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Endothelial Angiotensin II Generation Induced by Placenta-derived Factors From Preeclampsia

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Abstract

Hypersensitivity to angiotensin II contributes to the increased vasoconstriction in preeclampsia. In this study, we determined whether placenta-derived factors could affect endothelial cell angiotensin II generation. Our results showed that more angiotensin II was produced by endothelial cells treated with preeclampsia placental conditioned medium than the cells treated with normal conditioned medium or untreated controls. To determine which pathway, angiotensin-converting enzyme or nonangiotensin-converting enzyme angiotensin-generating enzyme/chymase, might be involved in preeclampsia conditioned medium induced angiotensin II generation, angiotensin-converting enzyme inhibitor captopril and chymotrypsin inhibitors were applied to the cell culture either separately or in combination. We found that chymotrypsin inhibitor, but not captopril, could attenuate the increased angiotensin II generation. To further test specific effects of the protease on endothelial cell angiotensin II generation, endothelial cells were grown in cell culture inserts and chymotrypsin was added to the upper chamber of the cell culture (apical exposure). The medium in the lower chamber (basal direction) was collected and measured for angiotensin II. Our results showed that apical exposure of endothelial cells to the protease resulted in a concentration-dependent increase in basal release of angiotensin II. Angiotensin II receptor-1 expression was also upregulated in cells treated with preeclampsia conditioned medium or chymotrypsin. This data suggest that placenta-derived factors may activate chymase-angiotensin pathway in endothelial cells. Moreover, increased endothelial cell basal release of angiotensin II in response to the protease stimulation further suggests that angiotensin II levels in the circulation may not necessarily reflect angiotensin II generation within the vascular wall.

Keywords

Angiotensin II; chymase; endothelium; placenta; preeclampsia

INTRODUCTION

Increased vascular response to angiotensin II (Ang II) in women with preeclampsia was described more than 30 years ago. In the early 70s, Gant and colleagues conducted a clinical trial to study the pressor response to Ang II in primigravid patients throughout pregnancy. They found that increased vascular sensitivity to Ang II occurs several weeks before maternal hypertension is detectable in those women who later develop preeclampsia during their pregnancy.^{1,2} Based on their findings, it was believed that hypersensitivity to Ang II might be the most effective predictor for preeclampsia. Although other studies showed that

neither renin, renin substrate (an Ang II precursor) nor Ang II were elevated in women with preeclampsia,^{3,4} Merrill et al actually did find increased Ang II levels in preeclamptic than in normal pregnancies,⁴ suggesting that the amount of Ang II being formed in preeclamptic patients is greater than that occurring in normal pregnant controls. The finding of the presence of Ang II receptor-1 (AT-1) agonistic autoantibody in the maternal circulation⁵ further supports the notion that increased vascular response to Ang II contributes to the increased vasoconstriction in women with preeclampsia. However, the cellular basis of Ang II-induced vascular hypersensitivity in preeclampsia is still not fully understood.

Chymase, a chymotrypsin-like serine protease (CLP), was originally found in mast cells⁶ and later found in the human heart and in the vascular tissue.^{7,8} By study of the Ang II generation in human heart tissue, Urata et al noticed that chymase is responsible for more than 80% of Ang II produced in the human heart, whereas only 10% to 20% of Ang II is generated through angiotensin-converting enzyme (ACE).⁷ Therefore, it has been considered that chymase is a potent non-ACE angiotensin-generating enzyme to convert Ang I to Ang II.⁷ Chymase also has an ability to convert big endothelin to endothelin-1, another strong vasoconstrictor in the vascular tissue and tracheal smooth muscle cells.⁹ These observations suggest that CLP/chymase may exert a great influence in regulating contractile activity in various tissues and in different cell types, including cardiac and bronchial airway tissues, as well as vascular and nonvascular smooth muscle cells.

Placenta-derived factors play an important role in regulation of vascular endothelial function and induce endothelial activation/dysfunction in preeclampsia. Factors derived from preeclamptic placentas may upregulate endothelial adhesion molecule expression and promote neutrophil and endothelial interaction/adhesion.¹⁰ Factors derived from preeclamptic placentas may also disturb endothelial junction molecules and increase endothelial permeability.¹¹ Recently, we reported increased CLP/chymase activity in preeclamptic placentas.¹² We also found that the protease activity was elevated in the maternal plasma from preeclamptic patients compared to that from women with normal pregnancies.¹³ To further determine whether CLP/chymase may contribute to Ang II generation in preeclampsia, we conducted this in vitro study to investigate whether placenta-derived factors could affect endothelial Ang II generation and to determine which Ang II generation pathway, ACE or CLP/chymase, is involved.

MATERIALS AND METHODS

Endothelial Cell Isolation and Culture

Endothelial cells were isolated from human umbilical cords (human umbilical vein endothelial cells, HUVECs) from normal term deliveries by collagenase digestion as described previously.¹⁰ Isolated cells were incubated with endothelial cell growth medium (BioWhittaker Inc, Walkersville, Md). Media were changed within 24 hours of isolation and then every 3 days. When cells were confluent, they were harvested with 0.01% trypsin/EDTA (Sigma; St Louis, Mo) and passed into 24 or 12 wells/plate, 25 cm² cell culture flask, or 6 wells/cell culture inserts based on the experiment need. Cells grown on glass coverslips were used for immunofluorescent staining. Cells grown in 12 wells/plate were used to study Ang II generation; cells grown in 25 cm² cell culture flasks were used for protein isolations; and cells grown on 6 wells/plate cell culture inserts were used to study basal release of Ang II, respectively. Only the primary passage (P1) cells were used in our experiments. The study was approved by the Institutional Review Board (IRB) for Human Research at Louisiana State University Health and Sciences Center (LSUHSC) at Shreveport, Louisiana.

Preparation of Placental-Conditioned Medium

Placental-conditioned media were prepared by culturing placental villous tissue for 48 hours as described previously.¹⁴ Briefly, placentas from normal and preeclamptic pregnancies were processed immediately after delivery. The criteria for normal pregnancy and preeclampsia were based on American College of Obstetricians and Gynecologists (ACOG) guidelines. Placental tissue was gently separated by sterile dissection from different cotyledons, excluding chorionic and basal plates, and washed repeatedly with phosphate-buffered saline (PBS) to remove blood. Villous tissue explants were incubated with serum-free Dulbecco's Modified Eagles Medium (DMEM) (Sigma). The incubation was carried out for 48 hours at 37°C in an incubator gassed with 95% air, 5% CO₂ (Forma Scientific Inc, Marietta, Ohio). Medium samples were collected at the end of incubation as conditioned medium and stored at -80°C until assay. A total of 16 placentas were used, 8 from normal term pregnant women, and 8 from women with preeclampsia. The mean gestational age was 39 weeks for the normal group and 34 weeks for the preeclamptic group.

Measurement of Ang II

Endothelial Ang II production was measured by enzyme immunoassay (EIA). The Ang II EIA kit was purchased from Cayman Chemical (Ann Arbor, Mich). The assay was performed following the manufacturer's instructions. The EIA kit consists of Ang II standard, anti-Ang II IgG tracer, glutaraldehyde, borane trimethylamine, Ellman's reagent, assay and wash buffers. The range of standard curve was 0.98 to 125 pg/mL. An aliquot of 100 µL of endothelial culture medium was assayed and all samples were tested in duplicate. Within assay variation was < 6%. The EIA plate was read at 405 nm by an auto plate reader (Molecular Devices, Sunnyvale, Calif).

Endothelial Chymase Expression

Endothelial chymase expression was examined by fluorescent staining and immunoprecipitation (IP) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (IB). For fluorescent staining, confluent endothelial cells grown on glass cover-slips were treated with normal or preeclamptic placental conditioned medium for 4 hours. The cells were fixed with 95% ethanol, permeabilized with 50% acetone, and then stained with monoclonal antibody against human chymotrypsin (Abcam, Cambridge, Mass). Cy3-labeled donkey anti-mouse IgG (H+L) was used as the secondary antibody (Jackson ImmunoResearch Laboratories Inc, Westgrove, Pa). Stained cell coverslips were examined by fluorescent microscope (Olympus IX71, Tokyo, Japan). Fluorescent images were recorded by a digital camera linked to a computer with PictureFrame software (Optonics Inc, Sunnyvale, Calif). For IP, after cells were treated with preeclamptic conditioned medium, total cellular protein was extracted by lysis buffer and then immunoprecipitated with Protein-A immunoprecipitation kit (Sigma). The immunoprecipitation procedure followed the manufacturer's instructions. An aliquot of 500 µg of total cellular protein per sample was used. The precipitated protein was then run on SDS-PAGE and transferred to a nitrocellulose membrane, which was probed with chymotrypsin antibody. The secondary antibody was horseradish-peroxidase linked antirabbit antibody. The bound antibody was visualized with an enhanced chemiluminescent (ECL) detection kit (Amersham Corp, Arlington Heights, Ill).

Angiotensin II Receptor-1 Protein Expression

Endothelial AT-1 expression was also examined by fluorescent staining and IB. Fluorescent staining procedures were the same as described above for chymotrypsin. Monoclonal anti-AT-1 antibody was purchased from Santa Cruz, San Diego, Calif. Fluorescein-isothiocyanate (FITC)-labeled donkey anti-mouse IgG (H+L) was used as the secondary

antibody. For IB, an aliquot of 10 µg of total cellular protein per sample was subjected to electrophoresis on 12% polyacrylamide gels using a Mini-protein 3 gel running system (Bio-Rad, Hercules, Calif) and then transferred to nitrocellulose membrane. The membrane was probed with a primary monoclonal antibody against AT-1 (Santa Cruz). The membrane was stripped and blocked before it was probed again with β-actin antibody (Sigma).

Statistical Analysis

Data are presented as mean ± SE and analyzed by analysis of variance (ANOVA) using computer software StatView (Cary, NC). Student-Newman-Keuls test was used as a post hoc test. A probability level of $P < .05$ was considered statistically significant.

RESULTS

Ang II Generation

To test whether placenta-derived factors could promote endothelial cells to generate Ang II, cells were exposed to placental conditioned medium for 6 hours and then Ang II was measured in the culture medium. As shown in Figure 1, cells treated with preeclamptic placental-conditioned medium produced significantly more Ang II than those treated with normal placental conditioned medium and untreated controls, 14.69 ± 3.55 versus 3.77 ± 1.45 , and 0.38 ± 0.25 pg/mL, $P < .01$, respectively.

To further determine whether ACE or CLP/chymase was more responsible than the other for the increased Ang II generation by endothelial cells stimulated with preeclamptic placental-conditioned medium, endothelial cells were pretreated with ACE inhibitor captopril or chymotrypsin inhibitor separately or in combination prior to being exposed to placental-conditioned medium. Angiotensin II production was then measured. Consistently, we found that endothelial cells treated with preeclamptic placental-conditioned medium produced significantly more Ang II than the cells treated with normal conditioned medium, $P < .01$ (Figure 2). There was no difference for Ang II generation in cells treated with normal placental-conditioned medium with or without chymotrypsin inhibitor or captopril in culture. However, the increased Ang II production by endothelial cells treated with preeclamptic conditioned medium was attenuated by chymotrypsin inhibitor or chymotrypsin inhibitor combined with ACE inhibitor captopril, but not by captopril alone, in culture, $P < .01$ (Figure 2). These data indicate that increased Ang II generation by endothelial cells induced by placenta-derived factor(s) was associated with endothelial CLP/chymase activation, whereas endothelial ACE might play an insignificant role.

Basal Release of Ang II

To further investigate the possibility of endothelial basal release of Ang II and to test the specificity of chymotrypsin-induced Ang II generation, endothelial cells were grown in the cell culture inserts (8 µm pore size). When cells were confluent, chymotrypsin at concentrations of 0.5, 1.0, 2.5, and 5.0 µg/mL was applied to the upper chambers (apical surface). After 24 hours of culture, medium in the lower chambers (basal direction) was collected and measured for Ang II production. Our results showed that Ang II concentrations in the lower chambers were dose-dependently increased with apical exposure of endothelial cells to chymotrypsin (Figure 3), $P < .01$.

CLP/Chymase Expression

Because chymotrypsin inhibitor could attenuate preeclamptic conditioned medium induced increases in endothelial Ang II generation, we next examined CLP/chymase expression in cells exposed to normal or preeclamptic placental-conditioned medium. Expression of

chymotrypsin was examined by staining of cells with fluorescent-labeled antibody. Our results showed that the protease expression was not detectable in control cells and in cells treated with normal placental-conditioned medium. In contrast, the protease expression was observed in cells exposed to preeclamptic placental-conditioned medium and positive expression was localized in the cytosol. Representative chymotrypsin expression in control cells and cells treated with preeclamptic placental conditioned medium is shown in Figure 4A and B. Increased chymotrypsin protein expression by endothelial cells treated with preeclamptic placental conditioned medium was further confirmed by immunoprecipitated/immunoblotted (IP/IB), as shown in Figure 4C.

Angiotensin II Receptor-1 Expression

We further tested whether factors derived from preeclamptic placentas had any effects on endothelial AT-1 expression, AT-1 expression was examined in endothelial cells treated with preeclamptic placental-conditioned medium. As shown in Figure 5A and B, positive AT-1 expression can be detected both in the membrane and in the nuclei of control cells (Figure 5A), but AT-1 expression was mainly localized in the nuclei of cells after stimulated with preeclamptic placental conditioned medium (Figure 5B). The translocation of the AT-1 receptor from membrane to nuclei indicates receptor activation after stimulation. Chymotrypsin induced endothelial AT-1 upregulation was also demonstrated by Western blot (Figure 5C).

DISCUSSION

In this study, we investigated effects of placental factors on endothelial Ang II generation. We found more Ang II was produced when cells were exposed to conditioned medium derived from preeclamptic than from normal placenta cultures. This observation suggests that factors produced by the preeclamptic placentas have a significant impact on endothelial Ang II generation. We further found that the increased Ang II generation by endothelial cells treated with preeclamptic placental-conditioned medium was significantly attenuated when chymotrypsin inhibitor was present in culture, however captopril had no effect. These findings indicate that endothelial CLP/chymase may play a dominant role in mediating Ang II generation induced by factors released from preeclamptic placentas.

Both ACE and non-ACE angiotensin generating enzyme chymase are present in the vascular endothelial cells. Both of them are able to convert Ang I to Ang II. The reason for preeclamptic placental factor induced endothelial Ang II generation is not clear. However, upregulation of CLP expression in endothelial cells when exposed to preeclamptic placental-conditioned medium supports the notion that increased endothelial CLP/chymase activity could lead to endothelial Ang II generation. This notion is also supported by enhanced chymase immunostaining found in maternal vessel endothelium from women with preeclampsia.¹³

The precise mechanism of upregulation of endothelial CLP expression induced by preeclamptic placental-conditioned medium is not known. It could be related to increased endothelial inflammatory response by factors released from the placenta. We previously reported that placenta-derived factor(s) from preeclampsia were able to induce endothelial activation by upregulation of endothelial adhesion molecule P-selectin and E-selectin expressions.¹⁵ Both P-selectin and E-selectin are involved in the early phases (capture and rolling process) of neutrophil/endothelial and platelet/endothelial interaction and adhesion. Moreover, the upregulated selectin expressions could be attenuated by chymotrypsin inhibitor, suggesting that placenta-derived factor(s) related to CLP were, at least in part, responsible for inducing endothelial activation in preeclampsia.¹⁵ In fact, CLP/chymase has been considered an inflammatory protease. Chymase is stored in granules of Weibel-Palade

bodies of endothelial cells.¹⁶ Activated endothelial cells could release the protease from Weibel-Palade bodies along with many other inflammatory mediators such as interleukin-8 (IL-8), P-selectin, and endothelin.¹⁷ These biological agents are well recognized to be involved in the enhanced inflammatory response in preeclampsia.¹⁸

Another significant finding of this study is the ability of basal release of Ang II by endothelial cells with apical exposure of the protease chymotrypsin in culture. Furthermore, the amount of basal released Ang II is directly related to the protease concentration added to the cell surface. This is very important because if this occurs in an in vivo situation such as in preeclampsia, as we observed in our in vitro study, elevated protease activity in the maternal circulation¹³ could mediate and promote endothelial Ang II generation through the chymase—Ang II pathway. Second, basal released Ang II could directly bind to AT-1 receptor located in the underlying vascular smooth muscle cells, and subsequently regulates vascular contractile activity. Therefore, the Ang II levels in the circulation may not necessarily reflect the increased conversion of Ang I to Ang II within the vascular wall. In other words, in situ or locally generated Ang II by endothelial cells may directly affect the vasoactivity of the underlying vascular tissue. Moreover, the protease could also upregulate endothelial AT-1 expression. The observation of AT-1 receptor trans-localization from membrane/cytosol to nuclei implicates the receptor activation after the protease stimulation. Presently, we do not know the role of the protease-induced endothelial AT-1 activation in preeclampsia. However, many downstream effects of AT-1 activation including increased oxidative stress have been considered to be involved in the vascular pathophysiology in preeclampsia.

We did not measure endothelial Ang I levels in this study. However, earlier studies have demonstrated that renin substrate, renin activity, and Ang I or Ang II generation can all be detectable in endothelial cells,^{19,20} which indicate that endothelial cells are able to generate Ang II intracellularly independent of the extracellular Ang I levels.^{19,20}

In summary, results from this study are important pertaining to the increased vasoactivity in preeclampsia. Our results point out the importance of the local effects of Ang II generation and vasoconstriction in systemic vasculature. If this is the case in an in vivo condition, our findings could be significant not only to preeclampsia but also to other cardiovascular diseases such as chronic hypertension and diabetes, because basal released Ang II by endothelial cells can directly bind to its receptor AT-1 in the underlying vascular smooth muscle cells and mediate vasoactivity of the vessel wall.

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Figure 1.

Angiotensin II (Ang II) generation by endothelial cells (ECs) treated with normal (NL) and preeclamptic (PE) placental-conditioned medium (CM). Endothelial cells treated with PE placental-CM produced significantly more Ang II than the cells treated with NL placental-CM and untreated controls, $P < .01$, respectively. Data are means from 8 independent experiments.

Figure 2.

Effects of angiotensin-converting enzyme (ACE) inhibitor captopril and chymotrypsin inhibitor (CI) on endothelial cell (EC) generation of Ang II induced by placenta-derived factors. There was no difference for Angiotensin II (Ang II) generation in EC treated with NL placental-conditioned medium (CM) with or without CI or captopril in culture. In contrast, the increased Ang II production by ECs treated with preeclamptic (PE) placental-CM was significantly attenuated by CI or CI combined with ACE inhibitor captopril, but not by captopril alone, $P < .01$. Data are means from 8 independent experiments.

Figure 3.

Basal directional release of Angiotensin II (Ang II) by endothelial cells (ECs) with apical exposure to protease chymotrypsin. In this experiment, ECs were grown in cell culture inserts. When cells were confluent, chymotrypsin at different concentrations was added to the upper chamber (apical) of the inserts. The medium at lower chamber (basal) was collected after 24 hours of culture and measured for Ang II. We found that Ang II concentrations in the lower chambers were dose-dependently increased with apical exposure of ECs to the protease, $P < .01$. Data are means from 6 independent experiments.

Figure 4.

Chymotrypsin-like serine protease (CLP)/chymase expression in cells (ECs) treated with placental-conditioned medium (CM). A and B: cells were stained with a fluorescent-labeled antibody. A: control cells; B: cells were treated with preeclamptic (PE) placental-CM. Bar = 10 μ m. C: endothelial chymotrypsin expression examined by immunoprecipitated/immunoblotted (IP/IB). Lanes 1 and 2 were control cells and lane 3 and 4 were cells treated with PE placental-CM. Cells treated with PE placental-CM exhibited increased chymotrypsin expression.

Figure 5. Angiotensin II receptor-1 (AT-1) expression in cells treated with preeclamptic (PE) placental-conditioned medium (CM) or protease chymotrypsin. A and B: cells were treated with PE placental-CM and stained with a fluorescent-labeled antibody. A: control cells; B: cells were treated with PE placental CM. Bar = 10 μm . C: cells were treated with different concentrations of chymotrypsin. The protease could up-regulate AT-1 expression.