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Reduced plasma levels of angiotensin-(1-7) and renin activity in preeclamptic patients are associated with the angiotensin I-converting enzyme deletion/deletion genotype

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Abstract

The relationship between preeclampsia and the renin-angiotensin system (RAS) is poorly understood. Angiotensin I-converting enzyme (ACE) is a key RAS component and plays an important role in blood pressure homeostasis by generating angiotensin II (Ang II) and inactivating the vasodilator angiotensin-(1-7) (Ang-(1-7)). ACE (I/D) polymorphism is characterized by the insertion (I) or deletion (D) of a 287-bp fragment, leading to changes in ACE activity. In the present study, ACE (I/D) polymorphism was correlated with plasma Ang-(1-7) levels and several RAS components in both preeclamptic (N = 20) and normotensive pregnant women (N = 20). The percentage of the ACE DD genotype (60%) in the preeclamptic group was higher than that for the control group (35%); however, this percentage was not statistically significant (Fisher exact test = 2.86, d.f. = 2, P = 0.260). The highest plasma ACE activity was observed in the ACE DD preeclamptic women (58.1 ± 5.06 *vs* 27.6 ± 3.25 nmol Hip-His Leu^{-1} min^{-1} mL^{-1} in DD control patients; P = 0.0005). Plasma renin activity was markedly reduced in preeclampsia (0.81 ± 0.2 *vs* 3.43 ± 0.8 ng Ang I mL plasma^{-1} h^{-1} in DD normotensive patients; P = 0.0012). A reduced plasma level of Ang-(1-7) was also observed in preeclamptic women (15.6 ± 1.3 *vs* 22.7 ± 2.5 pg/mL in the DD control group; P = 0.0146). In contrast, plasma Ang II levels were unchanged in preeclamptic patients. The selective changes in the RAS described in the present study suggest that the ACE DD genotype may be used as a marker for susceptibility to preeclampsia.

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- ACE polymorphism (I/D)
- Preeclampsia

Introduction

Preeclampsia is an idiopathic multisystem disorder specific to human pregnancy and puerperium and is associated with significant fetal and maternal morbidity (1). This disorder is characterized by high blood pressure and proteinuria which develops after 20 weeks of gestation in otherwise previously normotensive pregnant women (2).

Characteristically, pregnant subjects are normotensive or slightly hypotensive (3). During pregnancy, there is a progressive increase in different renin angiotensin system (RAS) components, such as circulating levels of angiotensinogen, renin and angiotensin II (Ang II) (3,4). The physiological consequences of the stimulated RAS in normal pregnancy are as yet unclear, as is the issue of how this system may be altered and contribute to hypertensive disorders during pregnancy (3).

In recent years, the recognition of angiotensin-(1-7) (Ang-(1-7)) as a mediating vasodilator of the RAS, along with the identification of the new angiotensin processing enzyme, angiotensin-converting enzyme 2 (ACE 2) (5), and the G protein-coupled receptor MAS as a receptor for Ang-(1-7) have contributed to the redefinition of the classical concept of the RAS (6). The dipeptidyl carboxypeptidase ACE is a key RAS component and plays an important role in blood pressure homeostasis by generating the vasoconstrictor peptide Ang II and by inactivating the vasodilator peptides bradykinin and Ang-(1-7) (7,8). Since Ang-(1-7) is an ACE substrate, clinical conditions in which ACE activity is elevated may result in lower plasma Ang-(1-7) levels (7,9). One such condition is related to ACE insertion/deletion (I/D) polymorphism (9,10).

The ACE (I/D) polymorphism is characterized by the presence (insertion (I) or deletion (D)) of a 287-bp fragment and has been identified in intron 16 of this gene (10). The presence of an ACE polymorphism in humans has been postulated from segregation analysis of plasma ACE levels, in which the D allele is associated with higher levels of ACE activity and shorter bradykinin life (9-11). Jalil et al. (9) observed that DD hypertensive subjects had higher ACE activity and lower Ang-(1-7) levels. The higher ACE activity in patients presenting the DD genotype may contribute to the hydrolysis of Ang-(1-7), in which vasodilatory actions oppose the vasoconstrictor effect of Ang II (8). It has been suggested that DD patients have an increased risk of left ventricular hypertrophy, myocardial infarction, nephropathy, and hypertension (9,11). However, data relating ACE polymorphism to preeclampsia are scarce. Moreover, the relationship between RAS components and ACE (I/D) polymorphism in preeclampsia has never been investigated, though a significant reduction in Ang-(1-7) has been observed in preeclamptic women (7).

In the present study, we tested the hypothesis that reduced plasma Ang-(1-7) levels in preeclamptic women are related to the DD genotype. For this purpose, ACE (I/D) polymorphism was correlated with plasma Ang-(1-7) levels and with several RAS components in both normotensive and preeclamptic women.

Subjects, Material and Methods

The study protocol was approved by the Ethics Committee of the Federal University of Minas Gerais and written informed consent was obtained from all patients. We investigated 20 hospitalized preeclamptic women and 20 normotensive pregnant women (both in the third trimester of pregnancy) regarding ACE activity, ACE (I/D) polymorphism, plasma renin activity (PRA), Ang II, and the endogenous ACE substrate, Ang-(1-7). The preeclamptic women (systolic blood pressure 172 ± 5 mmHg, proteinuria ≥+2) had no previous history of hypertension or renal, connective-tissue, or metabolic diseases. The control patients had no previous history of hypertension or renal, connective-tissue or metabolic diseases, and at time of delivery had normal blood pressure (115 ± 1 mmHg). Patients from both groups were on a regular sodium diet and were matched for age, gestational age, race, and laboratory data (Table 1).

Controls and preeclamptic women were lying down for 20 min before venous sam-

pling was performed. Samples were analyzed for PRA, ACE activity, ACE (I/D) polymorphism, Ang II, and Ang-(1-7), as described below.

Angiotensin peptides. Blood was collected in a cocktail of protease inhibitors (µL/mL of blood): 50 µL 7.5% EDTA, 10 µL 1 mM phenylmethylsulfonylfluoride, 50 µL 30 mM ortho-phenanthroline, 10 µL 1 mM p-hydroxy-mercury benzoate, and 20 µL 1 mM pepstatin A. Plasma was extracted using C 18 bond-elut phenylsilane cartridge (500 mG/3 mL-varian) pre-activated with a mixture of 20 mL 99.9% acetonitrile (ACN)/ 0.1% heptabutyric acid (HFBA, 0.1%) and 20 mL 0.1% HFBA in ultrapure water. The columns were then washed sequentially with a mixture of 3 mL 0.1% bovine serum albumin (0.1% BSA/0.1% HFBA), 10 mL 10% ACN/0.1% HFBA and 3 mL 0.1% HFBA. After sample application, the column was washed with 20 mL 0.1% HFBA and 3 mL 20% ACN/0.1% HFBA. The peptides were eluted with 3 mL 99.9% ACN/0.1% HFBA and the solvent was evaporated.

The angiotensins were determined by radioimmunoassay (all samples in the same assay, pg/mL plasma) by the method of Neves et al. (12). For Ang II, samples were reconstituted with a 0.9% NaCl, 0.1% BSA and 0.03% acetic acid. The Ang II antibody (kindly donated by S. Prennie, Cleveland Clinic Foundation, Cleveland, OH, USA) presented 100% cross-reactivity with Ang-(2-8), Ang-(3-8), and Ang-(4-8). Cross-reactivity of less than 0.001% was observed with Ang I and Ang-(1-7). The inter- and intra-assay levels of variability were 5.2 and 6.8%, respectively (13). Plasma Ang-(1-7) was determined using a polyclonal antibody with less than 0.08% cross-reactivity with Ang-(2-7) and Ang-(3-7), and less than 0.08% cross-reactivity with Ang-(4-7). Cross-reactivity with Ang I, Ang II, and amino-terminal fragments was less than 0.001%. The inter- and intra-assay levels of variability were 8.6 and 4.8%, respectively.

Plasma renin activity. PRA is defined as the rate of Ang I generation from an endogenous substrate, and was measured in plasma incubated at 37ºC in the presence of EDTA, phenylmethylsulfonyl fluoride and 8-OH quinoleine to prevent the degradation of the generated Ang I. The decapeptide was quantified by radioimmunoassay (12). The polyclonal Ang I antibody presented less than 0.001% cross-reactivity with Ang II and Ang-(1-7). The Ang I antibody cross-reacted 100% with the carboxyl-terminal fragments Ang-(2-10), Ang-(3-10) and Ang-(4-10). The inter- and intra-assay variability was 7.8 and 3.5%, respectively.

Angiotensin converting-enzyme activity. Plasma ACE activity was measured by a fluorometric method, as described by Santos et al. (14) using 5 mM hippuryl-histidine-leucine as the substrate. Duplicate plasma aliquots (10 µL) were incubated with 500 µL 5 mM Hip-His-Leu in 0.4 M sodium borate buffer, pH 8.3, containing 0.9 mM NaCl, for 15 min at 37ºC. The reaction was stopped by the addition of 1.2 mL 0.34 M NaOH. One

Table 1. Demographic and laboratory data of pregnant normotensive and preeclamptic women.

Characteristic	Normotensive pregnant	Preeclamptic pregnant
Age	23.9 ± 4.39	28.3 ± 5.4
Race		
Black	55%	50%
White	35%	35%
Mulatto	10%	15%
Gestational age (weeks)	34.5 ± 5.1	33.2 ± 4.6
Parity		
Primigravida	8/20 (40%)	8/20 (40%)
Multiparity	12/20 (60%)	12/20 (60%)
Systolic pressure (mmHg)	115 ± 1	172 ± 5*
Diastolic pressure (mmHg)	72 ± 6	114 ± 15*
Heart rate (bpm)	84 ± 9	83 ± 8
Hemoglobin (g/dL)	12.6 ± 0.8	12.9 ± 1.4
Hematocrit (%)	36.9 ± 1.4	37.6 ± 3.2
Creatinine (mg/dL)	0.79 ± 0.2	0.71 ± 0.2
Proteinuria (+)	Negative	(++)40%; (+++)40%; (++++)20%

Data are reported as means ± SD or percent.
*P < 0.05 compared to normotensive women (Student *t*-test).

hundred microliters of orthophthaldehyde (20 mg/mL in methanol) was added and after 10 min, 200 µL 3 N HCl was added at room temperature. After centrifugation at 800 *g* for 5 min, the fluorescence of the supernatant solution (365-nm excitation and 495-nm emission) was measured against water. Blanks were prepared by inverting the order of addition of enzyme and NaOH. A standard curve of 0.5 to 20 nmol His-Leu/tube was prepared for each assay. Enzyme activity is reported as nmol His-Leu min^{-1} mL^{-1}. Assays were carried out under conditions that provided constant velocity and constant enzyme-specific activity.

DNA extraction and PCR. Blood was drawn into EDTA-containing tubes and DNA was extracted by a standard phenol-chloroform isoamilic alcohol method (25:24:1) (15). To determine the ACE genotype, genomic DNA was amplified by PCR, initially using a pair of primers described by Rigat et al. (10) and subsequently, when necessary, with a pair of primers that recognize the insertion of specific sequences for confirmation of the specificity of the amplification reactions (15). Amplification with the former primer pair resulted in 490- and 190-bp amplification products, corresponding to the I and D alleles, respectively. PCR amplification employed 25-µL reactions (50-100 ng genomic DNA), 10 pmol of each primer, 0.5 mM each of deoxy-ATP, GTP, CTP, thymidine 5-triphosphate, 50 mM $MgCl_2$, 5 U/µL Taq DNA polymerase, 500 mM KCl, and 200 mM Tris-HCl buffer, pH 8.4, with 1 min denaturation at 94ºC, followed by 32 cycles of 30 s at 94ºC, 1 min at 58ºC (annealing) and 2 min at 74ºC (extension) in a thermal cycler. The amplification with the latter primer pair resulted in a 335-bp product, corresponding to the I allele and its reaction was performed as described above. The reaction consisted of 30 cycles of amplification (1 min of initial denaturation at 94ºC, 30 s of denaturation at 94ºC, 45 s of annealing at 67ºC, and 2 min of extension at 72ºC). These products were run on 1.5% agarose gel, stained with ethidium bromide and viewed with ultraviolet light.

Statistical analysis

Data are reported as means ± SEM. Statistical analysis was performed using the unpaired Student *t*-test and the Fisher test for the genotype analyses, with the level of significance set at $P < 0.05$ for both tests. Hardy-Weinberg equilibrium was checked by the χ^2 test.

Results

Profile of the renin-angiotensin system in preeclamptic and normotensive pregnant women

Twenty subjects were studied in each of the two groups. Plasma ACE activity was significantly higher in the preeclamptic group (51.8 ± 4.0 *vs* 29.3 ± 3.8 nmol Hip-His Leu min^{-1} mL^{-1} in the control group; P = 0.0002, Figure 1A). In contrast, plasma renin activity was markedly reduced in preeclamptic women (0.7 ± 0.1 *vs* 2.0 ± 0.4 ng Ang I mL $plasma^{-1}$ h^{-1} in the control group; P = 0.0071, Figure 1B). Plasma Ang-(1-7) was significantly reduced in preeclamptic women (16.9 ± 1.2 *vs* 21.6 ± 1.1 pg/mL plasma in the control group; P = 0.0063, Figure 1C), whereas plasma Ang II concentration did not differ between groups (54.1 ± 7.0 *vs* 66.4 ± 10.1 pg/mL plasma in the control group; P = 0.3225, Figure 1D).

Relationship between ACE I/D polymorphism and the renin-angiotensin profile in preeclamptic and normotensive pregnant women

ACE gene types in preeclamptic women included the insertion homozygote (II), deletion homozygote (DD) and insertion/deletion heterozygote (DI). Gene frequency in

the preeclamptic group was 5% (1/20) for II, 35% (7/20) for ID and 60% (12/20) for DD. In the control group, gene frequency was 5% (1/20) for II, 60% (12/20) for DI and 35% (7/20) for DD. Genotype distribution in preeclamptic patients (DD = 0.601; DI = 0.349 and II = 0.051; $\chi^2 = 0.00027$; P = 1.0) and the control group (DD = 0.423; DI = 0.455 and II = 0.123; $\chi^2 = 2.043$; P = 0.15) was in agreement with the Hardy-Weinberg equilibrium. The percentage of the ACE DD genotype (60%) was higher in the preeclamptic group than in the control group (35%), but the difference was not statistically significant (Fisher exact test = 2.86, d.f. = 2, P = 0.260).

The highest plasma ACE activity was observed in the ACE DD preeclamptic women (58.1 ± 5.06 *vs* 27.6 ± 3.25 nmol Hip-His Leu min^{-1} mL^{-1} in the DD control patients; P = 0.0005, Figure 2A).

Strikingly, reduced PRA activity was mainly associated with the ACE DD genotype in preeclamptic women (0.81 ± 0.2 *vs* 3.43 ± 0.8 ng Ang I mL $plasma^{-1}$ h^{-1} in DD normotensive patients; P = 0.0012, Figure 2B). Low PRA was also observed in preeclamptic women with the DI genotype (0.4 ± 0.1 *vs* 1.4 ± 0.4 ng Ang I mL $plasma^{-1}$ h^{-1}; P = 0.0006, Figure 2B).

The reduced plasma level of Ang-(1-7) was also associated with the ACE DD genotype in preeclamptic women (15.6 ± 1.3 *vs* 22.7 ± 2.5 pg/mL plasma in the DD control group; P = 0.0146, Figure 2C).

In contrast, plasma Ang II levels were unchanged in preeclamptic patients presenting the ACE DD genotype (42.77 ± 5.40 *vs* 51.27 ± 5.98 pg/mL plasma in DD normotensive patients; P = 0.3287, Figure 2D).

Discussion

The major finding of the present study was the detection of a significant reduction in plasma Ang-(1-7) levels, significantly higher plasma ACE activity and a marked reduction in plasma renin activity in preeclamptic women, especially in patients presenting the ACE DD genotype. In contrast, the plasma Ang II levels of preeclamptic women did not differ from those of normotensive pregnant subjects.

As stated above, there are few data describing the circulating levels of Ang-(1-7) in normotensive pregnant and preeclamptic

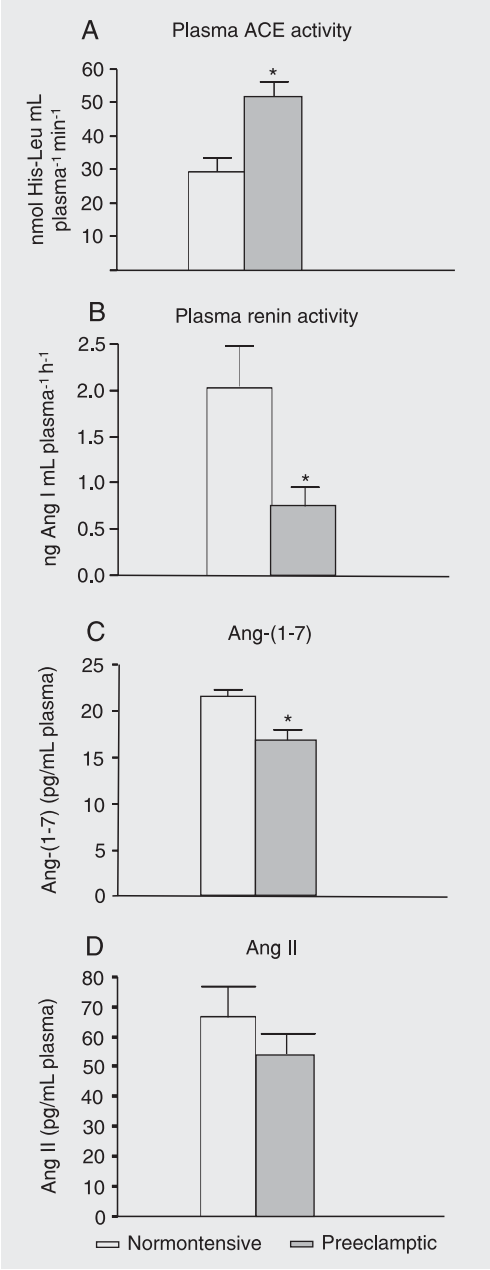

Figure 1. Plasma angiotensin-converting enzyme (ACE) activity (A), plasma renin activity (B), plasma angiotensin-(1-7) (Ang-(1-7)) levels (C), and plasma angiotensin II (Ang II) levels (D) of normotensive and preeclamptic pregnant women. *P < 0.05 compared to normotensive pregnant women (Student *t*-test).

women (7,16). Valdes et al. (16) reported a significant increase in the urinary excretion of Ang-(1-7) in pregnant normotensive women in comparison to normotensive non-pregnant women. However, Merrill et al. (7) observed a decrease in plasma Ang-(1-7) levels in preeclamptic women. Our data demonstrated a significant decrease in plasma Ang-(1-7) levels among preeclamptic women presenting the ACE DD genotype. This is further evidence of an important relationship between Ang-(1-7), blood pressure and pregnancy.

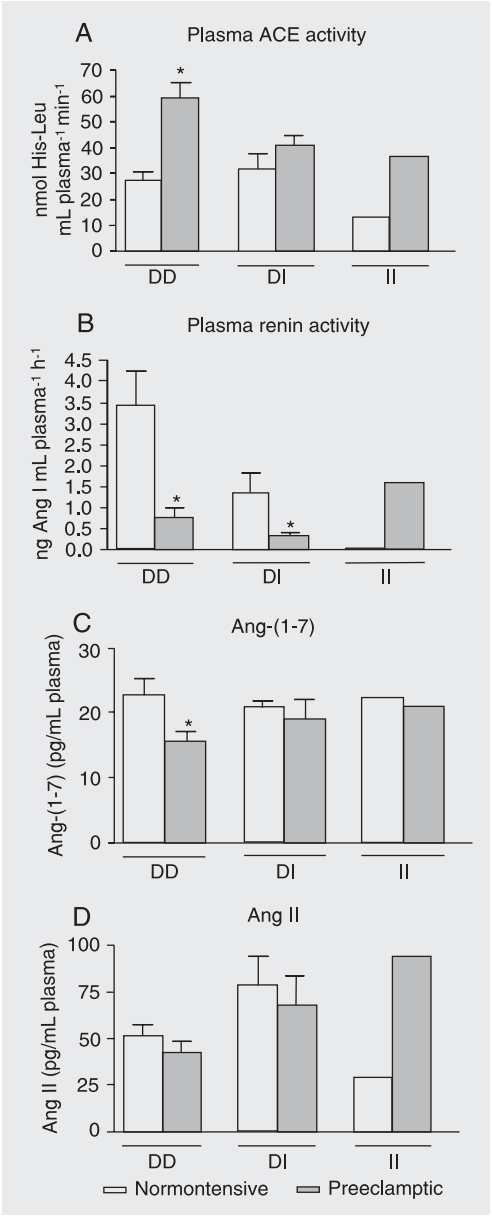

Figure 2. Plasma angiotensin-converting enzyme (ACE) activity (A), plasma renin activity (B), plasma angiotensin-(1-7) (Ang-(1-7)) levels (C), and plasma angiotensin II (Ang II) levels (D) in normotensive and preeclamptic pregnant women with DD, DI or II ACE genotypes. D = deletion; I = insertion. *P < 0.05 compared to normotensive pregnant women (Student *t*-test).

There are no studies concerning the influence of Ang-(1-7) on blood pressure levels in pregnant women. However, there is considerable experimental evidence of the vasodilator action of this hormone (8). A potent vasodilatory effect of Ang-(1-7) has recently been reported in various vascular beds, including the mesenteric, cutaneous, cerebral, and renal beds of Wistar rats (8,17). In humans, plasma Ang-(1-7) levels have been reported to show a negative correlation with blood pressure (18). Furthermore, a recent study has reported that the control of blood pressure in hypertensive patients treated with the dual ACE and neutral endopeptidase inhibitor Omopatrilat was associated with an increase in urinary Ang-(1-7) levels (19).

As cited above, Merrill et al. (7) demonstrated reduced levels of Ang-(1-7) in preeclamptic women when compared to normotensive pregnant women. In the present study, we observed for the first time that the greatest reduction of Ang-(1-7) in preeclamptic women was associated with the ACE DD genotype. In view of the putative counter-regulatory effects of Ang-(1-7) (8,17) and the diminished vascular reactivity to Ang II in normal pregnancy, a potential role of Ang-(1-7) in both the vasodilation of normal pregnancy and the pathologic vasoconstriction observed in preeclampsia should be considered.

Accordingly, Neves et al. (3) observed a significantly increased vasodilator response to Ang-(1-7) in the mesenteric vascular bed of pregnant rats when compared to non-pregnant rats (3). In a previous study, these investigators observed that estrogen replacement increased the vasodilatory response of Ang-(1-7) in ovariectomized rats (20). In this regard, estrogen may be a mediator of vascular changes during pregnancy, contributing to the vasodilation mediated by nitric oxide-releasing agonists, such as Ang-

(1-7), through the increase of endothelial nitric oxide synthase expression (21).

Ang-(1-7) is formed by Ang I and Ang II through the effect of tissue peptidases and is rapidly hydrolyzed, mainly by ACE (8). Thus, the decreased plasma Ang-(1-7) levels may be related to the increased ACE activity in pregnant preeclamptic women. However, a direct relationship between ACE activity, plasma Ang-(1-7) levels and preeclampsia is difficult to assume based on currently available data. For example, data related to ACE activity in pregnancy are still controversial. Some studies showed no difference in the plasma ACE activity between normotensive pregnant women and preeclamptic women (22,23). In contrast, other investigators observed higher ACE activity in preeclamptic women when compared to normotensive women (7,24), as also observed in our study. Also in keeping with our data, Gurdöl et al. (25) observed that the highest ACE activity in preeclamptic patients was associated with the ACE DD genotype. It is important to highlight that our data suggest that the influence of the RAS in preeclampsia appears to be more related to lower levels of Ang-(1-7) as a consequence of increased ACE activity than to changes in Ang II levels, which were not altered in our study, corroborating findings reported by Gordon et al. (4). It remains to be clarified whether changes in ACE activity and plasma Ang-(1-7) in preeclampsia result in a decreased contribution by other peptides, such as bradykinin, to vasodilator tonus.

Renin is another important RAS component. Longitudinal studies have demonstrated that PRA increases during pregnancy in normotensive women compared to the postpartum (26). Langer et al. (22) described significantly lower PRA in preeclamptic women compared to normotensive pregnant women. Our study corroborated these previous observations, showing that PRA was markedly reduced in preeclamptic women compared to the control group. A similar finding was reported by Merrill et al. (7). Importantly, our data suggest that this difference is mainly due to the presence of the D allele in preeclamptic women. There was also a reduction in PRA among preeclamptic women with the DI genotype, but this reduction was more pronounced among DD patients.

Weir et al. (27) demonstrated a significant reduction in plasma Ang II in preeclampsia, while other studies found no significant difference in plasma Ang II between preeclamptic women and pregnant normotensive women (4,22). This discrepancy in relation to Ang II could be explained by the blood collection conditions and different assay methodologies employed to measure this peptide (22). In the present study, no alteration in plasma Ang II was observed in preeclamptic women, despite a significant increase in plasma ACE activity. This suggests that plasma ACE, and probably tissue ACE as well, may not be a critical factor in determining plasma Ang II levels in such patients (5). Indeed, other enzymes such as chymase and ACE 2 also participate in Ang II metabolism (5).

Our data show significant and important changes in the RAS system in preeclamptic women presenting the ACE DD genotype, including a significant reduction in plasma Ang-(1-7), a significant increase in plasma ACE activity and a marked reduction in PRA. However, it should be pointed out that the present study has important methodological limitations: a small sample size, especially of patients with genotype II, and the fact that the racial background of our patients is mostly black-mulatto. Nevertheless, the selective changes in the RAS described in our study suggest that the ACE DD genotype may be used as a marker for susceptibility to preeclampsia. Further studies with a larger number of patients are needed to confirm this hypothesis.

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