitory curves for both antisera for bovine renin and endothelial renin-like activities are similar. This result indicates common immunoreactivity between the renal and endothelial enzymes. However, the possibility that other neutral nonimmunoreactive renin-like proteases contribute to endothelial renin-like activity cannot be excluded. Indeed, such enzymes have been described and characterized in the brain (Dzau et al., 1982a; Hussain et al., 1984).

Immunofluorescence microscopy confirmed the presence of immunoreactive renin within the cytoplasm of the cultured endothelial cells. We have previously reported a similar staining pattern in cultured vascular smooth muscle cells using anticanine renin antisera; such staining did not occur in cultured fibroblasts (Re et al., 1982). The presence of renin in vascular smooth muscle cells was shown to be due to in situ synthesis, and not to uptake from serum in growth media. Neither active nor inactive renins were detected in the diluted and deactivated calf serum. In the current study, biosynthetic radiolabeling experiments also provided evidence for biosynthesis and intracellular processing of renin in cultured vascular endothelial cells. Fur-
FIGURE 2. Immunofluorescence microscopy of bovine aortic endothelial cells. Panel A: phase-contrast image of cells fixed and permeabilized with 10% formalin and 0.1% NP-40. Panel B: after 30 minutes of incubation (37°C) with goat anticanine renin antiserum (GLN 816), cells were incubated with fluorescein-conjugated rabbit antigoat IgG antibodies, and viewed under epifluorescent illumination. Note granular cytoplasmic and more diffuse perinuclear staining patterns. Panel C: prior to the incubation outlined in panel B, the antirenin antiserum was preincubated with excess purified canine renin. Note loss of specific staining pattern. The measurement bar equals 50 μm.

FIGURE 3. Immunoprecipitation and SDS-polyacrylamide gel electrophoresis of [35S]methionine-labeled endothelial cell sonicates (panel A) after 3 hours, and (panel B) after a 3-hour labeling period and an additional 20 hours of incubation. Lane 1: nonimmune rabbit serum; lane 2: rabbit antihuman renin antiserum.

The prorenin synthesized by these cells has an apparent molecular weight of 64 ± 2 Kd, which is higher than that reported for human kidney or mouse submandibular gland prorenin. Sequence analysis of cDNA to mouse and human renin mRNA indicate that the protein moiety of prorenin has a molecular weight of 45-46 Kd (Panthier et al., 1982; Murakami et al., 1984). However, with the exception of the mouse submandibular gland renin, this protein is glycosylated, and the actual molecular weight is higher. Indeed, biosynthetic studies of human chorionic cells (Acker et al., 1982) and human tumoral juxtaglomemlar cells (Galen et al., 1984) indicate that human prorenin has a molecular weight of 55 Kd. The discrepancy in the size of prorenin from endothelial cells in this report and that from other species and tissues may be due to differences in biosynthetic processing and/or glycosylation. Such differences in tissue renin biosynthesis, if proven, may provide important insight into the biology and fate of various tissue renins. However, it should be pointed out that the molecular weight of bovine renal prorenin has not been reported. Thus, a direct comparison between bovine endothelial and renal renin is not possible.

The renin activity within these cultured bovine aortic endothelial cells is comparable, on a per cell basis, to that reported for cultured adrenal glomerulosa cells (unpublished data), and is greater than that present in cultured juxtaglomerular cells (Rightsel et al., 1982), neuroblastoma cells (Okamura et al., 1981; Fishman et al., 1981), and renal mesangial cells (Dzau and Kreisberg, 1983). Previous results with cultures of Wilm’s tumor (unpublished data) and vascular smooth muscle cells (Re et al., 1982)
have demonstrated somewhat greater quantities of active renin. Neither the precise subcellular localization of renin in endothelial cells nor the possibility of its secretion was examined in this study. Thus, it remains to be determined whether endothelial renin functions in the intracellular regulation of angiotensin I generation, or whether it is secreted for extra-cellular or cell surface reaction with angiotensinogen. The detection of angiotensin I in these cells suggests that the renin-angiotensinogen reaction may, at least in part, occur intracellularly. The origin of cellular angiotensinogen is not clear. There are at least two possibilities, i.e., it is synthesized intracellularly or is taken up from serum and internalized. The latter is less likely, since (1) angiotensinogen was not detectable in growth medium containing 10% calf serum, and (2) cellular angiotensinogen was detected after 48 hours of growth in serum-free medium.

Renin activity within vascular tissue extracts has been demonstrated previously (Gould et al., 1964; Swales and Thurston, 1973; Basso and Taguini, 1974; Thurston and Swales, 1977; Barrett et al., 1978; Thurston et al., 1979; Aguiler et al., 1981). Arterial renin activity has been measured in the rat and shown to have a longer half-life after nephrectomy compared with plasma renin (Rosenthal et al., 1969; Thurston et al., 1977). This activity theoretically could be synthesized locally or be derived from uptake of circulating renin, and there is evidence for both processes: Loudon et al. (1983) showed the uptake of plasma renin by the aortic wall in nephrectomized rats, whereas our present data, and that of Re et al. (1982), indicate de novo synthesis by cultured vascular endothelial and smooth muscle cells.

In vivo, the endothelial cell is ideally situated to monitor circulating vasoactive substances and transduce this information to underlying vascular smooth muscle cells. Recent data demonstrate that endothelial cells release factors, in response to a variety of substances, which can influence the contractile state of vascular smooth muscle cells (Chand and Altura, 1981; Cherry et al., 1982; Furchgott, 1983). Formation of All via the sequential action of renin and ACE activities in these cells could result in alterations in vascular tone either directly, through vasoconstrictive effects on smooth muscle, or indirectly, through the induction of endothelial prostaglandin synthesis (Gimbrone and Alexander, 1975).

Thus, bovine aortic endothelial cells in culture contain and synthesize renin. This activity, in association with endothelial ACE, suggests that a localized renin-angiotensinogen system is present within these vascular cells. This enzymatic pathway within the arterial wall may exert local control over vascular vasoconstrictive activity which is not reflected by circulating plasma renin levels.

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