Coronary microvascular endothelial cells cosecrete angiotensin II and endothelin-1 via a regulated pathway

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Kusaka, Yasuko, Ralph A. Kelly, Gordon H. Williams, and Imre Kifor. Coronary microvascular endothelial cells cosecrete angiotensin II and endothelin-1 via a regulated pathway. Am J Physiol Heart Circ Physiol 279: H1087–H1096, 2000.—Although endothelial cells produce angiotensin II (ANG II) and endothelin-1 (ET-1), it is not clear whether a single cell produces both peptides, with cosecretion in response to stimulation, or whether different subpopulations of endothelial cells secrete one or the other peptide, with secretion in response to different stimuli. Exposure of cultured coronary microvascular endothelial cells to cycloheximide for 60 min had no effect on ANG II or ET-1 secretion. This result suggested the existence of a preformed intracellular pool of ANG II and ET-1, which is a precondition for regulated secretion. Exposure of endothelial cells to isoproterenol, high extracellular potassium, or cadmium, all of which stimulate peptide secretion via different signaling pathways, significantly (P > 0.001) increased the secretion of both ANG II and ET-1 in a cell size-dependent manner. Sodium nitroprusside and S-nitroso-N-acetyl penicillamine significantly (P > 0.001) decreased ANG II and ET-1 secretion, whereas Nω-nitro-arginine-methyl ester enhanced it. The similar regulation of ANG II and ET-1 secretion and the presence of both peptides around individual endothelial cells indicate that the autocrine/paracrine regulation of cardiovascular function by endothelial cells is accomplished via cosecretion of ANG II and ET-1.

autocrine/paracrine regulation; parallel-acting messengers; intercellular communication via multiple messengers

TRADITIONALLY the cardiovascular effects of angiotensin II (ANG II) are attributed to blood-borne ANG II (33), whereas the effects of endothelin-1 (ET-1) are attributed to locally produced ET-1 secreted by endothelial cells and other cell types (58). Therefore, the concept that ANG II and ET-1 are independently synthesized and secreted peptides, playing independent but similar and often synergistic roles in cardiovascular regulation, is widely accepted (4, 17, 22, 46, 57). However, it is difficult to accept the assumption that temporally asynchronous and spatially distinct and variable changes in cardiovascular performance, including blood flow in various segments of the vascular bed, are modulated by a single pool of blood-borne ANG II. It is equally difficult to comprehend that the regulation of cardiovascular function in response to rapid changes in demand is by a relatively stable level of plasma ANG II or by a steady constitutive secretion of ET-1. Neither is a steady level of ANG II or ET-1 consistent with the requirements for specific temporal sequences and durations of growth regulatory factor exposure in cell cycle progression and/or hypertrophy of both the heart and the vasculature. In addition, it is well known that a continuous exposure of cells to ANG II or ET-1 leads to agonist-induced phosphorylation and inactivation of the ANG II and ET-1 receptors, which would suspend any further regulation (5, 20, 55).

These considerations are certainly not consistent with the documented role of ANG II and ET-1 in modulation of cardiovascular function. However, recent observations confirm the existence of local “tissue” sources of ANG II. Endothelial cells, cardiomyocytes, and vascular smooth muscle cells produce and secrete ANG II (29, 44), which, like ET-1, plays an important role in autocrine/paracrine regulation of cardiovascular function (14) and participates in the generation of cardiovascular dysfunction (9, 18, 39, 47, 52).

Autocrine/paracrine regulation of cardiovascular function is fundamentally different from regulation via blood-borne peptides. Intercellular communication, including neural, autocrine, and paracrine regulation, is accomplished more frequently than previously thought via simultaneous release of multiple, parallel-acting chemical messengers (34). At the target cell level, various cellular functions, including smooth muscle contractions, are mediated by interactive additive and/or synergistic signaling pathways initiated by a number of different agonists (2, 24, 49). The possibility that locally produced ANG II and ET-1 elicit additive and/or synergistic effects in the cardiovascular system is supported by the fact that the receptors of ANG II and ET-1 are coupled to similar G proteins. Consequently, the signaling pathways and the intracellular effects of ANG II and ET-1 are in fact similar. These observations led us to hypothesize that cosecretion of ANG II and ET-1 is an essential part of the autocrine and/or paracrine regulation of cardiovascular function.

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An important requirement for cosecretion of regulatory peptides from endothelial cells or other cell types is similar regulation of peptide production and secretion. Circumstantial evidence supporting stimulation-dependent secretion of ANG II and ET-1 includes the following: 1) proenin is activated exclusively within the regulated secretory pathway (3, 42); 2) the secretion of ANG II produced in the secretory granules of several cell types (6, 56) is rapidly modulated in response to stimulation or inhibition (30, 31); and 3) endothelial cells produce ET-1-containing granules (23), and ET-1 secretion is rapidly modulated in response to stimulation or inhibition (32, 38). In addition, endothelial cells respond to blood pressure- or blood flow-induced stretch or to stimulation by neurotransmitters and/or autocrine and paracrine factors by increases in cytosolic calcium and augmented signaling, which result in increased secretion via the regulated secretory pathway (8, 25, 35, 36, 45). Consequently, endothelial cells and other cell types respond to specific stimuli by releasing autocrine and paracrine factors, including ANG II and ET-1 (1, 12, 41). However, there are data to suggest that ET-1 may be released by a constitutive secretory pathway (see Ref. 28). Basolateral secretion of ET-1 from endothelial cells in tissue sections and possible rapid enzymatic degradation within the extracellular space by nonspecific extracellular peptidases during their slow diffusion into the media could result in an absent or blunted secretory response to stimulation. This secretory pattern mimics constitutive secretion.

To test our hypothesis that autocrine/paracrine regulation of cardiovascular function is accomplished by cosecretion of ANG II and ET-1 by coronary microvascular endothelial cells (CMECs), we incubated enzymatically dispersed single endothelial cells, obtained from primary cultures, on a transfer membrane in cell culture media. The secreted peptides were captured by the transfer membrane around secretory cells and were identified and quantitated by immunoblotting and video-image analysis. Under these conditions, peptide secretion does not end in rapid degradation, and each peptide-secreting cell is individually evaluated. This method yields cumulative values and permits the detection of two or more peptides in the same spot. Because 150–200 secretory cells were sufficient to test the effect of a given regulator on peptide secretion, we were able to compare the effect of a number of different regulatory agents acting via distinct signaling pathways on ANG II and ET-1 secretion by endothelial cells originating from the same small cell pool.

MATERIALS AND METHODS

Isolation and culture of CMECs. Methods for the isolation and culture of CMECs from adult rat ventricular tissue have been described previously (37). In brief, hearts are removed from male Sprague-Dawley rats (175–200 g body wt) after ether anesthesia and are subjected to retrograde perfusion with Krebs-Henseleit bicarbonate buffer for 5 min at 37°C. The atria, valvular tissue, and right ventricle are then removed, and the remainder of the left ventricle is immersed for 20 s in 70% ethanol to devitalize epicardial mesothelial and endocardial endothelial cells. The tissue is washed, and the outer one-third of the epicardial surface of the left ventricle is dissected away. The remaining ventricular tissue is finely minced and enzymatically dispersed with type 2 collagenase (0.2%) and trypsin (0.02%) in Hanks’ balanced salt solution without calcium. Dissociated cells are washed and resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 μg/ml). The cell suspension is plated onto laminin-coated (1 μg/cm²) culture dishes at a density of 2.5 × 10⁵ cells/cm². After 1 h, the medium is removed, and the preparation is washed once to remove loosely adherent cells and cell debris. DMEM (supplemented as just described) is added to the washed cells, and the dishes are placed in an incubator. The medium is changed every 3 days. CMEC cultures reach confluence within 7 days following isolation. At confluence, these primary cultures contain >90% endothelial cells, as identified by fluorescein-tagged antibody to the von Willebrand factor. Forty-eight hours after confluence all experiments, the left ventricle is replaced by DMEM with 1% bovine serum albumin (BSA).

Detection of ET-1 and ANG II secretion by single cells. ET-1 secretion was assessed by adaptation of a technique previously used to assess the secretion of ANG II from individual CMECs and adrenal zona glomerulosa cells (7, 19). In brief, confluent primary cultures of CMECs are incubated with collagenase D (1 mg/ml of DMEM) for 15 min, and cells are then gently scraped and mixed by repeated pipetting. The cell suspension is washed three times with medium 199 containing 4 mM KCl and 0.025% BSA and is then resuspended in fresh medium. After cell viability is confirmed by trypan blue exclusion, the suspension is diluted to a cell count of 3.0–4.0 × 10⁵ cells/25 μl. The amount of cell suspension needed for the experiment (usually 1–2 ml) is incubated for 45 min at 37°C in a humidified chamber containing a mixture of air and 5% CO₂. Aliquots of cell suspensions (25 μl) are dispensed and preincubated on a polyvinylidene difluoride (PVDF) transfer membrane for 30 min at 37°C in a humidified chamber containing air and 5% CO₂. Because of the hydrophobicity of the transfer membrane, the cell suspensions form stable droplets. The cells inside each droplet settle and attach to the membrane. Secretion is initiated after 30 min of preincubation by gentle mixing of test substances dissolved in 3 μl of medium into the droplets. Incubation is then continued for an additional 60 min. The final concentration of test substances added to the cell suspension is as follows: 1 μM isoproterenol, 7 μM cycloheximide, 10 mM potassium chloride, 2 μM cadmium chloride, 5 mM sodium nitroprusside (SNP), and 100 μM S-nitroso-N-acetyl penicillamine (SNAP). An aliquot of CMEC suspension (250 μl) is preincubated with 5 mM L-NAME for 60 min, 25-μl aliquots of the cell suspension are dispensed on the PVDF transfer membrane, and the assay continues as with other samples. The total exposure time of endothelial cells to l-NAME is 150 min.

Proteins and peptides secreted by the attached cells become irreversibly bound to the transfer membrane. After incubation for 90 min, the droplets are removed by gentle aspiration, and 25-μl aliquots of a serum-free protein-blocking solution (Dako, Carpinteria, CA) are added to the blots for 30 min to suppress nonspecific binding. The membranes are then incubated overnight at 4°C with rabbit antibody to either ET-1 or ANG II, diluted 1:500 in phosphate-buffered saline (PBS) containing 1% normal goat serum and 1% BSA (first antibody). After each step, the blots on the membrane are washed three times with Tris-buffered saline containing...
1% BSA. After the washing, a biotinylated antibody to rabbit IgG (second antibody; Vectastain ABC Kit), dissolved in Tris-buffered saline containing 1% normal goat serum and 1% BSA, is added to each membrane for 4 h. After another washing, a 25-μl volume of streptavidin-alkaline phosphatase complex (Vectastain ABC Kit) is added to the blots for an additional 60 min. A mixture of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium, dissolved in additional 60 min. A mixture of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium, dissolved in diethylamine buffer (pH 10.0) containing 5 mM MgCl₂, is used as an alkaline phosphatase substrate. Levamisole (1 mg/ml of diethylamine buffer) is added to the substrate to suppress endogenous alkaline phosphatase activity. To minimize cell displacement, reagents and washing fluid are removed with capillary tubing attached to a microperistaltic pump set to a slow flow rate. Reagents or washing fluids are delivered with gel-loader pipette tips, also at a slow rate. Finally, the membranes are air-dried and dry mounted under glass. Cells secreting ET-1 and ANG II appear as dark dots (cell bodies) surrounded by a colored halo of secreted peptides.

Cells on each membrane are examined with a Zeiss Axiosvert 10 microscope coupled to a video-image analysis system. At least 100 cells are analyzed for each condition in three experiments using Optimas image analysis software (Bukin, Edmonds, WA). The areas of halos are calculated by subtracting the cell area from the whole spot area. The integrated gray values (IGVs) of the pixels within the area boundary are individually determined. The mean color intensity of cells and halos, e.g., the gray value (GV), is calculated from the area and the IGV.

The specificity of antigen-antibody reaction in the halos depends on two factors: 1) the selectivity of the secretory process and 2) the specificity of primary antibodies. The steady intracellular supply of ANG II necessary for “on demand” secretion is maintained by a continuous peptide production within the regulated secretory pathway and a tightly controlled intracellular degradation of superfluous peptides within the lysosomes. This regulatory mechanism generates two distinct intracellular angiotensin peptide pools. The secretory granules, targeted to exocytosis, contain ANG II and residual amounts of precursor molecules. The lysosomal compartment contains the degradation products of the superfluous ANG I and ANG II. These peptides are not secreted. Consistent with this well-documented regulatory mechanism in endocrine cells, ANG II-producing cells in the adrenal zona glomerulosa contain a variety of degradation products of ANG I and ANG II, but secrete only ANG II and small amounts of precursor molecules (30). The highly selective process of regulated secretion renders the assessment of ANG II secretion relatively trouble free. Cross-reactions between the antibodies to ANG II and the precursor molecules of ANG II can be avoided by using carboxy-terminal-specific antibodies to ANG II, which do not cross-react with ANG I or angiotensinogen cosecreted with ANG II. Because acute stimulation or suppression of the secretory process does not affect secretion via a constitutive secretory pathway, constitutive secretion of angiotensinogen does not interfere with studies like ours that are based on secretory responses to stimulation or suppression. The rapid degradation of ANG II within the extracellular space by nonspecific peptidases is minimized by enzymatic dispersion of ANG II-secreting cells. Similar considerations apply for ET-1. In contrast, the large number and high concentration of various angiotensin peptides in autocrine/paracrine cells make it very difficult, or impossible, to assess cell or tissue content of ANG II by a similar immunocytochemical approach.

Double staining of halos for ANG II and ET-1. Enzymatically dispersed CMECs, suspended in 25 μl of medium, are incubated on PVDF transfer membranes as described above. After removal of the medium, autofluorescence is quenched by covering the blots with 0.1 M glycine in PBS for 10 min and application of blocking solution (DAKO) for 30 min. Subsequently, the blots are incubated overnight at 4°C with a mixture of rabbit antibody to ANG II and a mouse monoclonal antibody to ET-1 dissolved in PBS containing 1% BSA and 1% preimmune serum from the animal species used for the second antibody production. After the blots are washed three times as described above, Texas red-labeled goat antibody to rabbit IgG or fluorescein isothiocyanate (FITC)-labeled antibody to mouse IgG is applied. The order in which the blots are exposed to the second antibody depends on the intensity and stability of the fluorescence produced. In our hands, the application of Texas red followed by FITC produces better results. The preparations are then mounted in Vectashield. The cells and the cell halos are evaluated with a Zeiss Axiosvert 10 microscope equipped with two sets of Omega filters (Brattleboro, VT): one set for fluorescein (excitation wavelength (λexc) = 470 nm; emission wavelength (λem) = 540 nm) and the other for rhodamine (λexc = 546 nm; λem = 590 nm).

Sources of materials. DMEM, medium 199 without potassium chloride, sodium bicarbonate, FCS, Hanks’ balanced salt solution without calcium, trypsin-EDTA, laminin, BCIP, and nitroblue tetrazolium were obtained from GIBCO BRL (Grand Island, NY). Penicillin-streptomycin, BSA fraction V, trypsin, levamisole, and other reagents were purchased from Sigma (St. Louis, MO). Type 2 collagenase was obtained from Worthington (Freehold, NJ). PVDF transfer membranes (Immobilon-P) in a 96-well microtiter plate format (Multiscreen-IP) were purchased from Millipore (Bedford, MA). Protease-free BSA, collagenase D, and dispase were purchased from Boehringer-Mannheim (Indianapolis, IN). Serum-free protein-blocking solution was purchased from DAKO. Antibody to ET-1 was obtained from Peninsula Laboratories (Belmont, CA), antibody to ANG II was from IgG Corp. (Nashville, TN), mouse monoclonal antibody to ET-1 was from Biodesign International (Kennebunk, ME), and Vectashield and Vectastain ABC kits were from Vector Laboratory (Burlingame, CA).

Statistical analysis. The Kolmogorov-Smirnov test was used to assess the normality of frequency distribution. All correlations were calculated by the least-squares method. The significance of differences between control and experimental groups was calculated by means of the Mann-Whitney rank sum test or Kruskal-Wallis one-way analysis of variance on ranks.

RESULTS

ANG II and ET-1 secretion by CMECs under basal conditions. The presence of ANG II and ET-1 in the halos is indicated by a color reaction produced in response to sequential exposure of the transfer membrane first to an antibody to ANG II or ET-1 and then to a biotinylated second antibody, an avidin-biotinylated alkaline phosphatase complex, and an appropriate alkaline phosphatase substrate. The specificity of the color reaction is supported by the observation that if either preimmune serum or PBS containing 1% BSA is substituted for the first antibody, no halo is seen, although cell bodies are lightly stained. Saturation of the first antibody with the corresponding synthetic
peptide (ANG II or ET-1) also suppresses halo formation. No cross-reactivity of ANG II or ET-1 with antibody to the other peptide was observed.

As in our previous study with ANG II (7), there was a significant correlation ($P < 0.001$) between the concentration of ET-1 in test solutions and the intensity of the color reaction produced by immunoblotting (Fig. 1). Considering this correlation, one could assume that the GV of halos represent a quantitative measure of ANG II and ET-1 secretion from single cells. This assumption is not entirely correct. In contrast with the uniform pixel values and a peptide concentration-dependent increment of the GV in subsequent calibration immunoblots produced with peptide solutions, the secretion of ANG II and ET-1 from CMECs generates halos with a wide range of pixel values in the same halo. The histograms of individual halos (data not shown) indicate that areas with high pixel values appear adjacent to the secretory cells, whereas pixel values approaching background levels are common at the halo margins. Therefore, the GV (pixel value/unit area) reflect in this case only the mean pixel value of a halo and cannot be used directly to assess peptide concentration.

In Fig. 2, we show the correlations between the size of the secretory cells and the halo area or the halo GV produced by the same cell. It is well established that larger, hypertrophied cells secrete more peptide hormones and regulatory peptides than small or normalized cells. The significant ($P < 0.001$) correlation between cell size and halo area, generated by ANG II or ET-1, indicates that larger CMECs produce larger halos. Because the peptide “clouds” generated around secretory cells subsequent to exocytosis have a more or less uniform concentration gradient and a secretory output-dependent volume, and the halos are two-dimensional projections of these three-dimensional peptide clouds, the halo area reflects the magnitude of the secretory output. Whereas the GV provides a good measure of peptide concentration within a calibration immunoblot with uniform pixel values produced by a peptide solution, the rapid diffusion of peptides secreted by cells translates the differences in concentration to differences of halo areas. Therefore, the GVs of small halos are similar to the GVs of large halos, and there is no correlation between cell size and halo GV.

**Fig. 1.** Graph depicting the significant correlation ($P < 0.001$) between the concentration of endothelin-1 (ET-1) in test solutions applied to the polyvinylidene difluoride transfer membrane and the color intensity of the spots developed by the microimmunoblot technique used to identify and quantitate ET-1. GV, gray value.

**Fig. 2.** There is a significant ($P < 0.001$) correlation between the size of ANG II- (A) or ET-1-secreting (B) coronary microvascular endothelial cells (CMECs) and the area of halos produced around peptide-secreting cells. This result is consistent with observations that cell size and hypertrophy have an important impact on the magnitude of peptide hormone or regulatory peptide secretion. The lack of correlation between cell size and halo GV is certainly counter-intuitive. However, the halos generated by peptide-secreting cells contain a wide variety of pixel values, with maximal values adjacent to secretory cells and values approaching background levels at the margin of halos. Under these conditions, the GV is a reflection of the mean pixel values within the halo and not peptide concentration.
Virtually all the CMECs incubated on the PVDF transfer membrane produced halos. Occasionally, however, halos were detected either with multiple cells or without cell bodies. Cells without halos were observed with similar frequency to the frequency of halos without cell bodies, a result suggesting that the cells had been removed from the initial site of attachment during the procedure.

ANG II and ET-1 secretion by CMECs exposed to cycloheximide. Exposure of CMECs to 7 μM cycloheximide for 60 min did not significantly change the rate of basal secretion of ANG II or ET-1. The halo area and the halo area size distribution pattern were similar around control and cycloheximide-exposed cells (Table 1). The implication is that the CMECs contained sufficient amounts of preformed ET-1 and ANG II to sustain peptide secretion over this period.

**Table 1. Effect of a 1-h exposure to cycloheximide on ET-1 and ANG II secretion from rat CMECs**

<table>
<thead>
<tr>
<th></th>
<th>Median Halo Area, μm²</th>
<th>P</th>
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<tbody>
<tr>
<td>ET-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>109</td>
<td>221</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>108</td>
<td>244</td>
</tr>
<tr>
<td>ANG II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
<td>219</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>100</td>
<td>217</td>
</tr>
</tbody>
</table>

n, Number of cells counted per experimental condition; ET-1, endothelin-1; CMECs, coronary microvascular endothelial cells. All P values were calculated by the Mann-Whitney rank sum test.

Because the immunoblot assay makes it possible to evaluate individual secretory cells and cell halos, this assay provides an unconventional means of assessing different aspects of peptide secretion (Fig. 4). The halos produced around individual CMECs displayed remarkable heterogeneity in size. The significant (P < 0.001) correlation between the area of secretory cells and the area of halos produced around each cell indicated that under basal conditions larger CMECs secrete more ANG II and ET-1. Isoproterenol, extracellular potassium, and cadmium significantly increased the slope of the regression, a result suggesting that the secretion of ANG II and ET-1 in response to stimulation is also cell size dependent and that larger cells, by secreting more

**Table 2. Effect of isoproterenol on ET-1 and ANG II production by rat CMECs**

<table>
<thead>
<tr>
<th></th>
<th>Median Halo Area, μm²</th>
<th>P</th>
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<tbody>
<tr>
<td>ET-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>104</td>
<td>191</td>
</tr>
<tr>
<td>Isoproterenol (1 μM)</td>
<td>104</td>
<td>274</td>
</tr>
<tr>
<td>ANG II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>108</td>
<td>194</td>
</tr>
<tr>
<td>Isoproterenol (1 μM)</td>
<td>106</td>
<td>263</td>
</tr>
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</table>

n, Number of cells counted per experimental condition. All P values were calculated by the Mann-Whitney rank sum test.

![Fig. 3. Conventional approach shows that exposure of CMECs to 10 mM extracellular potassium, 2 μM cadmium, or 1 μM isoproterenol (see Table 2) significantly increases secretion of ANG II (A) and ET-1 (B) and documents an increase in the median halo area. The apparent changes in secretory output are significant but relatively modest for two distinct reasons. 1) The halo area is a two-dimensional projection of a three-dimensional space filled with ANG II or ET-1 secreted by endothelial cells, and a large increase in volume translates into a small increment in halo area. 2) Stimulation of ANG II and ET-1 secretion produces a small increment in secretory output from a large number of smaller cells and a larger increment in secretory output from larger cells (see Fig. 4). The conventional expression of secretory responses does not reflect the impact of the cell size-dependent heterogeneity of these responses.](http://ajpheart.physiology.org/)

ANG II and ET-1, play an augmented role in autocrine/paracrine modulation of cardiovascular function.

Consistent with the results presented in Fig. 4, stimulation by isoproterenol, extracellular potassium, or cadmium produced a significant shift to the right of the frequency distribution of halo areas (Fig. 5). This result suggested that a larger proportion of ANG II and ET-1 released in response to stimulation is provided by larger cells and halos.

Response of ET-1 and ANG II secretion to nitric oxide donors and nitric oxide synthase inhibition. Exposure to a nitric oxide (NO) donor, i.e., SNAP (100 μM) or

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Fig. 4. Unconventional approach shows that stimulation with 10 mM extracellular potassium (A and B, top), 2 μM cadmium (data not shown), and 1 μM isoproterenol (data not shown) increases secretion of ANG II (A) and ET-1 (B) by CMECs and documents that stimulation increases the slope of the regression line. This result indicates that larger cells secrete more ANG II and ET-1 in response to stimulation than normal size or smaller cells. In contrast, the nitric oxide donor sodium nitroprusside (SNP) suppresses the secretion of ANG II and ET-1 and decreases the intercept of the regression line (A and B, middle). Cycloheximide (A and B, bottom) has no effect on secretion.
SNP (5 mM), resulted in a significant decline in both ET-1 and ANG II secretion by CMECs, with a decreased median halo area (Table 3), a decreased intercept of the regression line (Fig. 4), and a left shift of the frequency distribution of halo areas (Fig. 5). Conversely, inhibition of endogenous NO synthase by L-NAME for 1 h before and during incubation resulted in increased secretion of both ANG II and ET-1 (Table 3) and a shift to the right of the frequency distribution curves (results not shown).

**Cosecretion of ANG II and ET-1 by CMECs.** Because virtually all the enzymatically dispersed CMECs incubated on a PVDF transfer membrane and tested for secretion of ANG II or ET-1 produced halos, the existence of subpopulations of cells that secrete only one or the other peptide is unlikely. Consistent with this interpretation were the results of double staining of the halos first with antibodies to ANG II and ET-1 and then with Texas red-labeled or FITC-conjugated secondary antibodies. The halos produced by this process displayed red and green fluorescence with proper filters, indicating the presence of both ANG II and ET-1. In the absence of the first antibodies (i.e., those to ANG II and ET-1) or in the presence of ANG II- or ET-1-saturated specific antibodies, no fluorescence was seen. The results of double staining were confirmed by double exposure of halos to color film with use of both rhodamine and FITC filters. The appearance of a yellow halo indicated the presence of both ANG II and ET-1 in the same area on the membrane (Fig. 6). In each of four independent experiments, more than 100 cells were examined. Because all halos examined displayed both red and green fluorescence, we concluded that endothelial cells cosecreted ANG II and ET-1.

**DISCUSSION**

By definition, autocrine and/or paracrine regulation of cardiovascular function implies that the pool of ANG II or ET-1 secreted by endothelial cells, vascular smooth muscle cells, or cardiomyocytes participates in autoregulation of cellular function or modulates the function of adjacent cells. Circulating pools of ANG II and ET-1 are not included in these regulatory loops. In fact, the concentration of ANG II and the ANG II basal rate of secretion are substantially higher in cardiovascular tissue than in plasma (31, 40). Pulsatile secretion of ANG II and ET-1 into the intercellular space, mediated by chemical messengers or stretch, further increases the gap between plasma levels and local intercellular concentrations of these peptides. In addition, extracellular nonspecific peptidases within the intercellular space could hamper the diffusion of regulatory peptides from the plasma to the target cells. This role of locally produced ANG II is clearly illustrated by targeted disruption of the membrane-spanning anchor domain of the angiotensin-converting enzyme (ACE) in mice. As a consequence of disruption of the normal
processing of nascent ANG II in the secretory pathway, these mice exhibit a phenotype similar to that of animals with a nonfunctional ACE gene, despite the presence of nearly normal plasma levels of ACE activity (15, 16). In contrast to ANG II, ET-1 (given its basolateral secretion from endothelial cells) has always been considered a locally produced and locally acting regulatory peptide.

Our results indicate that the regulation of ANG II and ET-1 secretion from endothelial cells is similar. Short-term exposure of endothelial cells to cycloheximide had no effect on ANG II or ET-1 secretion. This observation suggests that CMECs contain a preformed pool of both peptides, which is a critical requirement for stimulated “on-demand” secretion. Neurotransmitters, ion channel modulators, or autocrine- and paracrine-signaling autacoids that induce changes in cytosolic calcium or cAMP levels typically initiate regulatory peptide secretion via similar or different signaling pathways. High extracellular potassium levels promote insulin, ACTH, and regulatory peptide secretion and stimulate ANG II secretion from adrenal zona glomerulosa cells (30). As we have shown here, 10 mM extracellular potassium stimulates ANG II and ET-1 secretion by CMECs. Isoproterenol has been shown to induce ANG II secretion by endothelial cells via a regulated secretory pathway (54). Our results clearly indicate that isoproterenol induces the secretion of both ANG II and ET-1 by CMECs. Cadmium, in a concentration range of 100–300 μM, is known to inhibit L-type calcium channels and Na/Ca exchange in ventricular myocytes (26). However, at lower concentrations similar to those we used to induce ANG II and ET-1 secretion, cadmium appears to act as a receptor agonist and to mobilize calcium from intracellular stores via increased production of inositol 1,4,5-trisphosphate (51). This effect is consistent with the increased secretion of ANG II and ET-1 by CMECs in response to stimulation with cadmium. Supportive evidence includes the consequences of intraperitoneal administration of cadmium to male rats, i.e., induction of an increased number of ET-1-storing Weibel-Palade bodies in the rat aortic endothelium and enhancement of the release of ET-1 by exocytosis; these events result in elevation of the plasma ET-1 concentration (13).

NO decreases calcium influx and thereby the secretory output of peptides via a regulated secretory pathway. Papaverine and PGE1 also decrease calcium influx in smooth muscle cells (10,11) and decrease ANG II secretion from the vascular smooth muscle cells of the corpus cavernosum (31). These findings are consistent with our observation that NO significantly decreases the halo area produced by ANG II or ET-1 secretion by endothelial cells. NO may terminate the physiological effects of ET-1 by displacing ANG II and ET-1 from the respective receptors (21, 59). As a result of stimulation-dependent production, endothelial cells produce “puffs” of NO, and this pulsatile NO release may prevent sustained receptor desensitization by ANG II and ET-1 (59). The role of endogenous NO synthase activity in the regulation of ANG II and ET-1 secretion by CMECs is further supported by our observation that inhibition of NO generation by l-NAME results in a significant increase in ANG II and ET-1 secretion. This point may be relevant to the marked reduction of the effects of L-NAME by ACE inhibitors (53), which may increase NO production via bradykinin, with a known role in targeting of endothelial NO synthase to the caveolae of NO-producing cells in the cardiovascular system. Finally, mice with targeted disruption of endothelial NO synthase have been shown not only to be hypertensive but also to exhibit accelerated intimal proliferation in response to vascular injury and myocardial hypertrophy, effects that could be due in part to the unopposed actions of ANG II and ET-1 in this model (27,43,48).

Hypertrophied cells, regardless of their origin (e.g., species or organ), secrete increased amounts of peptide hormones and regulatory peptides in response to stimulation. Hypertrophy in the cardiovascular system, affecting cardiomyocytes and vascular smooth muscle
cells, plays an important role in cardiovascular remodeling and dysfunction. Our observations indicate that cultured CMECs display a lognormal size-distribution pattern, with a small proportion of larger cells. The significant correlation between the size of secretory cells and the size of halos produced around secretory cells indicates that both the secretion of ANG II and that of ET-1 from endothelial cells are cell-size dependent. Moreover, the increase in the slope of the regression line with stimulation suggests that larger cells secrete more ANG II and ET-1 in response to stimulation. The connection between hypertrophy and overproduction of ANG II by hypertrophied cells is further supported by our unpublished observation that, in the corpus cavernosum penis from impotent patients, a subpopulation of hypertrophied endothelial cells produces large halos, suggesting increased ANG II secretion. The corpus cavernosum penis is a modified vascular tissue, and ANG II plays a role in modulation of the vascular tissue function similar to that documented in other segments of the vascular system (31). The cell-size-dependent secretion of ANG II and ET-1 from endothelial cells further supports our hypothesis that the regulation of ANG II secretion is similar to the regulation of ET-1 secretion.

The formal demonstration that the halos produced around peptide-secreting CMECs contain both ANG II and ET-1 clearly supports the hypothesis that CMECs cosecrete ANG II and ET-1. One implication of these data is that either stimulatory or inhibitory signals affect the secretion of both peptides. Thus autocrine and paracrine regulation by endothelial cells or other cell types reveals an important pattern of intercellular communication: the employment of multiple, parallel-acting chemical messengers to mediate cellular responses (34, 50). Regulation via parallel-acting multiple messengers probably carries more information at a lower noise level and with a clearer semantic signal than regulation via a single messenger (34). The target cells respond to these messages by integration of signaling pathways initiated by multiple messengers. For example, smooth muscle contraction, Na+/Ca2+ exchanger activity, or cGMP formation is modulated by multiple signaling pathways with additive and/or synergistic effects (2, 24, 49).

Secretion of ANG II and ET-1 by CMECs is modulated by the same factors. The presence of ANG II and ET-1 in the same halos of peptide-secreting cells suggests that autocrine/paracrine regulation of cardiovascular function is accomplished by cosecretion of ANG II and ET-1.

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REFERENCES


