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ORIGINAL ARTICLE

Maternal iNOS genetic polymorphisms and hypertensive disorders of pregnancy

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Increased expression and activity of inducible nitric oxide synthase (iNOS) may contribute to the pathogenesis of pre-eclampsia (PE) and gestational hypertension (GH). However, no previous study has examined whether genetic polymorphisms in the *iNOS* gene are associated with PE or GH. We examined whether two functional, clinically relevant iNOS genetic polymorphisms (the $C^{-1026}A$ polymorphism, rs2779249, in the promoter region, and the G2087A polymorphism, rs2297518, in exon 16) are associated with GH or with PE. We studied 565 pregnant women: 212 healthy pregnant (HP), 166 pregnant with GH and 187 pregnant with PE. Genotypes were determined by real-time PCR, using the Taqman allele discrimination assay. The PHASE 2.1 program was used to estimate haplotype distributions in the three study groups. We found no significant association between the $C^{-1026}A$ polymorphism and PE or GH ($P>0.05$). However, we found the GA genotype and the A allele for the G2087A polymorphism at higher frequency in PE, but not in GH, compared with HP ($P<0.05$). The haplotype analysis showed no significant intergroup differences ($P>0.05$). These findings suggest that iNOS genetic variants may affect the susceptibility to PE, but not to GH.
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Introduction

Hypertensive disorders of pregnancy (HDP) are relatively common complications of pregnancy which increase maternal and fetal morbidity and mortality. Two important HDP include pre-eclampsia (PE) and gestational hypertension (GH). Although the etiologies of these conditions are not precisely known, it is now becoming clear that several pathogenetic mechanisms may be involved.[1–5] For example, an important mechanism possibly causing PE is abnormal cytotrophoblast differentiation leading to hypoperfusion of placenta, hypoxia and release of some soluble factors to the maternal circulation, thereby causing systemic endothelial dysfunction.[6–8] However, other mechanisms involving altered formation of nitric oxide (NO) have been implicated in HDP.[4,9–13] In fact, there is now important evidence that impaired endothelial-derived NO bioavailability contributes to HDP.[4,9–13]

NO is synthesized from L-arginine by at least three different nitric oxide synthases (NOS): neuronal, endothelial and inducible NOS, respectively.[14] Importantly, excessive amounts of NO may have deleterious effects. Indeed, increased oxidative stress has been reported in HDP, especially in PE, with increased levels of the highly reactive species superoxide reacting with NO to form peroxynitrite.[15] This reaction may be favored by increased iNOS expression because this enzyme produces huge amounts of NO, thus possibly contributing to PE.[16] However, while there is evidence that genetic polymorphisms commonly found in the *eNOS* gene may affect the susceptibility to HDP,[10,11,13] no previous study has examined whether genetic polymorphisms in the *iNOS* gene are associated with HDP in a relatively large number of patients, even though there is a pilot study implicating iNOS polymorphisms in PE.[17] This is an important hypothesis to be tested, especially because increased vascular iNOS expression and activity has been implicated in hypertension.[18]

In the present study, we examined whether two functional, clinically relevant iNOS genetic

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polymorphisms (the $C^{-1026}A$ polymorphism, rs2779249, in the promoter region, and the G2087A polymorphism, rs2297518, in exon 16) are associated with GH or with PE. These polymorphisms have been associated with hypertension[19,20] or with malignant neoplasias.[21,22] Moreover, we have also studied whether iNOS haplotypes combining the genetic variants for these polymorphisms are associated with HDP.

Materials and methods

Subjects

Approval for use of human subjects was obtained from the Institutional Review Board at the Faculty of Medicine of Ribeirao Preto, and informed consent was obtained from each participant.

All volunteers were consecutively enrolled in the Department of Obstetrics and Gynecology, University Hospital of the Faculty of Medicine of Ribeirao Preto. We studied 565 pregnant women: 212 healthy pregnant (HP), 166 pregnant with GH and 187 pregnant with PE. HDP were defined in accordance with the guidelines of the NHBPEP (National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy).[23] GH was defined as pregnancy-induced hypertension ($\geqslant$140 mm Hg systolic or $\geqslant$90 mm Hg diastolic on $\geqslant$2 measurements at least 6 h apart) in a woman after 20 weeks of gestation, and returning to normal by 12 weeks postpartum. PE was defined as increased blood pressure plus significant proteinuria ($\geqslant$0.3 g per 24 h) in a woman after 20 weeks of gestation. No women with pre-existing hypertension, with or without superimposed PE, were included in the present study.

At the time of clinic attendance, maternal venous blood samples were collected. Genomic DNA was extracted from the cellular component of 1 ml of whole blood and stored at $-20\,^{\circ}C$ until analyzed.

Genotyping for iNOS polymorphisms

Two clinically relevant polymorphisms in the *iNOS* gene were studied: the $C^{-1026}A$ (*rs2779249)* and the G2087A (*rs2297518*). Genotypes for these polymorphisms were determined by real-time PCR, using the Taqman allele discrimination assay (Applied Biosystems, Carlsbad, CA, USA). TaqMan PCR was performed in a total volume of 12 µl (3 ng of DNA, 1 × TaqMan master mix, 1 × assay mix, 900 nM of each primer and 200 nM of each probe) placed in 96-well PCR plates. The probes and the primers used in these genotypings were designed by Applied Biosystems. Fluorescence from PCR amplification was detected using Chromo 4 Detector (Bio-Rad Laboratories, Hercules, CA, USA) and analyzed with manufacturer's software.

Statistical analysis and study of iNOS haplotypes

The clinical characteristics of women with GH or PE were compared with those of HP women by Mann–Whitney U-test, χ^2 or Fisher exact, as appropriate. The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium, and differences in genotype and allele frequencies among groups were assessed using χ^2-tests or Fisher exact tests. A value of $P<0.05$ was considered statistically significant.

The Bayesian statistical based program PHASE (version 2.1, www.stat.washington.edu/stephens/software.html) was used to estimate the haplotype's frequencies in each group.[24,25] To further confirm these frequencies we have also used the Haplo.stats software (version 1.4.4, http://cran.r-project.org/web/packages/haplo.stats/index.html), which computes maximum likelihood estimates of haplotype probabilities. The possible haplotypes including genetic variants for the iNOS polymorphisms studied (C or A variants for the $C^{-1026}A$ polymorphism, and G or A variants for the G2087A polymorphism) were: H1 (CG), H2 (CA), H3 (AG) and H4 (AA). Differences in haplotype frequency were further tested using a contingency table. The minimum level of statistical significance was corrected for the number of comparisons made. Therefore, we considered significant a probability value of $P<0.05$/number of haplotypes ($P<0.05/4=0.0125$).

Results

Table 1 summarizes the characteristics of the 565 pregnant enrolled in the present study. We found no differences in age, ethnicity, smoking, % primigra-

Table 1 Demographic characteristics of study subjects

Parameters	*Healthy pregnant (n = 212)*	*Gestational hypertension (n = 166)*	*Pre-eclampsia (n = 187)*
Age (years)	26.5 ± 6.6	26.5 ± 6.8	26.9 ± 6.0
Ethnicity (% white)	74.3	72.3	67.0
Current smoking (%)	14.6	20.7	16.3
Primigravida (%)	46.7	38.9	43.3
BMI ($kg\,m^{-2}$)	29.7 ± 3.8	27.9 ± 6.3	26.3 ± 5.8
SBP (mm Hg)	112.7 ± 10.6	131.4 ± 16.5[a]	136.9 ± 21.6[a]
DPB (mm Hg)	72.9 ± 8.2	83.1 ± 13.0[a]	86.7 ± 12.41[a]
HR (beats per min)	82.8 ± 6.8	82.2 ± 7.9	81.7 ± 7.6
Fasting glucose ($mmol\,l^{-1}$)	4.04 ± 0.63	4.08 ± 0.47	4.14 ± 0.65
Hb ($g\,l^{-1}$)	120 ± 17	121 ± 12	119 ± 17
Hct (%)	35.6 ± 5.2	36.3 ± 3.5	36.2 ± 5.2
Creatinine ($\mu mol\,l^{-1}$)	61.9 ± 10.2	53.8 ± 10.3	58.1 ± 13.3
24-h-Pr ($mg\,24\,h^{-1}$)	ND	153 ± 76	1169 ± 1665[b]
Newborn weight (g)	3258 ± 575	3208 ± 595	2777 ± 816[a]
GAD (weeks)	39.7 ± 3.3	38.6 ± 4.0	35.6 ± 2.8[a]

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; GAD, gestational age at delivery; HR, heart rate; Hb, hemoglobin concentration; Hct, hematocrit; ND: not determined, however, with negative dipstick test; SBP, systolic blood pressure; 24-h-Pr, 24-h proteinuria.
Values are the mean ± s.d.
[a]$P<0.05$ vs healthy pregnant group.
[b]$P<0.05$ vs gestational hypertension group.

Table 2 Genotype and allele frequencies for iNOS polymorphisms in healthy pregnant, gestational hypertension and pre-eclampsia

Polymorphism	*Genotype or allele*	*Healthy pregnant* (n = *212*)	*Gestational hypertension* (n = *166*)	*OR (95% CI)*	P	*Pre-eclampsia* (n = *187*)	*OR (95% CI)*	P
$C^{-1026}A$								
Genotypes	CC	88 (41%)	67 (40%)	1.00 (reference)		67 (36%)	1.00 (reference)	
	CA	99 (47%)	78 (47%)	1.03 (0.67–1.60)	NS	97 (52%)	1.29 (0.84–1.97)	NS
	AA	25 (12%)	21 (13%)	1.10 (0.57–2.13)	NS	23 (12%)	1.21 (0.63–2.31)	NS
				$\chi^2 = 0.09$	NS		$\chi^2 = 1.39$	NS
Alleles	C	275 (65%)	212 (64%)	1.00 (reference)		231 (62%)	1.00 (reference)	
	A	149 (35%)	120 (36%)	1.05 (0.77–1.41)	NS	143 (38%)	1.14 (0.86–1.53)	NS
G2087A								
Genotypes	GG	155 (73%)	122 (73%)	1.00 (reference)		114 (61%)	1.00 (reference)	
	GA	53 (25%)	44 (27%)	1.05 (0.66–1.68)	NS	68 (36%)[a]	1.74 (1.13–2.69)	0.01
	AA	4 (2%)	0	0.10 (0.01–1.75)	NS	5 (3%)	1.13 (0.34–3.80)	NS
				$\chi^2 = 4.78$	NS		$\chi^2 = 6.41$	0.04
Alleles	G	363 (86%)	288 (87%)	1.00 (reference)		296 (79%)	1.00 (reference)	
	A	65 (14%)	44 (13%)	0.85 (0.57–1.29)	NS	78 (21%)[a]	1.47 (1.02–2.12)	0.04

Abbreviations: CI, confidence interval; NS, not significant; OR, odds ratio.
[a]$P<0.05$ vs healthy pregnant group.

Table 3 Estimated haplotype frequencies in healthy pregnant, gestational hypertension and pre-eclampsia

	Haplotype		*Healthy pregnant* (n = *212*)	*Gestational hypertension* (n = *166*)	*OR (95% CI)*	P	*Pre-eclampsia* (n = *187*)	*OR (95% CI)*	P
	$C^{-1026}A$	*G2087A*							
H1	C	G	0.59	0.59	1.00	NS	0.53	1.00	NS
H2	C	A	0.06	0.05	0.88 (0.41–1.89)	NS	0.08	1.42 (0.72–2.81)	NS
H3	A	G	0.26	0.28	1.09 (0.77–1.56)	NS	0.30	1.24 (0.88–1.76)	NS
H4	A	A	0.09	0.08	0.86 (0.50–1.50)	NS	0.09	1.04 (0.61–1.80)	NS
					$\chi^2 = 1.05$			$\chi^2 = 3.80$	

Abbreviations: CI, confidence interval; NS, not significant; OR, odds ratio.

vida, body mass index, heart rate, fasting glucose, hemoglobin, hematocrit and creatinine levels when HP, GH and PE women were compared (Table 1; all $P>0.05$). However, as expected, higher systolic and diastolic blood pressures were found in the PE group compared with the HP group (Table 1; both $P<0.05$). Importantly, most hypertensive patients were receiving pharmacological therapy (methyldopa in most cases). Lower newborn weights and gestational ages at delivery were found in the PE group compared with the HP group (Table 1; $P<0.05$). Significant proteinuria was found in PE women.

The distribution of maternal genotypes for the two polymorphisms studied here showed no deviation from Hardy–Weinberg equilibrium (all $P>0.05$). We found no significant differences in genotype or in allele distributions for the $C^{-1026}A$ polymorphism when the PE or the GH groups were compared with the HP group (Table 2; all $P>0.05$). However, the genotype and allele frequencies for the G2087A polymorphism were different in PE subjects as compared with HP subjects. Indeed, the GA genotype and the A allele were more commonly found in PE subjects compared with the HP group (Table 2; both $P<0.05$). However, no significant differences were found in genotypes and alleles distributions when the GH group was compared with the HP group (Table 2; all $P>0.05$).

The haplotype analysis showed no significant differences in overall distributions of haplotypes frequencies when the GH or the PE group was compared with the HP group (Table 3; all $P>0.05$).

Discussion

The most important finding of the present study was that the A allele for the G2087A polymorphism increases the susceptibility to the development of PE, but not GH. This is the first study to report a significant association between maternal iNOS genotypes and PE, and the first study to examine whether iNOS polymorphisms are associated with GH. Our findings are relevant because the search for genetic markers associated with HDP may allow early detection of increased susceptibility to disease conditions associated with increased morbility and mortality.[10,26,27]

Our findings implicating the G2087A polymorphism in the susceptibility to PE align with previous studies showing that this polymorphism affects the susceptibility to other disease conditions including diabetes, prostate cancer and non-Hodgkin lymphoma.[21,22,28] Interestingly, although iNOS is mainly regulated at the transcriptional level,[14] this polymorphism in exon 16 results in an amino acid substitution from serine to leucine, which increases iNOS activity and promotes excessive NO formation and inflammation.[21] Therefore, the significant association that we found between the A allele for the G2087A polymorphism and PE can be explained by increased tissue iNOS activity in subjects carrying this allele. This suggestion is supported by experimental evidence indicating that increased iNOS activity contributes to the pathogenesis of hypertension in spontaneously hypertensive rats.[18] The use of an iNOS inhibitor (aminoguanidine) prevented the development of hypertension and this finding was associated with lowered nitro-tyrosine levels, which may reflect blunted NO and lower peroxinitrite formation with this iNOS inhibitor.[18] Further supporting our suggestion, endothelial cells exposed to plasma from pre-eclamptic pregnant increased NOS activity,[29] and therefore it is highly probable that increased NO formation in a context of increased oxidative stress promotes the formation of peroxinitrite and an inflammatory state with vascular damage.[16] Indeed, a cascade of inflammatory mechanisms may be activated as a result of increased iNOS activity leading to increased vascular peroxinitrite levels. For example, matrix metalloproteinases may be activated by peroxynitrite,[30] and these enzymes may have a role in the pathogenesis of PE.[16] These possible alterations may be more relevant in PE patients carrying the A allele for the G2087A polymorphism than in GH patients, because the iNOS polymorphisms studied here were not associated with GH. It remains to be determined whether these alterations are associated with disease severity or whether they may help to explain why PE patients are at increased risk of developing cardiovascular diseases.[31]

The promoter region of the *iNOS* gene is complex. Because iNOS transcription is the most important level of iNOS regulation,[14] genetic polymorphisms that modify the promoter region of this gene are expected to significantly affect iNOS expression and therefore modulate disease susceptibility. In fact, the iNOS promoter region has been widely studied and genetic polymorphisms in this region have been associated with several diseases including hypertension.[32] However, the lack of significant association between the $C^{-1026}A$ polymorphism and PE or GH reported here suggests that this iNOS polymorphism may have no impact on iNOS biology in HDP. To our knowledge, no previous study has examined whether the $C^{-1026}A$ polymorphism is associated with HDP. Our negative findings with respect to this polymorphism are unexpected because this polymorphism was associated with susceptibility to hypertension,[20,32] and the A allele for this polymorphism was associated with approximately fivefold increases in iNOS promoter transcriptional activity compared with the C allele.[20] Our results may suggest that HDP may not critically involve alterations of transcriptional activity associated with this polymorphism in the promoter region of *iNOS* gene, even though this may well be the case of clinical hypertension.[20] However, we have not examined molecular mechanisms in the present study, and this hypothesis remains to be proved.

We found no significant associations between iNOS haplotypes and PE or GH. Although haplotype analysis is valued as a more powerful and interesting approach than the analysis of single polymorphisms, it may be less informative when a causal connection between genetic variations and a phenotype is really driven by a single polymorphism.[33,34] This may well be the case of the present study, because we found significant association between the G2087A polymorphism and PE.

Our study focused on the effects of maternal iNOS genotypes on the susceptibility to PE or GH. It should be clear that fetal genotypes for iNOS polymorphisms may have more important contribution to the risk of developing these diseases. However, this hypothesis remains to be tested.

In conclusion, our results indicate that the G2087A polymorphism affects the susceptibility to PE, but not to GH. Whereas we found lack of significant associations between iNOS polymorphisms and GH, possibly suggesting that different pathophysiological mechanisms are involved in these hypertensive disorders, further studies are required.

What is known about this topic

- Experimental studies showed that increased iNOS (inducible nitric oxide synthase) activity has a role in the pathophysiology of hypertension.
- Although genetic polymorphisms in the *iNOS* gene have been associated with hypertension, no previous study has examined whether functional iNOS polymorphisms affect the susceptibility to gestational hypertension or to pre-eclampsia.

What this study adds

- Whereas the genotype for the G2087A (rs2297518) polymorphism in the *iNOS* gene affects the susceptibility to pre-eclampsia, the $C^{-1026}A$ (rs2779249) polymorphism does not.
- Both iNOS polymorphisms are not associated with gestational hypertension.

Conflict of interest

The authors declare no conflict of interest.

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