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Elevated Plasma Chymotrypsin-like Protease (Chymase) Activity in Women with Preeclampsia

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Abstract

Objective—Chymase, a chymotrypsin-like protease, is a non-ACE angiotensin II (Ang II) generating enzyme. We determined if maternal chymotrypsin-like protease/chymase activity was increased in women with preeclampsia (PE).

Methods—Maternal plasma was extracted from venous blood of healthy nonpregnant women, women with normal and preeclamptic pregnancies. Chymotrypsin-like protease/chymase activity was measured by a colorimetric assay. Maternal vessel chymotrypsin-like protease/chymase expression was examined by immunohistochemistry.

Results—Maternal plasma chymotrypsin-like protease/chymase activity was significantly higher in women with PE than in nonpregnant and normal pregnant women: 0.181 ± 0.011 vs. 0.097 ± 0.05 ($p < 0.01$) and 0.132 ± 0.013 ($p < 0.05$) $\mu\text{kat/mL}$. Chymotrypsin-like protease/chymase activity was markedly reduced 24 hours postpartum in women with PE, $p < 0.05$. Enhanced chymotrypsin-like protease/chymase expression was observed in vascular endothelium in women with PE compared with those in normal pregnancies.

Conclusions—Elevated maternal chymotrypsin-like protease/chymase activity and enhanced protease immunostaining in the maternal vessel endothelium may constitute the exacerbated inflammatory state and account for the increased vascular Ang II sensitivity in PE.

Keywords

Chymotrypsin-like protease/chymase; Ang II; Endothelium; Preeclampsia

INTRODUCTION

Chymase is a chymotrypsin-like serine protease found in mast cells with broad tissue and species expression. Chymase, like angiotensin converting enzyme (ACE), is able to convert angiotensin I (Ang I) to Ang II. Therefore chymase is a non-ACE angiotensin-generating enzyme. Importantly, it has been demonstrated that chymase is responsible for approximately 70% to 80% of Ang II generated in human heart tissue (1) and it is also a

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Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

major serine protease response for ACE-independent production of Ang II in human arteries (2). Studies also showed that chymase can produce endothelin-1 from big endothelin and interleukin-1 β from its precursor (3,4). Most recently, we reported that chymase gene is present in the human placental trophoblasts and that the open reading frame of the chymase gene in trophoblasts is 100% homologous to that reported in human heart tissue (5,6). We also found that chymase expression was upregulated and chymase activity was increased in placental trophoblasts from women complicated by preeclampsia (6), a unique hypertensive and multiple system disorder of human pregnancy.

In the early 1970s, Dr. Gant and colleagues showed an increase in vascular sensitivity to Ang II in women with preeclampsia (7,8). They conducted a clinical trial to test the pressor response to Ang II in primigravitate patients throughout pregnancy. They found that increased vascular sensitivity to Ang II occurs several weeks before maternal hypertension occurs in those women who later develop preeclampsia during their pregnancy (7,8). Therefore, it was considered that hypersensitivity to Ang II might be the most effective predictor for preeclampsia. However, subsequent studies showed that plasma renin, renin substrate and Ang II levels were not increased in the maternal circulation of women with preeclampsia (9). A recent study by Merrill et al (10) found that although the absolute levels for Ang II in the maternal plasma were not increased in preeclampsia, the ratio of Ang II/Ang I was higher in women with preeclampsia than that in women with normal pregnancies, suggesting that the amount of Ang II being formed in preeclamptic patients is greater than would occur in normal pregnancies. However, the basis of the hypertension arising during pregnancy in preeclampsia is still largely unknown.

To investigate the possible role of chymase during preeclampsia, in the present study we measured chymotrypsin-like protease/chymase activity in the maternal plasma samples in women with normal pregnancies, and in women with pregnancies complicated by preeclampsia. Samples from nonpregnant healthy women were measured as a control. In addition, we also examined chymase immunoreactivity of the maternal vessels in subcutaneous fat tissues obtained during Cesarean section from normal and preeclamptic pregnant women.

MATERIALS AND METHODS

Sample Collection and Patient Information

Venous blood was drawn from women diagnosed with normal pregnancies and those with preeclampsia when admitted to the Labor and Delivery Unit at the main hospital of Louisiana State University Health Sciences Center-Shreveport (LSUHSC-S), LA, after consent form was obtained. In some patients, subcutaneous fat tissues were obtained at the time of cesarean section from normal pregnant women and from women with preeclampsia. Normal pregnancy is defined as pregnancy with normal blood pressure (<140/90 mm Hg), no proteinuria, and absence of obstetrical and medical complications. Preeclampsia is defined as a maternal blood pressure of 140/90 mm Hg or higher on two separate readings at least 6 hours apart with proteinuria >300 mg/24 h after 20 weeks of gestation. Preeclampsia is defined severe if one or more of the following criteria is present: maternal blood pressure $\geq$ 160/110 mm Hg; proteinuria >3 + or >5 g/24 h; oliguria of less than 500 mL in 24 hours; intrauterine growth restriction, and presence of persistent headache or visual disturbances. Venous blood was also obtained from nonpregnant, nonsmoking, healthy female volunteers. Plasma were extracted from blood samples by centrifugation and stored at -80°C freezer until analysis. This study was approved by the Institutional Review Board (IRB) for Human Research at LSUHSC-S at Shreveport, LA.

Measurement of Plasma Chymotrypsin-like Protease/Chymase Activity

Plasma chymotrypsin-like protease/chymase activity was measured colorimetrically using MeO-Suc-Arg-Pro-Tyr-pNA (S2586), a chymotrypsin protease substrate, (DiaPharma Group Inc., West Chester, OH) according to the manufacturer's instruction. Aliquots of plasma 50 μ L were incubated with equal volumes of Tris/Ca⁺⁺ buffer (100 mol/L Tris and 960 mmol/L NaCl at pH 8.3), at 37°C for 5 minutes. S2586 was added to the sample to a final concentration of 2 μ M. After 15 minutes of incubation, the reaction was stopped by adding 25 μ L of 20% acetic acid to each well. The plate was then read at 405 nm with a microplate reader (Molecular Devices, Sunnyvale, CA). The enzyme activity was calculated according to a modified formula from DiaPharma method procedure: $\mu\text{kat/mL} = \{[\text{OD}_{450} \text{ nm (sample)} - \text{OD}_{450} \text{ nm (background)}] \times 2.31 \times 200\} / 1000$. The normal and preeclamptic samples were tested in the same time and within assay variation was < 5%. All samples were tested in duplicate.

Examination of Chymotrypsin-Like Protease Immunoreactivity in Maternal Vessels

Fresh subcutaneous fat tissues were fixed with Zamboni buffer containing 2% formaldehyde, 0.1 M phosphate buffer and 15% saturated picric acid and embedded with Immuno-Bed solution (Polysciences Inc., Warrington, PA). Tissue sections (5 micron) were subjected to immunohistochemical staining using monoclonal antibody specific against human chymotrypsin (Novus Biologicals Inc., Littleton, CO) and biotinylated secondary antibody (ABC staining system, Santa Cruz, San Diego, CA) according to manufacturer's instructions. Stained slides were counterstained with Gill's formulation hematoxylin. Stained tissue slides were viewed using an Olympus microscope (Olympus IX71), and images were captured by a digital camera and recorded into a microscope-linked PC computer. Tissue sections stained with secondary antibody only were used as a negative control.

Statistical Analysis

Data are presented as mean $\pm$ SD for the demographic data and mean $\pm$ SE for the chymotrypsin-like protease/chymase activity. Paired *t*-test, analysis of variance (ANOVA), and a post hoc test, the Student-Newman-Keuls test, were used for statistical analysis (StatView, Cary, NC). A probability level of $p < 0.05$ was considered statistically significant.

RESULTS

Maternal Plasma Chymotrypsin-like Protease/Chymase Activities

Chymotrypsin like protease/chymase activity was measured in a total of 72 plasma samples from 13 non-pregnant healthy women, 23 normal pregnant women, and 36 women complicated by preeclampsia. Demographic data for nonpregnant, normal pregnant, and preeclamptic pregnant women including maternal age, racial status, gestational age, parities, maternal blood pressure and mode of delivery is shown in Table 1. All patients in the preeclampsia group were clinically diagnosed with severe preeclampsia.

Chymotrypsin-like protease/chymase activities were significantly higher in the antenatal plasma samples from women with preeclampsia, $0.181 \pm 0.011 \mu\text{kat/mL}$, than those in nonpregnant women, $0.097 \pm 0.05 \mu\text{kat/mL}$ ($p < 0.01$), and in women with normal pregnancies, $0.132 \pm 0.013 \mu\text{kat/mL}$ ($p < 0.05$), respectively (Figure 1). Plasma chymotrypsin-like protease/chymase activity was not different between nonpregnant women and women with normal pregnancies.

Blood samples were also obtained 24 hours after deliveries from 11 of 23 normal pregnant and 15 of 36 preeclamptic pregnant women. Plasma chymotrypsin-like protease/chymase activities in these plasma samples were also measured. Our results showed that the protease activity was significantly reduced 24 hours postpartum in plasma from women with preeclampsia, $p < 0.01$ (Figure 2). The plasma chymotrypsin-like protease/chymase activity was not significantly different in normal pregnant women before and after delivery.

Endothelium Chymotrypsin-like Protease Immunostaining

Subcutaneous fat tissues were obtained from 4 normal and 5 preeclamptic pregnant women during Cesarean section delivery. Chymotrypsin-like protease/chymase immunoreactivity in maternal vascular endothelium was examined by immunohistochemical staining of subcutaneous fat tissue sections. Three sections were stained from each patient. A total of 70 to 100 vessels per patient were counted. Our results showed that chymase staining was barely visible in vessels of normal pregnant samples, whereas intense staining was clearly observed in 40% to 50% of vessels from preeclamptic pregnancies. Figure 3 shows representative protease staining in maternal vessels from one normal (A) and one preeclamptic (B) pregnant woman. Figure 3C shows positive CD31 staining as a marker of vascular endothelium.

DISCUSSION

The role of chymotrypsin-like protease/chymase in normal physiology is not well understood. However, chymase has been implicated in the pathogenesis of several cardiovascular diseases including heart failure, cardiac hypertrophy, and diabetes mellitus (11–13). Recent studies also showed that advanced glycation end-products, markers of oxidative stress, can activate a chymase-dependent angiotensin II-generating pathway in diabetic complications (14). Because chymase is bound to heparin, it is resistant to endogenous inhibitors such as α_1 -antitrypsin or α_1 -antichymotrypsin (15). This could be a reason for the persistent effects of chymotrypsin-like protease/chymase in cardiovascular diseases.

In the present study, we found that chymotrypsin-like protease/chymase activity was found to be significantly increased in the maternal circulation of women with preeclampsia (compared with those from nonpregnant women and from women with normal pregnancies). Moreover, we observed intense staining for chymotrypsin-like protease in the maternal vascular endothelium in tissue samples from women with preeclampsia. Although the role of chymase in preeclampsia is largely unknown, our findings of elevated circulating chymotrypsin-like protease/chymase activity and enhanced staining of chymotrypsin-like protease in the maternal vascular endothelium provide the first compelling evidence for a possible role of this non-ACE Ang II generating enzyme in the pathophysiology of preeclampsia.

It is well known that vessel pressor response to Ang II is increased during preeclampsia, and this increase occurs before any other clinical symptoms become evident in those women who later develop preeclampsia during their pregnancies (8). Circulating Ang II levels are not higher in preeclampsia compared to that in normal pregnancies and several studies even showed that the renin-angiotensin system (RAS) activity was reduced in women with preeclampsia (9,10). Although recent findings of the presence of circulating autoantibody to Ang II receptor 1 (AT1) and increased AT1 receptor activity in preeclampsia may shed some light and provide an explanation of the increased Ang II pressor response (16,17), the relationship between circulating Ang II levels and the increased vessel pressor response to Ang II in preeclampsia is still not completely understood. Chymase was found in vascular smooth muscle cells (18). It is known that vascular endothelium generates most of the

circulating Ang II. In fact, the Ang II levels in the circulation may not necessarily reflect increased Ang I-II conversion within the vascular wall. Our finding of enhanced chymotrypsin-like protease/chymase immunoreactivity in vascular endothelium suggests a possible contribution of chymase in Ang II formation within the vascular wall in preeclampsia. Therefore, the signal of endothelial-derived Ang II to the underlying vascular smooth muscle cells (VSMCs) and the sensitive pressor response of the VSMCs to the locally generated Ang II may explain the dysregulation of vascular contractile function seen in preeclampsia.

In fact, chymotrypsin-like protease/chymase has been considered as an inflammatory protease. This notion is supported by the following data: first, chymase-like immunoreactivity localized within endothelial cell Weibel-Palade bodies (19) provides a convincing rationale that activated endothelial cells release chymotrypsin-like protease/chymase. Weibel-Palade bodies contain a subset of molecules including the cytokine interleukin-8 (IL-8), the adhesion molecule P-selectin, and the vasoconstrictor endothelin (20). Preeclampsia is an endothelial disorder during pregnancy (21). Activated/dysfunctioned endothelial cells are able to release these inflammatory molecules into the extracellular compartment upon stimulation. These biological agents are well recognized to be involved in the enhanced inflammatory response during preeclampsia (22). Second, chymase has been found to be a potent chemoattractant for human monocytes and neutrophils (23); chymase also stimulates migration of lymphocytes and purified T-cells (23). Therefore, enhanced chymotrypsin-like protease/chymase immunoreactivity in the maternal vascular endothelium would be expected to contribute to the increased neutrophil infiltration of the maternal vasculature found in women with preeclampsia (24). Lastly, genomic analysis of human heart chymase revealed that sequences in the 5'-untranslated regions include the κ B site for the binding of the nuclear protein factor NF- κ B and the binding site for phorbol ester reactive element (5). Currently there is no information available for the transcriptional factor NF- κ B expression in endothelial cells during preeclampsia, but enhanced NF- κ B and phorbol ester reactive element expression are well recognized as general cellular responses to inflammatory stimuli.

The origin of circulating chymotrypsin-like proteases has not been defined during pregnancy. Such protease could come from different sources including placental trophoblast cells and activated endothelial cells in the systemic vasculature, in addition to mast cells. Most recently, we have demonstrated that chymase gene was present in placental trophoblasts, which were able to release chymotrypsin-like proteases. We also observed enhanced chymotrypsin-like protease activity in placental tissues from women with preeclampsia (6). In fact, our results which show reduced chymotrypsin-like protease/chymase activity following delivery in women with preeclampsia strongly support the concept that placental trophoblast cells could possibly be a source of this pressor generating protease during preeclamptic pregnancy. Studying the cellular localization and distribution of chymase in human heart tissue, Urata et al found that not only cardiac mast cells, but also mesenchymal interstitial cells and endothelial cells were the cellular sources and storage depots for chymase (19). Therefore, it is likely that activated endothelial cells may release chymotrypsin-like protease/chymase into the circulation upon activation and degranulation.

In our study, most of the patients in the preeclamptic group delivered before term. One question raised is the different gestational age between normal pregnancies and those completed by preeclampsia. However, we do not believe that higher levels of the protease activity in women with preeclampsia were due to an early gestational age, because reduced protease activities were noticed in women with preeclampsia following delivery but no significant change in normal pregnancies, which suggest that elevated chymotrypsin-like protease/chymase activity is associated with the disease process in this pregnancy disorder.

At present, we do not know the precise role of increased chymotrypsin-like protease/chymase activity in women with preeclampsia. However, given the established link between chymase and Ang II generation and chronic inflammatory responses, our findings of elevated chymotrypsin-like protease/chymase activity and enhanced protease immunostaining in the maternal vascular endothelium now link chymase, a non-ACE Ang II generating enzyme, to the inflammatory aspects of preeclampsia. We also conjecture that chymotrypsin-like protease/chymase and its signaling cascade between endothelium and underlying vascular tissue may, at least in part, contribute to the increased pressor response in maternal vasculature during preeclampsia.

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Figure 1. Maternal plasma chymotrypsin-like protease/chymase activities in healthy non-pregnant females, women with normal pregnancies and women with preeclampsia. The mean activity for chymotrypsin-like protease/chymase was significant higher in women with preeclampsia than in nonpregnant and normal pregnant controls, $*p < 0.05$; $**p < 0.01$, respectively.

Figure 2.

Maternal plasma chymotrypsin-like protease/chymase activities at admission and 24 hours after delivery in normal pregnant women and in women with preeclampsia. Plasma chymotrypsin-like protease/chymase activities were significantly reduced postpartum in women with preeclampsia. $**p < 0.01$. No significant changes for the chymotrypsin-like protease/chymase activities were observed in normal pregnant women before and after delivery.

Figure 3.

Representative chymotrypsin-like protease/chymase immunostaining of the maternal vessels in subcutaneous fat tissues from normal and preeclamptic pregnancies. Enhanced protease expression was observed in vessel endothelium from women with Preeclampsia (*B*) compared with that in normal pregnant women (*A*). *C*: positive CD31 staining as a positive marker for vessel endothelium. Arrow = vessels; bar = 20 micron, respectively.

Table 1

Demographic data for the study subjects.

	Non-pregnant (n = 13)	Normal Pregnancy (n = 23)	Preeclampsia (n = 36)	P value
Maternal age (years)	28 ± 7	23 ± 4	24 ± 6	0.43
Racial status				
White	5	2	10	–
Black	6	21	24	–
Other	2		2	–
Gestational age (weeks)		39 ± 2	34 ± 4	< 0.01
Nulliparity		4 (17%)	19 (53%)	< 0.01
Blood pressure (mmHg)				
Systolic		112 ± 8	162 ± 13	< 0.001
Diastolic		61 ± 8	101 ± 14	< 0.001
Mode of delivery				
Vaginal		19	16	–
Cesarean section		4	19	–

Data expressed as mean ± SD.