



Original

Genotype of the CYBA promoter –930A/G, polymorphism C677T of the MTHFR and APOE genotype in patients with hypertensive disorders of pregnancy: An observational study

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ABSTRACT

Background and objective: Hypertensive disorders of pregnancy could be favoured by polymorphisms in genes affecting vascular physiology. The aim of our work was to study several variants in the genes regulating oxidative stress, plasma lipids metabolism and endothelial function (observational study).

Material and methods: We studied the –930A/G polymorphism of the CYBA gene promoter, the apolipoprotein E (APOE) genotype and the methylene-tetrahydrofolate reductase (MTHFR) gene C677T polymorphism in 134 healthy pregnant women, 266 pregnant with non-proteinuric hypertension (NPH) and 184 patients with preeclampsia (PE).

Results: The GG genotype of the CYBA gene promoter was present in 32.1% of the control population, 38.7% of patients with NPH ($P=0.19$) and 21.2% of the women with PE ($P=0.03$). A higher frequency of $\epsilon 3/\epsilon 4$ and $\epsilon 4/\epsilon 4$ genotypes of APOE was observed in patients with PE or NPH compared with controls ($P<0.01$). There were no significant differences detected in genotype or allele distribution of the MTHFR, C677T polymorphism. APOE $\epsilon 3/\epsilon 4$ and $\epsilon 4/\epsilon 4$ genotypes had a worse lipoprotein profile characterized by higher plasma values of total cholesterol ($P<0.05$) and triglycerides ($P<0.005$). Despite no differences in MTHFR C677T polymorphism distribution, higher levels of plasma homocysteine were observed in patients with PE than in patients with NPH or controls.

Conclusions: CYBA and APOE polymorphism showed a different distribution in the groups studied, while no differences were observed in MTHFR C677T polymorphism. APOE genotype was associated with changes in lipid and lipoprotein profiles in pregnant women.

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Genotipo del promotor del CYBA, polimorfismo C677T del gen de la MTHFR y genotipos de la APOE en diferentes trastornos hipertensivos del embarazo: estudio observacional

RESUMEN

Fundamento y objetivo: Nos propusimos valorar en un estudio observacional si algunos polimorfismos en genes que regulan el estrés oxidativo, los niveles de homocisteína y el metabolismo de los lípidos, podrían predisponer a diferentes trastornos hipertensivos del embarazo.

Material y métodos: Estudiamos el polimorfismo –930A/G del gen promotor del CYBA, el genotipo de la apolipoproteína E (ApoE) y el polimorfismo C677T del gen de la metileno tetrahidrofolato-reductasa (MTHFR) en 134 embarazadas sanas, 266 embarazadas con hipertensión no proteinúrica (HNP) y 184 pacientes con preeclampsia (PE).

Resultados: El genotipo GG del promotor del gen del CYBA estuvo presente en el 32,1% de la población de control, en el 38,7% de las pacientes con HNP ($p=0,19$) y el 21,2% de las mujeres con PE ($p=0,03$). Los pacientes con PE o HNP, en comparación con los controles, mostraron una mayor frecuencia de genotipos $\epsilon 3/\epsilon 4$ y $\epsilon 4/\epsilon 4$ ($p<0,01$). No hubo diferencias significativas en la distribución por genotipos del polimorfismo C677T del gen de la MTHFR. Los genotipos $\epsilon 3/\epsilon 4$ y $\epsilon 4/\epsilon 4$ de la ApoE mostraron un peor perfil lipoproteico caracterizado por un aumento de colesterol total ($p<0,05$) y triglicéridos ($p<0,005$).

Palabras clave:

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A pesar de no haber diferencias en la distribución de la mutación C677T del gen de la MTHFR, se observó un aumento de los niveles de homocisteína plasmática en los pacientes con PE.

Conclusiones: Los polimorfismos del gen del CYBA y de la ApoE mostraron una distribución diferente en los grupos estudiados que no se observó en el polimorfismo C677T del gen MTHFR.

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Introduction

Essential hypertension is an important clinical problem due to difficulties in control, a high prevalence, target organ damage and an associated increase in cardiovascular risk.^{1–3} During pregnancy there are also several hypertensive disorders complicating gestation, ranking from mild and transient hypertension to severe preeclampsia-eclampsia syndrome. These disorders account for approximately 15% of pregnancy-related deaths, represent the second-leading cause of morbidity and mortality during pregnancy and are also a major risk factor for fetal mortality and morbidity.⁴

The cause of hypertensive disorders of pregnancy remains elusive but both an environmental and a genetic origin have been proposed.⁵ In this respect, several authors studied polymorphisms in genes involved in vascular pathologies, often with contradictory results.^{6–8} In general, efforts to identify gene variants associated with a susceptibility to hypertensive disorders of pregnancy have focused on a single polymorphism and most studies have compared normal pregnancy with preeclampsia-eclampsia, ignoring non-proteinuric disorders. The size and heterogeneous ethnic origin of the population under study could also limit the significance of this type of studies.⁹

Factors producing maternal endothelial cell dysfunction are prominent in pregnancy-related hypertensive disease. Both the uncontrolled formation of free radicals¹⁰ and other mechanisms¹¹ have been shown to play a role in preeclampsia. Therefore, genes implicated in the regulation of redox state are good candidates in genetic association studies of the disease.⁹ Also, maternal dyslipidemia could contribute to endothelial damage through the formation of atherosclerotic lesions.¹²

In the present work we study three polymorphisms of candidate genes potentially involved in endothelial damage processes. The polymorphisms studied were the –930A/G polymorphism in the CYBA gene promoter, the apolipoprotein E genotype and the C677T polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene. We found a higher prevalence of the GG variant in the CYBA gene, more subjects carrying the apoE4 allele and more frequency of the TT variant of the MTHFR gene, in patients with any type of hypertensive disorder of pregnancy.

Material and methods

Subjects, clinical and laboratory examinations

This is a sample of consecutive cases recruited between January and December of 2007 at the Women Hospital of University Hospitals Virgen del Rocío in Seville, Spain. All the pregnant women who accepted to participate and signed the informed consent were included. The populations studied were 584 Caucasian women from the South of Spain: 134 had normal healthy pregnancies, 266 pregnant women had non-proteinuric hypertension (NPH) and 184 patients had preeclampsia (PE). They were included in the study at diagnosis and evaluated during periparturition. Patients were also evaluated 4 months after delivery in order to confirm or reclassify the diagnosis according to the

guidelines of the Working Group on High Blood Pressure in Pregnancy.⁴ In the control group, the existence of hypertension or proteinuria in a previous pregnancy was considered criteria for exclusion. Hypertension was established by measuring blood pressure 3 times in a seated position after having relaxed 5 min in supine position. Blood samples were drawn after an overnight fast of 12 h, during the third trimester, and plasma lipids, creatinine, uric acid, sodium, potassium, ALT, urinary excretion of proteins red cell, leucocytes and platelet count were measured using conventional methods. Homocysteine was measured by HPLC with fluorescent detector using the kit of Bio-Rad based on the method of Ueland et al.¹³

Anamnesis, physical examination and several tests including urinary concentrations of catecholamines, plasma aldosterone, plasma renin activity and an abdominal ultrasonography (including Doppler study of the renal artery flow) excluded causes of secondary hypertension in the group of chronic hypertensive patients.

The procedures followed in this study were in accordance with institutional guidelines, all the subjects gave informed consent and our local Ethical Committee approved the study protocol.

Genetic polymorphisms

The –930A/G promoter polymorphism in the CYBA (p22phox) gene, coding for the alpha polypeptide of cytochrome b-245, was determined as previously reported with some modifications.¹⁴ The specific genomic DNA amplification was performed with two specific oligonucleotides; 5' GGA AAC CAC CAA GTG CCT CGG ATG G 3' and 5' TCT GCA CCC TGA TGC TAC CAA GGA C 3'. Ten µL of a 1/10 dilution of blood genomic DNA was amplified in a buffered solution containing 1 mM MgCl₂, 0.4 µM of each primer and 300 U/mL of Taq Polymerase (Roche Diagnostics). The PCR conditions were a denaturation step of 95 °C for 10 min, followed by 35 cycles of amplification (94 °C during 1 min; 57 °C during 30 s; 72 °C during 1 min) and a final amplification for 10 min at 72 °C. The genotypes resulting from the A to G substitution at position –930 were assessed by BbvI endonuclease digestion using 30 µL of PCR product analyzed in a 2% agarose gel. The expected size of the fragments was 504 and 85 bp for the GG genotype, and a 589 bp fragment for A/A genotype. APOE polymorphism was genotyped using the polymerase chain reaction (PCR) by amplification of the polymorphic fragment of the APOE gene and digestion of the PCR product with the restriction enzyme CfoI according to previous studies.¹⁵ Finally, the analysis of the C677T mutation in the MTHFR gene was performed by PCR and *HinfI* digestion, as previously described.¹⁶ All the mentioned polymorphisms were analyzed by L. Bellido and J. Luna, with the supervision of a senior investigator (P. Garcia de Frutos). All of them were blinded of the type of pregnancy (normal, 242089447NPH242089447AQanuradha2420894471725559799AQ: Please check if "NPH o PE" is correct. o PE) that they were analyzing.

Statistical analysis

All data are expressed as the mean ± SD and a *P* value lower than 0.05 was considered statistically significant. For comparison of means, ANOVA or Student's *t* test was used for all normally distributed variables, and the Kruskal–Wallis test for non-normally

distributed variables. Previously, normality of distribution was studied using the Kolmogorow–Smirnov test. Categorical variables were analyzed using the chi-square test and the odds ratio (OR) and confidence intervals (CI) were calculated. The SPSS 15.00 statistical software was used.

Results

The characteristics of the studied population are described in Table 1. As expected, mean age and body mass index (BMI) were higher in patients with NPH, while mean number of pregnancies, mean neonatal weight and mean week at delivery were lower in patients with PE.

In 314 consecutive women of the 584 studied pregnant women we had the biochemical parameters shown in Table 2. Plasma values of total cholesterol, triglycerides, homocysteine and uric acid were higher in the group of PE women compared to control and NPH groups. The higher value of mean ALT, as well as the wide range of values, was indicative of the presence of subjects with

HELLP syndrome in this group. Homocysteine was also higher and non-normally distributed in the PE group.

Table 3 shows the genotype frequencies distribution in this sample which is in agreement with Hardy–Weimberg equilibrium.

As shown in Table 3, the GG genotype of the promoter of the CYBA gene was higher in NPH patients, where it was found in 103 of 266 patients with NPH (38.7%), compared with only 43 of 134 controls (32.1%), although the difference was not significant. In contrast, PE patients had a lower proportion than controls of GG carriers (39 of 184 patients, 21.1%; OR=0.57, CI=0.33–0.97, P=0.03). The allele frequencies were not significantly different between patient groups and controls.

The allele E4 of the apolipoprotein E is related with a worse lipid composition of plasma, insulin resistance and vascular risk. For this reason we compared the carrier of the genotypes ε3/ε4 and ε4/ε4 with the remainder subjects. A higher frequency of the APOE genotypes ε3/ε4 and ε4/ε4 was observed in patients with hypertensive disorders of pregnancy, being 24.4% and 15.0% of PE and NPH patient groups, compared to 13.4% of controls (see Table 3). Logistic analysis indicated that the ε3/ε4 and ε4/ε4 genotypes were significantly associated with PE with an OR=2.09 (95% CI=1.15–3.78, P=0.015).

As mentioned before, plasma values of total cholesterol and triglycerides were available in 314 consecutive pregnant women, 70 of them were carriers of the ε3/ε4 or ε4/ε4 genotypes and 244 carried any other genotype. The ε3/ε4 and ε4/ε4 carriers had higher plasma values of total cholesterol and triglycerides (respectively, in mmol/L: 6.56±0.98 vs. 6.21±1.43, P<0.05 and 2.97±1.35 vs. 2.51±1.06, P<0.005).

Finally, as also shown in Table 3, and in spite of observing increased values of homocysteine in the group of patients with PE, there were no statistically significant differences among groups in the MTHFR gene polymorphism distribution.

Table 1
General characteristics of the studied population

	Controls (n=134)	NPH (n=266)	PE N=184)
Age (years)	28.8±5.3	31.0±4.7	29.7±5.8
BMI (Kg/m ²)	27.5±4.9	31.8±5.8	28.4±5.1
Week of delivery	38.4±4.3	38.2±2.3	35.2±3.8
Number of pregnancy	2.3±0.6	1.7±1.2	1.3±0.9
Neonatal weight (g)	3272±874	3196±2100	2189±831

The results were significant in an ANOVA test with a P<0.001.

Table 2
Biochemical characteristics of the studied subjects (n=314)

	Controls	NPH	PE
Total cholesterol (mg/dL)	222.2±46.8	237.0±49.3	246.5±66.0
Triglycerides (mg/dL)	147.3±65.35	214.1±92.8	261.8±128.6
ALT (UI/L)	11.0±0.5	21.6±16.0	52.0±144.3
Uric acid (mg/dL)	3.7±1.0	4.5±1.4	6.2±1.8
Homocysteine (μM/L)	6.3±1.7	7.6±2.7	11.1±10.6

Table 3
Genetic polymorphisms of several genes involved plasma lipid profile, plasma levels of homocysteine and oxidative stress and in a sample of 584 pregnant women

	Controls (n=134)	NPH n=266)	O.R. (95% C.I.) P	PE (n=184)	O.R. (95% C.I.) P
APOE polymorphism					
ε3/ε4 ε4/ε4	18	40	1.14 (0.63–2.06) 0.764	46	2.09 (1.15–3.78) 0.015
ε2/ε2 ε2/ε3 ε3/ε3	116	226		139	
MTHFR C677T variant					
TT	21	36	0.84 (0.47–1.50) 0.549	27	0.92 (0.50–1.71) 0.874
CT+CC	113	230		157	
CYBA -930A/G variant					
GG	43	103	1.34 (0.86–2.07) 0.226	39	0.57(0.33–0.97) 0.03
AG+AA	91	163		145	

Discussion

This study is the first report of the genotype distribution of the –930A/G polymorphism of the human CYBA gene promoter in pregnancy-related hypertensive disorders. The CYBA protein is a major component of NADPH oxidase, and has been proposed to be linked to the capacity of the endothelium to respond to redox damage. Previous studies have described that this genetic variant in the CYBA gene promoter is associated with hypertension in rats and humans.^{14,17} Furthermore, patients with

essential hypertension with the GG genotype of the –930A/G polymorphism exhibited greater CYBA mRNA and protein levels and NADPH oxidase activity than AA/AG hypertensive patients, although this was not the case in normotensive subjects.¹⁸ A recent large population study has confirmed that the GG genotype confers susceptibility for hypertension in the male population.¹⁹ In our study, the higher proportion of GG carriers in the NPH group than in healthy controls could be in line with these results, although it did not reach significance. More surprising was the lower incidence of GG genotypes in the PE group. This would suggest that the higher NADPH oxidase activity associated with the GG genotype is a protective factor for preeclampsia since this genotype is less prevalent in PE. It is possible that the CYBA function is necessary to maintain the balance between the signaling effects of reactive oxygen species in pregnancy.²⁰ Further studies are needed to confirm the observed association.

Another remarkable finding of this study is that the genotypes $\epsilon 3/\epsilon 4$ and $\epsilon 4/\epsilon 4$ were more prevalent among non-proteinuric hypertension and preeclampsia than in normal pregnancies. An association of the $\epsilon 4$ allele with higher LDL values is well established, although its implication in vascular diseases is not so straightforward.^{21–23} We have also previously described that in essential hypertension, the presence of the $\epsilon 4$ allele is related to insulin resistance, and to a worse plasma lipid profile due to higher levels of LDL-cholesterol and VLDL triglycerides and to lower values of plasma HDL-cholesterol.²⁴ It is possible that the $\epsilon 4$ allele-related predispositions to hypertension, hyperlipidemia and insulin resistance syndrome explains the finding of a higher proportion of carriers of the $\epsilon 4$ allele in pregnancy-related hypertensive disorders than in normal pregnancies. In agreement with this, our results show that subjects with the $\epsilon 4$ allele had a worse plasma lipid profile than carriers of other genotypes. Previous studies have not found an association between APOE genotypes and PE,^{25,26} probably due to a smaller sample size (103 and 49 women with PE). Furthermore, these studies did not have an NPH group.

The C677T polymorphism of the MTHFR gene has been examined in more than 30 studies with widely divergent results.²⁷ The finding of an increased plasma concentration of homocysteine in preeclamptic women and absence of relation with the C677T polymorphism in the MTHFR gene has also already been described.²⁸ This is principally due to the fact that C677T MTHFR polymorphism is rather common in populations, and an example of predominance of environmental vs. genetic interaction.²⁹ However, measurements of plasma values of homocysteine might be of interest since it allows selecting a group of patients where the diet supplementation with folic acid might be considered.³⁰

In conclusion, the CYBA and APOE polymorphism showed a different distribution in the groups studied, while no differences were observed in MTHFR C677T. APOE $\epsilon 3/\epsilon 4$ and $\epsilon 4/\epsilon 4$ genotypes had a worse lipoprotein profile characterized by higher plasma values of total cholesterol and triglycerides. Despite no differences in MTHFR C677T polymorphism distribution, higher levels of plasma homocysteine were observed in patients with PE than in patients with NPH or controls.

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The authors have no conflicts of interest to declare.

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