Hypertension Research (2010) 33, 814–818

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ORIGINAL ARTICLE

The effect of p22^{phox} −930A/G, A640G and C242T polymorphisms of NADPH oxidase on peripheral and central pressures in healthy, normotensive individuals

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The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase produces superoxide, thus regulating redox state in the vessel wall. Three single-nucleotide polymorphisms (SNPs; −930A/G, A640G and C242T) of the p22^{phox} subunit have been associated with hypertension; however, their role in peripheral and central pressures in normotensive individuals has not been addressed. A total of 210 healthy, normotensive individuals were studied. Genotypes for the −930A/G, A640G and C242T polymorphisms were determined by polymerase chain reaction. Peripheral pressures were measured by mercury sphygmomanometer and aortic pressures by a validated device using applanation tonometry. Both peripheral and central pressures differed across −930A/G genotypes. G allele carriers showed higher levels of peripheral systolic blood pressure (PSBP; AA: 113 ± 12, GG/AG: 119 ± 12 mm Hg; $P<0.01$) and peripheral diastolic blood pressure (AA: 70 ± 9, GG/AG: 73 ± 10 mm Hg; $P<0.05$). Regarding central pressures, AA homozygotes had lower central systolic blood pressure (CSBP; AA: 103 ± 12, GG/AG: 108 ± 12 mm Hg; $P<0.01$) and central diastolic blood pressure (AA: 71 ± 9, GG/AG: 74 ± 10 mm Hg; $P<0.05$). In multiple linear regression analysis, presence of the G allele (AG or GG) independently predicted CSBP. Blood pressure levels did not differ across A640G and C242T genotypes. The −930A/G polymorphism of p22^{phox} is a determinant of peripheral and central pressures in normotensive individuals. The G allele is associated with higher blood pressure in the brachial artery, as well as in the aorta. These findings further elucidate the role of this polymorphism in the regulation of blood pressure. In contrast, the A640G and C242T SNPs do not influence peripheral and central pressures in normotensives.
Hypertension Research (2010) **33,** 814–818; doi:10.1038/hr.2010.78; published online 27 May 2010

Keywords: aorta; blood pressure; NADPH oxidase; polymorphism; reactive oxygen species

INTRODUCTION

Excess production of reactive oxygen species (ROS) in the vascular milieu has been linked to the development and progression of hypertension.[1] The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is a membrane-bound enzyme responsible for the production of ROS. A number of studies have established its role as a major source of vascular superoxide anion ($^{\bullet}O2^{-}$) in the hypertensive setting.[2,3]

The role of this enzyme has been the subject of extensive genetic research, and a number of single-nucleotide polymorphisms (SNPs) of the genes that encode its subunits have been reported to confer increased propensity for elevated levels of blood pressure.[4,5] The *CYBA* gene encodes the essential p22^{phox} subunit of NADPH oxidase; substitution of adenosine (A) for guanosine (G) in position −930 or 640, as well as cytidine (C) for thymidine (T) in position 242 have been reported to be more frequent among hypertensives, suggesting a possible role for these SNPs in the development of essential hypertension.[3,6]

However, a putative contribution of these polymorphisms to the regulation of blood pressure in a non-hypertensive setting has not yet been addressed. Furthermore, correlations with central blood pressures have not been investigated. Central aortic pressures are physiologically more relevant than peripheral pressures, accurately reflecting the afterload of the left ventricle. Furthermore, they predict cardiovascular risk independently of brachial pressures.[7] Anti-hypertensive medications change peripheral and central pressures in a disparate manner and this may explain superiority of specific classes of agents in outcome trials.[8,9] To this end, we sought to investigate a possible contribution of the −930A/G, A640G and C242T SNPs of the p22^{phox} subunit of NADPH oxidase to arterial blood pressure levels in a population of healthy, normotensive individuals. The effect on both peripheral (brachial) and central

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Received 21 October 2009; revised 10 March 2010; accepted 28 March 2010; published online 27 May 2010

(aortic) pressures was studied in addition to the effect on arterial wave reflections.

METHODS

Study population

The study population consisted of 210 Caucasian subjects of Greek origin, who were randomly selected from the employees of two large industries producing food and cosmetics. They were from various socioeconomic categories, performing office and manual work.

Participants with coronary artery disease, cerebrovascular disease, peripheral artery disease, systemic inflammatory disease and on medication (anti-inflammatory agents, including aspirin and corticosteroids, anticoagulants) were excluded from the study. Exclusion criteria also included hypertension (values of blood pressure, as measured on two different occasions, $>140/90$ mm Hg, or use of antihypertensive drugs), diabetes mellitus (fasting glucose >126 mg per 100 ml, or use of antidiabetic agents) and dyslipidemia (history or use of lipid-lowering agents).

Study design

The study was conducted in the morning in a quiet, temperature-controlled room at 23 °C. Subjects underwent a standardized medical history and medical examination, anthropometric measurements, laboratory tests and a 12-lead electrocardiogram and examination after abstaining from caffeine, ethanol and flavonoid-containing beverages for at least 12 h before the study. Blood pressure was measured twice on the right arm with a mercury sphygmomanometer and the average of two blood pressure values spaced by 1 min was used in the analyses. The first and fifth phase of the Korotkoff sounds were used to identify systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively. Pulse pressure was calculated as SBP minus DBP. After a 20-min rest period in the supine position, baseline measurements of arterial function indices were taken, followed by blood sampling for full blood count, glucose, creatinine and lipoprotein profile. Levels of oxidized low-density lipoprotein (oxLDL) were measured in a subgroup of our population ($n=105$) using a competitive enzyme-linked immunoassay (Mercodia AB, Uppsala, Sweden). Weight and height were measured and body mass index was calculated. Estimated glomerular filtration rate was calculated using the Cockroft–Gault equation. All participants gave their informed consent to participate in the study, which was approved by the local ethics committee.

Measurement of aortic pressures and wave reflection indices

Central pressures were calculated using a validated, commercially available system (SphygmoCor; AtCor Medical, Sydney, Australia), which uses the principle of applanation tonometry and appropriate acquisition and analysis software for non-invasive recording and analysis of the arterial pulse, as previously described.[7,10,11] The radial pulse wave was transformed into the central pulse wave of the aorta through pulse wave analysis and use of a validated transfer function. Mean blood pressure was calculated as the integral of the aortic pressure waveform.

The augmentation index (AIx) of the central (aortic) pressure waveform was measured as a composite measure of the magnitude of wave reflections and arterial stiffness, which affects the timing of wave reflections.[12] Augmented pressure is the pressure added to the incident wave by the returning reflected wave, and represents the increased afterload that the left ventricle must cope with. AIx is defined as augmented pressure divided by central pulse pressure and is expressed as a percentage.[7,10,11] Larger values of AIx indicate increased wave reflections from the periphery and/or earlier return of the reflected wave as a result of increased pulse wave velocity (because of increased arterial stiffness), and vice versa. AIx was averaged from 10 to 12 successive waves and was corrected for a steady heart rate of 75 beats min^{-1}.[13]

Genotyping

Peripheral blood was drawn, collected in EDTA and stored at −20 °C. DNA was extracted using a commercially available kit (QIamp DNA Blood Midi Kit; Qiagen, Valencia, CA, USA). The −930A/G, A640G and C242T *CYBA* gene polymorphisms were analyzed in all subjects using restriction fragment length polymorphism as previously described.[6,14] Genotypes were determined according to the pattern of the bands on electrophoresis by two independent investigators.

Statistical analysis

The Hardy–Weinberg equilibrium was tested using χ^2-test. Statistical normality of the data was checked using the Kolmogorov–Smirnov test. The distributions of high-density lipoprotein, triglyceride and oxLDL values were skewed; they were $\log_{10}$ transformed and became normally distributed. Continuous variables are presented as mean ± s.d. Categorical variables are reported as frequencies and group percentages. A test for linearity was used to evaluate the trend of the investigated variables. Differences across study groups were examined using Student's *t*-test or one-way between-group analysis of variance followed by Bonferonni correction for continuous variables; the χ^2-test for independence was used for comparison of categorical variables. Seven multiple linear regression analyses with stepwise selection were conducted to determine the variables independently associated with peripheral, central and mean blood pressures. Peripheral systolic and diastolic blood pressure, peripheral pulse pressure, central systolic and diastolic blood pressure, central pulse pressure and mean blood pressure were the dependent variables of each model, respectively. Independent variables of the models included presence of the G allele (AG or GG genotypes) for the −930A/G SNP, age, sex, smoking, body mass index, total cholesterol and estimated glomerular filtration rate. All statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL, USA). A two-tailed *P*-value of <0.05 was regarded as significant.

Sample size calculation was based on the hypothesis that a difference of 10 mm Hg in PSBP would be detected across genotypes. Previous studies of our group have shown an s.d. for PSBP of 13 mm Hg. On the basis of these assumptions, 32 subjects per genotype group (96 in total) were required to provide the study with 80% power at the 5% level of significance. We recruited additional subjects ($n=210$) for confidence.

RESULTS

The characteristics of the study population are shown in Table 1. Distribution of alleles was consistent with the Hardy–Weinberg equilibrium for all SNPs ($\chi^2=2.74$, $P=0.10$; $\chi^2=0.39$, $P=0.53$; and $\chi^2=0.06$, $P=0.80$ for −930A/G, A640G and C242T, respectively).

For −930A/G, the G allele frequency was 0.50 and the prevalence of AA, AG and GG genotypes was 27.6, 44.3 and 28.1%, respectively. Both PSBP and CSBP differed between the three genotypes, with AA subjects having lower pressures than GG and AG subjects. Presence of the G allele (GG/AG genotypes) resulted in higher peripheral (PSBP, peripheral diastolic blood pressure and peripheral pulse pressure) and central pressures (CSBP, central diastolic blood pressure and central pulse pressure) compared with the AA genotype. Mean blood pressure was similarly lower in AA homozygotes (Figure 1). No differences were detected regarding AIx; oxLDL levels did not differ either. In multivariate linear regression analyses, presence of the G allele of the −930A/G polymorphism was an independent predictor of CSBP levels (Table 2).

For the A640G polymorphism, the G allele frequency was 0.47 and the prevalence of AA, AG and GG genotypes was 29.5, 47.6 and 22.9%, respectively. For the C242T polymorphism, the T allele frequency was 0.42 and the prevalence of CC, CT and TT genotypes was 33.3, 49.5 and 17.2%, respectively. Neither peripheral nor central blood pressure levels differ across genotypes of the two SNPs. Similarly, oxLDL levels were not different between groups.

DISCUSSION

This is the first study, to the best of our knowledge, to explore the effect of the −930A/G, A640G and C242T polymorphisms of the *CYBA* gene on a healthy, normotensive population. In addition, the effect of these genetic loci on central pressures had not been

Table 1 Characteristics of the study group stratified by genotypes of the −930A/G, A640G and C242T polymorphisms

		−930A/G				*A640G*				*C242T*			
	Total population (n=210), 100%	*AA genotype (n=58), 27.6%*	*AG genotype (n=93), 44.3%*	*GG genotype (n=59), 28.1%*	*P-value**	*AA genotype (n=62), 29.5%*	*AG genotype (n=100), 47.6%*	*GG genotype (n=48), 22.9%*	*P-value**	*CC genotype (n=70), 33.3%*	*CT genotype (n=104), 49.5%*	*TT genotype (n=36), 17.2%*	*P-value**
Males/females (%)	139/71 (66.2%/33.8%)	33/25 (57%/43%)	66/27 (71%/29%)	40/19 (67.8%/32.2%)	NS	39/23 (62.9%/37.1%)	67/33 (67%/33%)	33/15 (68.8%/31.2%)	NS	46/24 (65.7%/34.3%)	67/37 (64.4%/35.6%)	26/10 (72.2%/27.8%)	NS
Age, years	41±8	40±9	41±9	42±8	NS	41±8	41±8	41±9	NS	43±9	40±8	40±9	NS
Smokers (%)	111 (52.9%)	33 (56.9%)	50 (53.8%)	28 (47.5%)	NS	32 (51.6%)	56 (56%)	23 (47.9%)	NS	37 (52.9%)	53 (51%)	21 (58.3%)	NS
Pack-years	22.2±17.2	26.9±20.8	18.4±14.4	23.6±16.6	NS	23.2±17.4	22.4±18.7	20.2±12.7	NS	23.2±17.4	22.4±18.7	20.2±12.7	NS
Peripheral systolic blood pressure, mm Hg	117±12	113±12	118±13	119±11	<0.05	118±11	116±13	118±14	NS	117±13	117±12	117±12	NS
Peripheral diastolic blood pressure, mm Hg	72±10	70±9	73±10	74±9	NS	73±9	72±10	73±11	NS	73±11	72±8	72±10	NS
Peripheral pulse pressure, mm Hg	45±8	43±8	46±7	45±7	NS	45±6	44±8	45±8	NS	45±7	44±9	45±7	NS
Central systolic blood pressure, mm Hg	106±12	103±12	107±13	108±11	<0.05	107±11	106±12	107±14	NS	107±13	106±12	105±13	NS
Central diastolic blood pressure, mm Hg	73±10	71±9	74±10	75±9	NS	73±9	73±10	74±11	NS	74±11	73±8	73±10	NS
Central pulse pressure, mm Hg	33±7	31±7	34±8	33±6	NS	34±7	33±7	33±7	NS	33±7	33±8	32±5	NS
Mean blood pressure, mm Hg	88±10	86±10	90±11	90±10	<0.05	89±9	88±10	89±12	NS	89±12	88±9	88±11	NS
Heart rate, beats per min	66±9	67±7	66±9	66±9	NS	66±8	66±9	67±8	NS	67±9	66±9	66±8	NS
Augmentation index, %	21±13	21±13	20±12	20±14	NS	21±13	20±13	21±14	NS	22±13	21±13	18±12	NS
Total cholesterol, mg per 100 ml	194±42	195±46	195±37	192±45	NS	193±40	191±42	203±44	NS	194±46	192±39	199±42	NS
LDL, mg per 100 ml	132±38	132±43	133±33	129±41	NS	129±35	129±39	141±41	NS	132±42	129±35	137±40	NS
Oxidized LDL, U l^{-1}[a]	72.7±32.7	79.5±43.7	70.2±26.5	71.1±30.8	NS	82.1±33.2	69.4±34.6	67.6±25.4	NS	66.8±25.8	77.7±40.5	73.5±25.8	NS
HDL, mg per 100 ml	47±11	47±10	47±11	46±12	NS	47±13	45±10	50±12	<0.05	46±12	48±11	47±10	NS
Triglycerides, mg per 100 ml	92±52	92±56	92±54	92±44	NS	89±46	96±61	88±37	NS	94±58	94±53	82±33	NS
Fasting glucose, mg per 100 ml	89±12	87±12	89±13	90±10	NS	88±10	89±14	88±9	NS	88±13	90±12	86±10	NS
Creatinine, mg per 100 ml	1.0±0.2	1.0±0.1	1.0±0.2	1.0±0.1	NS	1.0±0.2	1.0±0.1	1.0±0.1	NS	1.0±0.2	1.0±0.1	1.0±0.1	NS
eGFR, ml min^{-1} per 1.73 m^2 of body surface area	106±25	108±29	103±23	107±23	NS	106±22	107±27	103±22	NS	102±30	107±24	108±15	NS
Height, m	1.72±0.09	1.71±0.09	1.73±0.09	1.73±0.10	NS	1.72±0.08	1.73±0.09	1.71±0.10	NS	1.71±0.09	1.73±0.09	1.74±0.08	NS
Weight, kg	77±14	75±16	78±14	79±13	NS	77±13	77±14	78±15	NS	76±12	77±15	79±15	NS
BMI, kg m^{-2}	26.0±4.0	25.4±3.9	25.9±3.6	26.4±3.1	NS	26.0±3.5	25.6±3.4	26.4±4.1	NS	25.9±3.2	25.8±3.6	26.1±4.0	NS

Abbreviations: BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant.
[a]Oxidized LDL was measured in a subset (n=105) of the original population.
**P*-value refers to ANOVA between the three genotypes.

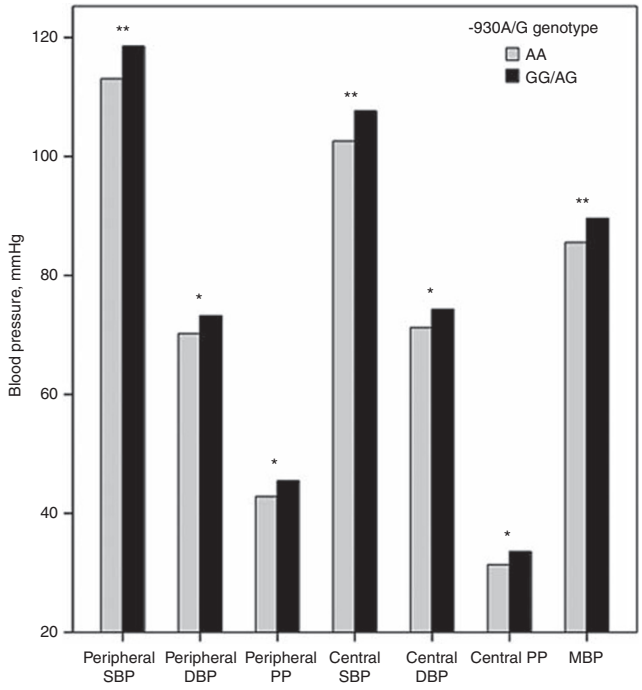

Figure 1 Blood pressure levels for −930A/G genotypes. $*P<0.05$; $**P<0.01$. SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MBP, mean blood pressure.

Table 2 Multivariate linear regression analysis for central systolic blood pressure

	Unstandardized coefficient β (s.e.)	*Standardized coefficient β*	*95% Confidence interval*	P-*value*
Presence of the G allele	3.371 (1.610)	0.123	0.198 to 6.545	0.037
Sex (female)	−5.924 (1.521)	−0.229	−8.923 to −2.925	<0.001
Age, years	0.712 (0.086)	0.484	0.542 to 0.881	<0.001

Adjusted R^2=0.324. Age, sex, smoking status, eGFR, $\log_{10}$ BMI, cholesterol and presence of the G allele (GG/AG) were forced as independent variables into the model; stepwise regression was used.

addressed hitherto in any population, neither hypertensive nor normotensive. We have shown that carriage of the G allele of the −930A/G SNP is linked with higher, yet within the normal range, levels of both peripheral as well as central pressures.

NADPH activation may participate in the regulation of blood pressure through different mechanisms mediated by ROS. Indeed, upregulation of superoxide ($^{\bullet}O2^{-}$) production leads to inactivation of nitric oxide. Moreover, peroxynitrite ($ONOO^{-}$) and hydrogen peroxide (H_2O_2) production pave the way for media hypertrophy. NADPH oxidase, the key enzyme responsible for ROS production in arteries, consists of six subunits.[15–17] The $p22^{phox}$ subunit is essential for the formation of a functional NADPH oxidase complex in all tissues. The *CYBA* gene, which encodes this subunit, has been shown to have several genetic polymorphic loci. Genetic variation has been linked to presence of hypertension,[3] cerebrovascular disease[18] and atherosclerosis.[19] SNPs at positions −930, 640 and 242 of the gene modulate superoxide production and are linked to presence of hypertension as well as increased coronary artery disease risk among hypertensives.[3,4,20] The −930A/G SNP is located at the promoter region of the gene, and regulates transcriptional activation. Hypertensive GG homozygotes show enhanced transcription of the *CYBA* gene, increased production of the $p22^{phox}$ subunit, upregulation of NADPH oxidase activity and, ultimately, increased ROS production.[21] The A640G, located at the 3′-untranslated region of the gene, influences $p22^{phox}$ synthesis,[22] whereas C242T modifies the binding of heme due to a replacement of histidine by tyrosine at amino acid position 72.[19]

Our findings regarding central pressures are particularly interesting. Aortic pressures are pathophysiologically more relevant than peripheral pressures, inasmuch as they are the pressures that the left ventricle must cope against.[7] Prospective studies have established their role as markers and predictors of disease independently of brachial pressures. Moreover, corroborative data point to the differential effects of antihypertensive drugs at the aortic level despite similar effects at the periphery, and this may explain superiority in outcome of certain class of agents.[8,9,16] Given the fact that the blood pressure-lowering agents that improve central hemodynamics also reduce oxidative load, we incorporated central blood pressures and wave reflection measurements in the design of our study. Indeed, the −930A/G SNP influenced aortic blood pressure levels, and GG/AG genotype independently predicted CSBP levels after adjusting for multiple confounders.

Interestingly, the role of the −930A/G polymorphism seems to extend beyond the regulation of blood pressure. It has been recently linked to propensity for insulin resistance,[23] as well as increased carotid intima-media thickness in young smokers.[24] Moreover, advanced age increases expression of $p22^{phox}$, with a concomitant increase in superoxide anion production from the endothelium and the adventitia in a rodent model.[25] Taken together, these results raise the possibility that carriers of the G allele may develop a greater risk of cardiovascular mishap on account of exposure to multiple vascular injurious stimuli.

Scant experimental data exist with regard to the role of the $p22^{phox}$ polymorphisms in normotensives. San José *et al.*[21] have addressed the role of −930A/G in blood pressures both within and above the normal range. Although in hypertensive patients GG homozygotes produced excess superoxide, as a consequence of $p22^{phox}$ upregulation, protein levels and NADPH activity did not differ across the three genotypes in the normotensive group. This is in keeping with our finding that oxLDL levels did not differ among genotypes of the −930A/G, as well as for A640G and C242T. These findings when interpreted together with our results underscore the need for further research, incorporating $^{\bullet}O2^{-}$ measurements, in the subject.

Regarding dominance, the exact significance of G and A alleles for the −930A/G SNP, however, await clarification, because of the fact that non-uniform results are reported in various populations with different risk factors. In our study, presence of the G allele (GG/AG genotypes) conferred higher blood pressures among normotensives. In the setting of hypertension, only GG genotypes showed higher NADPH activation,[21] whereas in young non-smokers it was the AA genotype that was associated with increased carotid intima-media thickness.[24]

The advent of NADPH oxidase inhibitors breaks new ground pertaining to potential clinical applications. Apocynin[26], gp91ds-tat[27] and protein kinase C inhibitors[28] have been shown to decrease blood pressure; nevertheless, they have not been implemented in clinical practice.[15] Dihydropyridines[29] and blockade of the renin-angiotensin-aldosterone axis by means of angiotensin-converting inhibitors[30] or angiotensin receptor blockers[31] have been shown to effectively inhibit NADPH activation and concomitant production of ROS. The reduction of superoxide production through NADPH inhibition is ascribed as an additional pleiotropic effect of statins.[32]

A limitation of our study was that levels of superoxide anion, as an index of NADPH activity, were not measured; such analyses would require electron spin resonance spectrometry or a chemiluminescence assay. Similarly, mRNA or protein levels of $p22^{phox}$ were not determined. It should be emphasized that the population of our study consisted solely of Caucasians, and thus our results may not be directly extrapolated to different groups. Further research in large and diverse populations of various ethnic backgrounds and risk factor profiles is warranted to explore the effect of the *CYBA* gene SNPs and haplotypes on blood pressure levels.

In conclusion, we have shown that the −930A/G polymorphism at the promoter region of the *CYBA* gene that encodes the $p22^{phox}$ subunit of NADPH oxidase is independently correlated with brachial and aortic blood pressure levels in a normotensive population. Presence of the G allele confers higher blood pressure levels when compared with AA homozygotes. In contrast, the A640G and C242T SNPs do not influence peripheral and central blood pressures in normotensive individuals. These findings lend further support to the role of $p22^{phox}$ SNPs in blood pressure regulation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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