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ORIGINAL ARTICLE

eNOS haplotype associated with hypertension in obese children and adolescents

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bjective: The aim of our study is to investigate whether genetic polymorphisms in the endothelial nitric oxide synthase (*eNOS*) gene (in the promoter region $T^{-786}C$, in exon 7 (Glu298Asp) and in intron 4 (4b/4a)) or eNOS haplotypes are associated with hypertension in obese children and adolescents.
Methods: We genotyped 175 healthy (controls), 110 normotensive obese and 73 hypertensive obese children and adolescents. Genotypes were determined by Taqman allele discrimination assay and real-time PCR, and by PCR followed by fragment separation by electrophoresis. We compared the distribution of eNOS genotypes, alleles and haplotypes in the three study groups of subjects. We have also measured whole-blood nitrite concentrations.
Results: The 4a4a genotype for the intron 4 polymorphism was more common in normotensive obese and hypertensive obese ($P<0.02$). No significant differences were found in allele distributions for the three eNOS polymorphisms. However, the haplotype combining the C, 4b and Glu variants for the three polymorphisms was more common in hypertensive obese than in normotensive obese or control children and adolescents (odds ratio = 2.28 and 2.79, respectively; 95% confidence interval: 1.31–4.31 and 1.39–5.64, respectively; both $P<0.00625$). This haplotype was not associated with significantly different nitrite concentrations ($P>0.05$).
Conclusions: Our findings suggest that the eNOS haplotype, C b Glu, is associated with hypertension in obese children and adolescents. Further studies examining the possible interactions of eNOS haplotypes with environmental factors and other genetic markers involved in the development of obesity and its complications are warranted.
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Keywords: children; eNOS; nitric oxide; haplotypes; hypertension

.ntroduction

Obesity has reached epidemic proportions worldwide, and is now occurring at younger ages.[1,2] This condition is the most common cause of hypertension in children.[3] In this context, there is strong evidence that nitric oxide (NO) is critically involved in obesity and its clinical consequences, including cardiovascular disease, diabetes, and especially, hypertension.[4–6] Recent studies have shown that obesity reduces NO bioavailability in adolescents, and this alteration has been linked to important cardiovascular diseases.[7,8] Indeed, endothelium-derived NO has a major role in vascular homeostasis, causes vasodilation, prevents platelet and leukocyte adhesion, and inhibits vascular smooth muscle cell migration and proliferation.[9]

Recent studies have shown that polymorphisms in the gene encoding endothelial nitric oxide synthase (eNOS), the most important enzyme that synthesizes NO in the endothelial cells, affect NO formation,[10,11] drug responses,[12–14] and are associated with cardiovascular diseases.[15] Three clinically relevant polymorphisms in this gene have been widely studied: a single-nucleotide polymorphism (SNP) in the promoter region ($T^{-786}C$, rs 2070744), an SNP in exon 7 (G894T, rs 1799983) and the variable number of tandem repeats (VNTRs) in intron 4.[15] Importantly, studies have found associations between polymorphisms or haplotypes of the *eNOS* gene and hypertension in black and white subjects, in patients with type 2 diabetes mellitus and in pregnant women.[16–20] However, very few studies took into consideration the possible interaction of eNOS polymorphisms and

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obesity,[21] and no previous work has studied these polymorphisms in obese children and adolescents with or without hypertension. This interaction may be very relevant because obesity may interact with eNOS polymorphisms and affect NO formation.[22]

In this study, we compared the distribution of variants for the three eNOS polymorphisms mentioned above in healthy children and adolescents with those found in normotensive obese and in hypertensive obese children and adolescents. In addition, we also examined the association of eNOS haplotypes with these conditions. We hypothesized that eNOS haplotypes would be associated with hypertension in obese children and adolescents. To improve the validity of our findings, we have also measured nitrite concentrations in whole-blood samples because nitrite levels reflect endogenous NO formation.[10,12,23]

Methods

Subjects

Approval for use of human subjects in this study was obtained from the Institutional Review Board at the Federal University of Juiz de Fora, Brazil. Parents and children were informed as to the nature and purpose of the study. Parents gave their written consent and children gave their verbal consent.

Study populations consisted of 110 normotensive obese and 73 hypertensive obese subjects recruited from the Endocrinology Ambulatory of the Adolescent and Child Institute at Juiz de Fora and from the Childhood Endocrinology Ambulatory of the IMEPEN Foundation at Juiz de Fora. The control group consisted of 175 healthy children and adolescents recruited from the local community.

All children underwent thorough physical examination. Height was measured to the nearest 0.1 cm by using a wall-mounted stadiometer. Body weight was measured with a digital scale to the nearest 0.1 Kg. Body mass index was calculated as the weight in kilograms divided by height in meters squared. Obesity was defined as body mass index greater than the 95th percentile, matched according to age and sex.[24] Systolic (SBP) and diastolic blood pressures (DBP) were measured at least three times and the presence of hypertension was defined as SBP and/or DBP exceeding the 95th percentile.[25]

At the time of clinic attendance, venous blood samples were collected and genomic DNA was extracted from the cellular component of 1 ml of whole blood by a salting-out method and stored at −20 °C until analyzed.

Measurement of nitrite concentrations in whole-blood samples

To measure whole-blood nitrite concentrations in triplicate, venous blood samples were collected into standard Vacutainer tubes (Becton-Dickinson, Sao Paulo, Brazil) containing heparin and immediately mixed with a nitrite preservation solution in a 5:1 dilution as previously described.[12] Briefly, this solution contains 0.8 M ferricyanide and 1% NP-40. The samples were analyzed for their nitrite content using an ozone-based chemiluminescence assay, as previously described.[26] The samples were deproteinated with methanol (1:1) and centrifuged at 14 000 *g* for 5 min. Then, 200 µl supernatant was were injected into a solution of acidified triiodide, purging with in-line nitrogen with a gas-phase chemiluminescence NO analyzer (Sievers Model 280 NO Analyzer, Sievers, Boulder, CO, USA).[27] Approximately 8 ml of triiodide solution (2.0 g of potassium iodide and 1.3 g of iodine dissolved in 40 ml of water with 140 ml of acetic acid) were placed in the purge vessel into which the samples were injected. The triiodide solution reduced nitrites to NO gas, which was detected by the NO analyzer.

Laboratory analyses

Glucose concentrations and lipid parameters (total cholesterol, triglycerides, high-density lipoprotein cholesterol) were determined in plasma and serum, respectively, with routine enzymatic methods using commercial kits (Labtest Diagnostic, SA, Lagoa Santa, Brazil). Low-density lipoprotein concentration was calculated according to the Friedewald formula.

Genotype determination

Three clinically relevant polymorphisms of *eNOS* gene were studied:[14,19] the T-786C polymorphism in the 5′-flanking region of *eNOS* gene, VNTRs (27-bp repeat) polymorphism in intron 4 and the Glu298Asp polymorphism in exon 7. Genotypes for the T-786C and for the Glu298Asp polymorphisms were determined by Taqman Allele Discrimination assay and real-time PCR on Chromo 4 Detector (Bio-Rad Laboratories, Hercules, CA, USA). Genotypes for the VNTR polymorphism in intron 4, however, were determined by PCR and fragment separation by electrophoresis in 8% polyacrylamide gels as previously described.[28,29]

Statistical analysis

The clinical characteristics of normotensive obese, and hypertensive obese children and adolescents were compared with those of control children and adolescents by one-way analysis of variance followed by the Tukey's multiple comparisons test. The categorical variables were compared between groups by χ^2-tests. The distribution of genotypes for each polymorphism was assessed for deviation from the Hardy–Weinberg equilibrium, and differences in genotype frequency and in allele frequency between groups were assessed using χ^2-tests. A value of $P<0.05$ was considered statistically significant.

Haplotypes were inferred using the Bayesian statistical-based program PHASE version 2.1 (http://www.stat.washington.edu/stephens/software.html) to estimate the haplotype frequencies in each ethnic group.[30] The possible

haplotypes including genetic variants of three polymorphisms in the *eNOS* gene studied (T-786C, intron4 and Glu298Asp) were H1 (T b Glu), H2 (T b Asp), H3 (C b Glu), H4 (C b Asp), H5 (T a Glu), H6 (T a Asp), H7 (C a Glu) and H8 (C a Asp). Differences in haplotype frequency were further tested using a contingency table, and a value of $P<0.00625$ (0.05/number of haplotypes, 8) was considered significant to correct for the number of comparisons made.

Results

The clinical and laboratorial characteristics of the study groups are presented in Table 1. As expected, subjects in the normotensive obese and hypertensive obese groups had higher body mass index than controls ($P<0.05$; Table 1). Hypertensive subjects had higher arterial blood pressure than normotensive obese and controls ($P<0.05$; Table 1). In addition, normotensive and hypertensive obese subjects had higher total cholesterol and low-density lipoprotein cholesterol compared with controls (all $P<0.05$; Table 1).

Tables 2 and 3 show eNOS *e*NOS genotypes and alleles frequencies in the three study groups. The distribution of genotypes for each polymorphism showed no deviation from Hard–Weinberg equilibrium (all $P>0.05$). As significant interethnic differences exist in the distribution of *e*NOS polymorphisms,[28,29] we carried out two different analyses. The first analysis included black and white children and adolescents, whereas the second analysis took into consideration only white children and adolescents, which corresponded to 50–55% of the subjects. As both analyses have produced very similar conclusions, we report here the results corresponding to the first analysis.

We found significant differences in the distribution of *e*NOS genotypes, and the 4a4a genotype for the intron 4 polymorphism was more common in normotensive obese and hypertensive obese than that in the control group ($P<0.01$; Table 2). In addition, the AspAsp genotype for the Glu298Asp polymorphism was more common in normotensive obese than in controls ($P<0.02$; Table 2). No significant differences were found in allele distributions for the three *e*NOS polymorphisms (all $P>0.05$; Table 3).

We estimated eNOS haplotypes frequencies including the three polymorphisms for the three study groups and we found significant differences in the distributions of eNOS haplotypes ($P<000625$; Table 4). Specifically, the haplotype including the C b Glu variants was more frequent in hypertensive obese children and adolescents when compared with normotensive obese and controls (18% vs 7 and 8%, respectively; odds ratio = 2.8 and 2.3, respectively, both $P<0.00625$; Table 4). The haplotype including the C and Asp

Table 1 Demographic characteristics of study participants.

rameters	*Controls*	*Normotensive obese*	*Hypertensive obese*
N	175	110	73
Age (years)	11.9 ± 3.1	10.4 ± .2.6*	12.2 ± 3.3
Ethnicity (% white)	51	53	55
BMI (kg m^{-2})	18.3 ± 2.9	26.0 ± 4.4*	28.1 ± 6.6*
SBP (mm Hg)	105.7 ± 11.5	111.3 ± 9.9*	127.5 ± 17.5*,**
DPB (mm Hg)	65.7 ± 9.0	70.8 ± 8.7*	77.4 ± 11.0*,**
Fasting glucose (mg per 100 ml)	82.9 ± 11.4	85.7 ± 9.6	84.0 ± 11.1
Total cholesterol (mg per 100 ml)	142.7 ± 41.4	147.6 ± 40.1	146.8 ± 39.4
LDL cholesterol (mg per 100 ml)	74.6 ± 23.0	89.3 ± 31.5*	93.8 ± 31.5*
HDL cholesterol (mg per 100 ml)	44.1 ± 9.8	39.1 ± 10.1*	37.8 ± 12.1*
Triglycerides (mg per 100 ml)	77.0 ± 28.7	88.2 ± 43.3*	91.6 ± 44.7*
Nitrite (nM)			

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure. *$P<0.05$ vs controls. **$P<0.05$ vs normotensive obese group. Values are the mean ± s.d. or the median (interquartile range).

Table 2 Genotype frequencies of the endothelial nitric oxide synthase polymorphism in controls, normotensive obese and in hypertensive obese children and adolescents

	Genotype	*Controls, % (N)*	*Normotensive obese, % (N)*	P-*value*	OR (95% CI)	*Hypertensive obese, % (N)*	P-*value*[a]	OR[a] (95% CI)	P-*value*[b]	OR[b] (95% CI)
$T^{-786}C$	TT	0.514 (90)	0.473 (52)	—	1.00 (reference)	0.438 (32)	—	1.00 (reference)	—	1.00 (reference)
	TC	0.411 (72)	0.418 (46)	NS	1.11 (0.67–1.83)	0.425 (31)	NS	1.21 (0.68–2.17)	NS	1.10 (0.58–2.06)
	CC	0.074 (13)	0.109 (12)	NS	1.60 (0.68–3.76)	0.137 (10)	NS	2.16 (0.86–5.42)	NS	1.35 (0.52–3.49)
Intron 4	4b, 4b	0.600 (105)	0.545 (60)	—	1.00 (reference)	0.671 (49)	—	1.00 (reference)	—	1.00 (reference)
	4a, 4b	0.383 (67)	0.364 (40)	NS	1.05 (0.63–1.73)	0.247 (18)	NS	0.58 (0.30–1.07)	NS	0.55 (0.28–1.08)
	4a, 4a	0.011 (2)	0.082 (9)	0.01	7.88 (1.65–37.67)	0.082 (6)	0.01	6.43 (1.25–33.02)	NS	0.82 (0.27–2.45)
	4a, 4c	0.006 (1)	0.009 (1)	NS	1.75 (0.11–28.51)	0.000 (0)	NS	0.71 (0.03–17.77)	NS	0.41 (0.02–10.23)
Glu298Asp	Glu, Glu	0.589 (103)	0.627 (69)	—	1.00 (reference)	0.616 (45)	—	1.00 (reference)	—	1.00 (reference)
	Glu, Asp	0.366 (64)	0.373 (41)	NS	0.96 (0.58–1.57)	0.342 (25)	NS	0.89 (0.50–1.60)	NS	0.94 (0.50–1.74)
	Asp, Asp	0.046 (8)	0.000 (0)	0.02	0.09 (0.01–1.54)	0.041 (3)	NS	0.86 (0.22–3.39)	NS	10.69 (0.54–212.1)

Abbreviations: CI, confidence interval; NS, not significant; OR, odds ratio. [a]Hypertensive obese compared with controls. [b]Hypertensive obese compared with normotensive obese.

Table 3 Allele frequencies of the endothelial nitric oxide synthase polymorphisms in controls, normotensive obese and in hypertensive obese children and adolescents

	Alleles	*Controls, % (N)*	*Normotensive obese, % (N)*	*P-value*	*OR (95% CI)*	*Hypertensive obese, % (N)*	*P-value*[a]	*OR*[a] *(95% CI)*	*P-value*[b]	*OR*[b] *(95% CI)*
$T^{-786}C$	T	0.720 (252)	0.682 (150)	—	1.0 (reference)	0.651 (95)	—	1.00 (reference)	—	1.00 (reference)
	C	0.280 (98)	0.318 (70)	NS	1.20 (0.83–1.73)	0.349 (51)	NS	1.38 (0.91–2.09)	NS	1.15 (0.74–1.79)
Intron 4	4b	0.791 (277)	0.727 (160)	—	1.00 (reference)	0.795 (116)	—	1.00 (reference)	—	1.00 (reference)
	4a	0.206 (72)	0.268 (59)	NS	1.42 (0.96–2.11)	0.205 (30)	NS	1.00 (0.62–1.61)	NS	0.70 (0.43–1.16)
	4c	0.003 (1)	0.005 (1)	NS	1.73 (0.11–27.89)	0.000 (0)	NS	0.79 (0.03–19.65)	NS	0.46 (0.02–11.38)
Glu298Asp	Glu	0.771 (270)	0.814 (179)	—	1.00 (reference)	0.788 (115)	—	1.00 (reference)	—	1.00 (reference)
	Asp	0.229 (80)	0.186 (41)	NS	0.77 (0.51–1.78)	0.212 (31)	NS	0.91 (0.57–1.46)	NS	1.18 (0.70–1.98)

Abbreviations: CI, confidence interval; NS, not significant; OR, odds ratio. [a]Hypertensive obese compared with controls. [b]Hypertensive obese compared with normotensive obese.

Table 4 Estimated haplotypes frequencies in controls, normotensive obese and in hypertensive obese children and adolescents

	Haplotype	*Controls (N = 350)*	*Normotensive obese (N = 220)*	*P-value*	*OR (95% CI)*	*Hypertensive obese (N = 146)*	*P-value*[a]	*OR*[a] *(95% CI)*	*P-value*[b]	*OR*[b] *(95% CI)*
H1	T b Glu	0.49	0.47	—	1.00 (reference)	0.46	—	1.00 (reference)	—	1.00 (reference)
H2	T b Asp	0.09	0.04	NS	0.41* (0.18–0.93)	0.09	NS	1.04 (0.51–2.10)	NS	2.52 (0.99–6.41)
H3	T a Glu	0.13	0.17	NS	1.33 (0.81–2.18)	0.09	NS	0.65 (0.33–1.31)	NS	0.49 (0.24–1.01)
H4	T a Asp	0.01	—	NS	0.33 (0.02–6.91)	0.02	NS	3.83 (0.62–23.44)	NS	10.84 (0.55–213-3)
H5	C b Glu	0.08	0.07	NS	0.85 (0.43–1.66)	0.18	0.001*	2.28* (1.31–4.31)	0.0005*	2.79* (1.39–5.64)
H6	C b Asp	0.13	0.15	NS	1.18 (0.71–1.96)	0.07	NS	0.55 (0.26–1.16)	NS	0.47 (0.22–1.02)
H7	C a Glu	0.07	0.10	NS	1.57 (0.83–2.97)	0.06	NS	1.00 (0.44–2.27)	NS	0.64 (0.28–1.46)
H8	C a Asp	—	—	—	—	0.03	0.004*	12.76* (1.46–111.3)	NS	17.03 (0.92–313.2)

Abbreviations: CI, confidence interval; *N*, number of haplotypes; NS, not significant; OR, odds ratio. [a]Hypertensive obese compared with controls. [b]Hypertensive obese compared with normotensive obese . *$P < 0.00625$ (or 0.05/8).

alleles was found only in hypertensive obese subjects, but not in the other study groups ($P < 0.00625$; Table 4).

Although we found the *e*NOS haplotype C b Glu associated with hypertension in obese children and adolescents, we found no significant differences nitrite concentrations when the different haplotype groups were compared (Figure 1).

Discussion

This was the first study to compare the distribution of *eNOS* haplotypes in normotensive obese and in hypertensive obese children with those found in healthy controls. We found that the C b Glu haplotype is more frequent in hypertensive obese children when compared with normotensive obese and healthy controls. This finding suggests that this *eNOS* haplotype may increase the susceptibility to hypertension in obese children and adolescents.

NO is a major player in the regulation of the cardiovascular system, and reduced NO bioavailability has been linked to hypertensive disorders.[8] Our genotype findings showed for the first time that the 4a4a genotype for the intron 4 eNOS polymorphism is more common among obese children (normotensive or hypertensive). It remains to be determined whether this particular genotype is involved in the reduction of NO production that has been described in obese children.[7] In addition, although we found that the AspAsp genotype for Glu298Asp polymorphism is more common in normotensive obese than in control children and adolescents, the examination of combinations of polymorphisms (haplotypes) rather than only one polymorphism provides much improved information.[31]

There is clear evidence of a genetic contribution to the variability in NO formation.[10,18] Interestingly, previous studies reported an association between the C b Glu haplotype and reduced circulating concentrations of NO products.[10,11] Consistent with these findings, our results suggest that this particular eNOS haplotype (C b Glu) is associated with increased susceptibility to hypertension in obese children and adolescents. Altogether, these findings suggest that the susceptibility to hypertension in obese children and adolescents could be explained, at least in part, by reduced NO formation, which has been shown in carriers of the C b Glu haplotype. In this study, we tried to demonstrate functional implications of the eNOS polymorphisms studied here. However, our results showed no effects of eNOS polymorphisms or haplotypes on nitrite

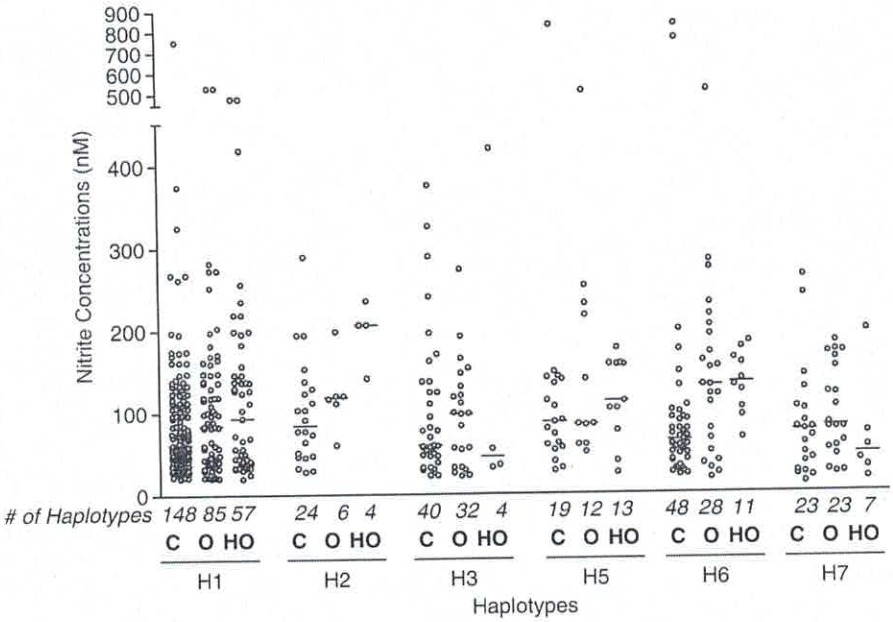

Figure 1 Whole-blood nitrite concentrations in the different eNOS haplotype groups. The H4 and H8 haplotype groups are not shown because very few or no subjects had this haplotype. The bar indicates the median. No significant differences were found.

concentrations. Although nitrite concentrations reflect endogenous NO formation,[10,12,23] it is possible that other factors in children may interact with eNOS polymorphisms and neutralize the effects that have been shown in adults.

It is clear that obesity and hypertension in children and adolescents are not entirely explained by genetic factors. Indeed, other risk factors may modify the effects of *eNOS* polymorphisms on the risk of developing cardiovascular diseases.[32,33] Moreover, hypertension and obesity are complex diseases that surely involve many genes in addition to the *eNOS* gene.[34] Therefore, it is probable that studies examining the association between eNOS haplotypes and different cardiovascular conditions may reflect different interactions between these polymorphisms and different environmental factors.

Previous studies showed molecular mechanisms that may help to explain clinical findings. For example, a recent study showed that the transcription efficiency of the *eNOS* gene is apparently haplotype dependent.[35] The VNTR in intron 4 (27 bp repeats) regulates eNOS expression through small interference RNA (sirRNA), and endothelial cells from carriers of the allele with five repeats express higher quantities of sirRNA, thus leading to lower eNOS expression when compared with those carrying the allele with four repeats.[36] Finally, the single-nucleotide polymorphism in exon 7 (G894T, rs 1799983), which corresponds to a guanine to thymine conversion in the position the 894 of the *eNOS* gene, leads to a glutamine to aspartate substitution in the 298 position of the protein, and probably affects the amounts of eNOS located within the caveolae, thus affecting eNOS activity and NO production.[37] Further studies are necessary to explain how the particular combination of eNOS variants C b Glu may predispose to hypertension in obese children and adolescents.

In conclusion, our findings suggest that the *eNOS* haplotype C b Glu is associated with hypertension in obese children and adolescents. Further studies examining the possible interactions of eNOS haplotypes with environmental factors and other genetic markers involved in the development of obesity and its complications are warranted.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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