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GENETIC VARIATION IN THE α_{1A}-ADRENERGIC RECEPTOR AND PHENYLEPHRINE-MEDIATED VENOCONSTRICTION

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Abstract

There is large interindividual variability and ethnic differences in phenylephrine-mediated vasoconstriction. We tested the hypothesis that genetic variation in *ADRA1A*, the α_{1A} adrenergic receptor gene, contributes to the variability and ethnic differences. We measured local dorsal hand vein responses to increasing doses of phenylephrine in 64 Caucasians and 42 African-Americans and genotyped for 32 *ADRA1A* SNPs. The ED_{50} ranged from 11 to 5442 ng/min, and the E_{max} ranged from 13.5% to 100%. The rs574647 variant was associated with a trend towards lower $logED_{50}$ in each race and in the combined cohort (P=0.008). Additionally, rs1079078 was associated with a trend to higher $logED_{50}$ in each race and in the combined cohort (P=0.011). Neither variant accounted for the ethnic differences in response. None of the *ADRA1A* haplotypes was associated with the outcomes. In conclusion, *ADRA1A* variants do not contribute substantially to the marked interindividual variability or ethnic differences in phenylephrine-mediated venoconstriction.

Keywords

Alpha-1A adrenergic receptor; Genetics; Vasoconstriction; Phenylephrine; Ethnicity

Introduction

The sympathetic nervous system regulates blood pressure and vascular resistance, and its effects are mediated through adrenergic receptors (AR). Vascular α_1-and α_2-ARs are

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expressed in arterial resistance and venous capacitance vessels, where they regulate blood pressure by maintaining vascular tone through vascular smooth muscle contraction.[1, 2] Vascular α_1-ARs appear to be the prime mediators for vascular smooth muscle contraction.[3, 4]

There is large interindividual variability in the vasoconstrictive response of arterial and venous vessels to the infusion of α-AR agonists,[5–7] much of which is genetically determined.[8,9] The two main α-AR subgroups, α_1-AR and α_2-AR, differ in their signal transduction pathways (coupling to the Gq or Gi subtypes of G-proteins, respectively), but share a common final effector pathway (myosin light chain kinase activation, myosin phosphorylation and cross bridging with actin filaments) to mediate vascular smooth muscle contraction.[1, 10, 11] Studies finding no correlation between an individual's sensitivity of venoconstriction in response to a selective α_1-AR agonist (phenylephrine) and a selective α_2-AR agonist (dexmedetomidine),[12, 13] which share the distal effector pathway, suggested that an important part of the interindividual variability is likely to be explained by differences in the distinctive proximal α_1-AR – and α_2-AR-signaling pathways (e.g., the membrane-bound adrenergic receptors and coupling). In support of this interpretation, we found ethnic differences between whites and blacks in vascular sensitivity to α_1-AR agonists[5, 7] but not to the α_2-AR agonist dexmedetomidine.[6] Thus, genetic variation in the α_1-AR could contribute to the interindividual variability in sensitivity to α_1-agonists and to the observed ethnic differences.

Of the three α_1-AR subtypes (α_{1A}, α_{1B} and α_{1D}), the α_{1A}-AR is the predominant subtype expressed in human vascular smooth muscle.[1] There is great genetic variation in *ADRA1A*, the gene encoding the α_{1A}-AR, with distinct differences between whites and blacks in single nucleotide polymorphism (SNP) prevalence and haplotype structure.[14] There is little information about the functional consequences of *ADRA1A* genetic variation on agonist-induced vasoconstriction. In an earlier study, we found that a common *ADRA1A* non-synonymous SNP (rs1048101) resulting in Arg347Cys, formerly called Arg492Cys, did not affect phenylephrine-mediated venoconstriction using the hand vein model.[15] However, the effect of the many other *ADRA1A* SNPs and haplotype blocks on agonist-induced vasoconstriction has not been defined.

We therefore set out to study venous responses to phenylephrine in a large cohort of healthy Caucasian and African American subjects using the dorsal hand vein model and define the association between vascular sensitivity and *ADRA1A* genotypes and haplotypes. We chose the dorsal hand vein model since it is a reproducible model for the study of local venous responses to vasoactive substances *in vivo* that elicits only minimal systemic cardiovascular changes, and is less invasive compared to studies in arterial vessels.[6, 7, 16, 17] Our hypothesis was that genetic variation in *ADRA1A*, represented by 32 SNPS, affects phenylephrine-mediated venoconstriction and contributes to interindividual and ethnic differences in agonist-mediated venoconstriction.

METHODS

Subjects

Between February 2002 - April 2004 and between May 2009 – January 2011, we recruited healthy subjects to participate in phenylephrine hand vein studies. These cohorts contributed data to previous publications,[7, 12, 15] which describe the study population and procedures in detail. For the current study, we included normotensive male and non-pregnant female Caucasians and African Americans aged 18–45 years who took no medications for at least 2 weeks and abstained from alcohol and caffeine for at least 5 days before the study. Each subject received a diet containing 150 mmol/day of sodium, 70 mmol/day of potassium, and 600 mmol/day of calcium for at least 4 days prior to the study day. Studies were performed in the morning after an overnight fast in a temperature-controlled room at the Vanderbilt Clinical Research Center. The Institutional Review Board of Vanderbilt University Medical Center approved the study protocol, and all subjects provided written informed consent.

Venous response to phenylephrine

Venous responses were measured in a dorsal hand vein with a linear variable differential transformer (LVDT) as previously described.[7] In summary, a 24-gauge intravenous cannula was inserted into a suitable right dorsal hand vein and kept patent with saline solution infused at a flow rate of 0.4 mL/min. A LVDT (MHR 100; Shaevitz Engineering, Pennsaken, NJ) was mounted on the dorsum of the subject's hand. A second intravenous cannula was inserted for blood sampling into the antecubital vein of the contralateral arm. After 30 minutes of saline infusion, a blood sample was taken for the determination of baseline plasma catecholamines and for genotyping. We determined the baseline vein diameter while a sphygmomanometer cuff around the upper arm was inflated to 50 mm Hg to induce venous filling. After 3 stable baseline measurements, we assessed vein constriction in response to increasing doses of phenylephrine. Phenylephrine (Elkins-Sinn, Cherry Hill, NJ) was infused through the cannula with a syringe infusion pump (Harvard Apparatus, Holliston, MA) at increasing dose rates (range, 12–12,000 ng/min). The infusion at each dose rate lasted 7 minutes, and the vein diameter was measured during the last two minutes of the infusion. The total flow rate through the vein was kept constant at 0.4 mL/min throughout the various phenylephrine dilutions. Heart rate and blood pressure were continuously monitored with a bedside cardiac monitor (Dinamap MPS; Johnson and Johnson Medical, Tampa, FL).

Analysis of hand vein response to phenylephrine

Venoconstriction was expressed as the percentage reduction in vein diameter from average baseline measurements, plotted against dose rates in individual semi-logarithm dose–response graphs, and analyzed using a sigmoid dose–response model with variable slope (GraphPad Prism 4.03, GraphPad, La Jolla, CA). The software provided the dose that produced 50% of maximal constriction ($ED_{50,}$ representing sensitivity to the drug) and the maximal venoconstriction ($E_{max,}$ representing maximum response) for each subject.

Determination of plasma catecholamine concentrations

Blood was collected into cooled heparinized tubes that were placed on ice until centrifuged at 4°C for 10 minutes at 3000 rpm. Plasma was separated and stored at −20°C in previously cooled tubes containing 40μL of reduced glutathione (6%) until assayed. Norepinephrine and epinephrine concentrations were measured by high-performance liquid chromatography using electrochemical detection with dihydroxybenzylamine as internal standard.[18]

Genotyping

We genotyped 32 *ADRA1A* SNPs, including 23 previously described tagSNPs defining four *ADRA1A* haplotype blocks[14] and 9 additional SNPs common in Caucasians and/or African Americans (supplementary Table 1). We genotyped these SNPs using the Sequenom platform (MassArray, San Diego, CA), except for two SNPs (rs13261054 and rs1353446) which were genotyped using allelic discrimination by TaqMan 5′-nuclease assays on an ABI 7900 HT real-time PCR system (Applied Biosystems, Foster City, CA) using validated TaqMan probes.[19] For quality control, we included negative and positive controls with each genotyping run. Quality-control procedures included examination of marker and sample genotyping efficiency, allele-frequency calculations, and tests of Hardy-Weinberg equilibrium (HWE).

Statistical analysis

Our cohort was a convenience sample consisting of participants in previous studies,[7, 12, 15] and the sample size was not based on a priori calculations. Our primary outcome was drug sensitivity (expressed as ED_{50}), and drug efficacy (E_{max}) was the secondary outcome. ED_{50} values were not normally distributed and were therefore expressed as geometric means with 95% confidence intervals (CIs) following log-transformation. Other data were expressed as mean and standard deviation (SD) or median and interquartile range (IQR) as appropriate. Genotype distributions were tested for deviation from Hardy-Weinberg equilibrium using a χ^2 test with one degree of freedom.

We compared the outcome, phenylephrine sensitivity ($LogED_{50}$) among genotypes in a single marker analysis using one-way analysis of variance (ANOVA), first in each ethnic group separately and then in the combined cohort. We then adjusted for potential confounders that were associated with the outcome in our previous analyses[7] (sex, BMI, resting norepinephrine and, for analyses of the combined cohort, ethnicity) using a multiple linear regression model. To explore the contribution of genetic markers to ethnic differences in phenylephrine sensitivity, we performed these regression analyses in the whole cohort combined, with ethnicity as additional covariate, with and without the genetic markers of interest. For all genetic analyses, we assumed an additive genetic model, coding the genotypes according to the number of variant alleles (0–2). In a sensitivity analysis, we also used a dominant model for variants with few homozygote subjects to reduce the influence of potential outliers. The secondary outcome, phenylephrine efficacy (E_{max}), was compared among genotypes using the non-parametric Kruskal-Wallis test. Race-specific haplotypes were derived with the R package haplo.stats which was also used to assess overall differences in the outcomes among the haplotypes.[20, 21] Other statistical analyses were

performed using SPSS software (v. 21, IBM® SPSS® Inc., Chicago, IL). All analyses were two-tailed, and a P-value < 0.05 was considered significant.

RESULTS

Demographic parameters and outcomes

Among 116 subjects studied, we excluded 10 (DNA not available or Asian ethnicity), and the final cohort included 106 subjects (64 Caucasians and 42 African Americans). Table 1 shows demographic data, baseline cardiovascular parameters, and outcome measures, as previously described.[7] African-Americans had a higher BMI (P = 0.007) and a higher resting heart rate (P = 0.005). There was wide interindividual variability in response to phenylephrine, with the range of ED_{50} spanning three log units (11 to 5442 ng/min; geometric mean, 245 ng/min; 95% CI, 190 to 318 ng/min), and the E_{max} ranging from 13.5% to 100% (median, 87%; IQR, 76% to 96%).

Genotyping

We genotyped 32 *ADRA1A* SNPs (Supplementary Table S1). Minor allele frequencies were in the expected range, and all genotypes conformed to Hardy-Weinberg equilibrium in each ethnic group. We did not identify any carrier of the rs1496126 variant.

Genetic variants and sensitivity to phenylephrine

The rs574647 was associated with a trend towards lower $logED_{50}$ both in Caucasians (β= −0.25; 95% CI, −0.51 to 0.02; adjusted P=0.064) and African Americans (β=−0.40; 95% CI, −0.79 to −0.02; adjusted P=0.039), and the statistical evidence for this association was stronger in the combined cohort (β=−0.29; 95% CI, −0.50 to −0.08; adjusted P=0.008). Geometric mean ED_{50} was 76% lower in subjects with two minor alleles (ED_{50}=69 ng/min; 95% CI, 10 to 489 ng/min; n=5) compared to those with no minor allele (ED50=289 ng/min; 95% CI, 207 to 405 ng/min; n=57; Figure 1).

Additionally, the rs1079078 variant was associated with a trend to higher $logED_{50}$ both in Caucasians (β=0.23; 95% CI, −0.01 to 0.47; adjusted P=0.055) and African Americans (β=0.31; 95% CI, −0.04 to 0.67; adjusted P=0.082), and the association was stronger in the combined group (β=0.25; 95% CI, 0.06 to 0.44; adjusted P=0.011). Thus, subjects with two variant alleles (n=6) had a geometric mean ED_{50} of 742 ng/min (95% CI, 345 to 1,595 ng/min), a 3.9-fold higher value compared to the 67 subjects with no minor allele (192 ng/min; 95% CI, 138 to 267 ng/min; Figure 2). In a sensitivity analysis, after combining heterozygous and homozygous variant carriers into one group assuming a dominant mode of inheritance, the associations between rs574647 and the rs1079078 with $logED_{50}$ in the whole cohort remained significant(adjusted P=0.037 and 0.030, respectively).

The association between rs13270252 and $LogED_{50}$ was in opposite directions in the two ethnic groups and was not significant in the combined group (adjusted P=0.69) (Table 2, Supplementary Table S2). The rs580739 variant was significantly associated with $LogED_{50}$ in African Americans (adjusted P=0.005) but not in Caucasians or in the combined cohort (both P>0.10). Moreover, the functional non-synonymous variant rs1048101, resulting in the

Arg347Cys polymorphism, was not associated with $LogED_{50}$ in any of the ethnic subgroups or the combined group (all P > 0.35).

Genetic variants and efficacy of phenylephrine

None of the 32 *ADRA1A* SNPs was associated with E_{max} in the ethnic subgroups, while rs17333700 was marginally associated with lower E_{max} in the combined group (P = 0.040, Table 3; lementary Table S3; Supplementary Figure 1).

ADRA1A haplotypes and outcome measures

Based on a previously published *ADRA1A* haplotype analysis, [14] we divided the *ADRA1A* gene into four blocks and inferred haplotypes for each block separately in each ethnic group (Supplementary Tables S4). Haplotype frequencies were overall similar to those previously published. Phenotype-haplotype analyses did not provide any evidence for an overall association between outcomes ($LogED_{50}$ or E_{max}) and haplotypes in any of the 4 haplotype blocks (Supplementary Tables S5 and S6).

Ethnic difference in ED_{50} and *ADRA1A* variants

As previously described,[7] geometric mean ED_{50} was 45% lower in African Americans than in Caucasians (172 ng/min and 310 ng/min in African Americans and Caucasians respectively; adjusted P= 0.004; Table 1). Since the four *ADRA1A* variants associated with logED50 (rs13270252, rs580739, rs574647 and rs1079078) had significantly different prevalences in Caucasians and African Americans, we examined whether these variants contributed to ethnic differences in ED_{50}. In the combined cohort, using a multiple linear regression model without genotypes (with $logED_{50}$ as dependent variable and sex, BMI, ethnicity, and resting plasma norepinephrine concentrations as covariates), black ethnicity was associated with lower $LogED_{50}$ (β=−0.36; 95% CI, −0.61 to −0.12; P=0.004). Adding the four genotypes to the model increased the coefficient of variation R^2, a measure of the variation in the outcome explained by the model, from 12.9% to 18.4%, but the ethnic difference remained unaffected (β=−0.36; 95% CI, −0.64 to −0.08; P=0.013), suggesting that these genotypes did not contribute substantially to the greater phenylephrine sensitivity of African Americans compared to Caucasians.

DISCUSSION

Our study is the first to systematically examine the effect of the genetic variation in *ADRA1A* on vascular responses to an α_1-AR agonist. Our major findings are that genetic variation in *ADRA1A* explains only little of the large interindividual variability in phenylephrine-mediated venoconstriction, and that it does not account for the ethnic differences in α_1AR-mediated venoconstriction. In addition, our study provided some evidence for an association of two *ADRA1A* SNPs (rs574647 and rs1079078) with increased and decreased sensitivity to phenylephrine-mediated venoconstriction, respectively.

Family studies have found large heritability in venous response to α-AR agonists.[8, 9] Veins express different α_1-AR subtypes, and the α_{1A}-AR subtype has the highest receptor density and greatest affinity for α_1-AR agonists.[3, 22] However, little is known about the effect of

genetic variability in *ADRA1A* on α_1-AR-mediated venoconstriction. We previously studied the effect of a single functional, non-synonymous *ADRA1A* SNP (rs1048101, resulting in Arg347Cys), in the hand vein model in 74 subjects (52 Caucasians, 5 African Americans and 17 other ethnicities), finding no association with phenylephrine-mediated venoconstriction.[15] In our current study, with a larger sample size, we confirmed this finding. Moreover, in a systematic approach using 32 SNPS selected to better capture *ADRA1A* genetic variation, we found that it contributed only little at best to the great variability in phenylephrine-mediated venoconstriction and to its ethnic differences. Thus, our findings are compatible with previous studies failing to show consistent associations of *ADRA1A* polymorphisms with more complex phenotypes to which α_1-mediated vasoregulation is thought to contribute, e.g. blood pressure response to stress[23] and hypertension.[24–30]

We found some evidence for an association of two intronic SNPs - rs574647 and rs1079078 - with increased and decreased sensitivity to phenylephrine-mediated venoconstriction, respectively. Subjects with two minor rs574647 variant alleles had a geometric mean ED_{50} that was 76% lower than that of subjects without any variant allele. The rs1079078 variant was more prevalent in Caucasians, and subjects with two variant alleles had a 3.9 fold higher geometric mean ED_{50} (reflecting lower α_{1A}-AR sensitivity to phenylephrine-mediated venoconstriction) compared to subjects with no variant allele. We are not aware of previous studies showing functional associations of these variants. Moreover, they may be only markers of functional haplotypes rather than SNPs primarily responsible for the observed effect. However, haplotype analysis did not provide evidence for functional effects of the haplotypes defined by these two variants.

We have previously shown increased sensitivity of venous[7] and arterial[5] α_1-AR-mediated vasoconstriction in African Americans compared to Caucasians. In view of the ethnic differences in *ADRA1A* genetic variation,[14] we therefore examined the hypothesis that genetic variants in α_1-AR account for these ethnic differences. However, in the present study, the four variants associated with phenylephrine ED_{50} did not explain the ethnic difference in ED_{50}. Thus, after excluding genetic variation in α_1-AR, it remains unclear which factors contribute to the large interindividual variability in α_1-AR mediated venoconstriction, and to the ethnic differences in particular. Yet, in view of the strong heritability of venous responsiveness to α-AR agonists, genetic variation in other candidate genes remains a likely cause.[8, 9]

In contrast to phenylephrine-mediated vasoconstriction, we previously did not find ethnic differences in α_2-AR mediated venoconstriction in the hand vein model,[6] suggesting that differences in the proximal signaling pathway specific to the α_1-AR pathway account for ethnic differences in phenylephrine sensitivity. Thus, taken together with our current findings, genes encoding proteins involved in the early signal transduction cascade below the α_1-AR level, e.g. in receptor coupling, would be reasonable candidates for explaining interindividual and ethnic differences in α_1-AR mediated responsiveness.

There are several limitations to our study. Phenylephrine is not selective for the α_{1A}-AR subtype, but acts as an agonist also at venous α_{1B}-AR and α_{1D}-AR. However, there is no

selective α_{1A}-AR agonist available for use in humans. Moreover, although α_{1B}- and α_{1D}-ARs are also expressed in human veins,[31, 32] α_{1A}-ARs are thought to be the prime mediators of vascular constriction.[4] Secondly, our findings were derived using the dorsal hand vein model, and may therefore not automatically be extrapolated to other venous or arterial vascular beds, or to more complex and multifactorial phenotypes such as blood pressure. However, we found similar ethnic differences in α_1-AR-mediated vasoconstriction both in venous and arterial vascular beds, suggesting that responsiveness in the dorsal hand vein model may to a certain degree also reflect that in arterial vascular beds. Finally, although our sample size was rather large for a translational study, some genotype groups were small, and we could not account for the multiple comparisons required by the large number of *ADRA1A* variants. Thus, we consider our findings regarding the association of the rs574647 and rs1079078 variants with responsiveness to phenylephrine preliminary rather than conclusive. Our findings should be validated in a separate cohort, and much larger cohorts would be necessary for systematic, non-candidate gene-based approaches such as genome-wide association studies.

In conclusion, we found some evidence for an association of sensitivity to phenylephrine-mediated venoconstriction with two intronic *ADRA1A* SNPs (rs574647 and rs1079078), but overall, genetic variation in *ADRA1A* does not contribute substantially to the large interindividual variation in phenylephrine-mediated venoconstriction and ethnic differences in response between African Americans and Caucasians. Future studies exploring additional candidate genes involved in the α_1-AR signal transduction pathway (e.g., those involved in receptor coupling) or genes encoding other α_1-AR subtypes will be of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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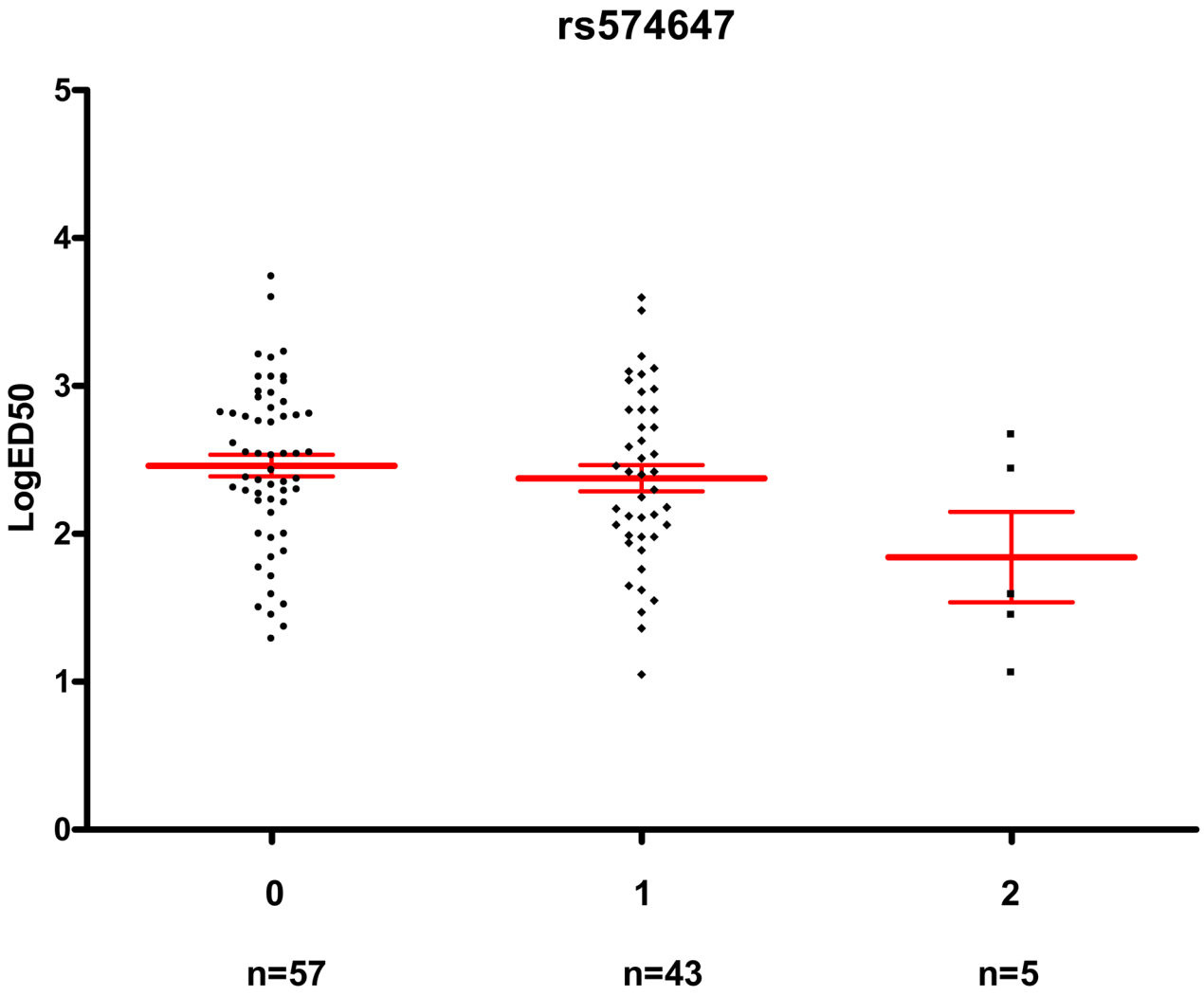

Figure 1. rs574647 genotype and its association with sensitivity to phenylephrine (ED_{50})

The horizontal lines represent the mean, and the whiskers represent the standard error of the mean. Subjects with variant alleles had significantly lower $LogED_{50}$ (unadjusted P=0.064, adjusted P=0.008).

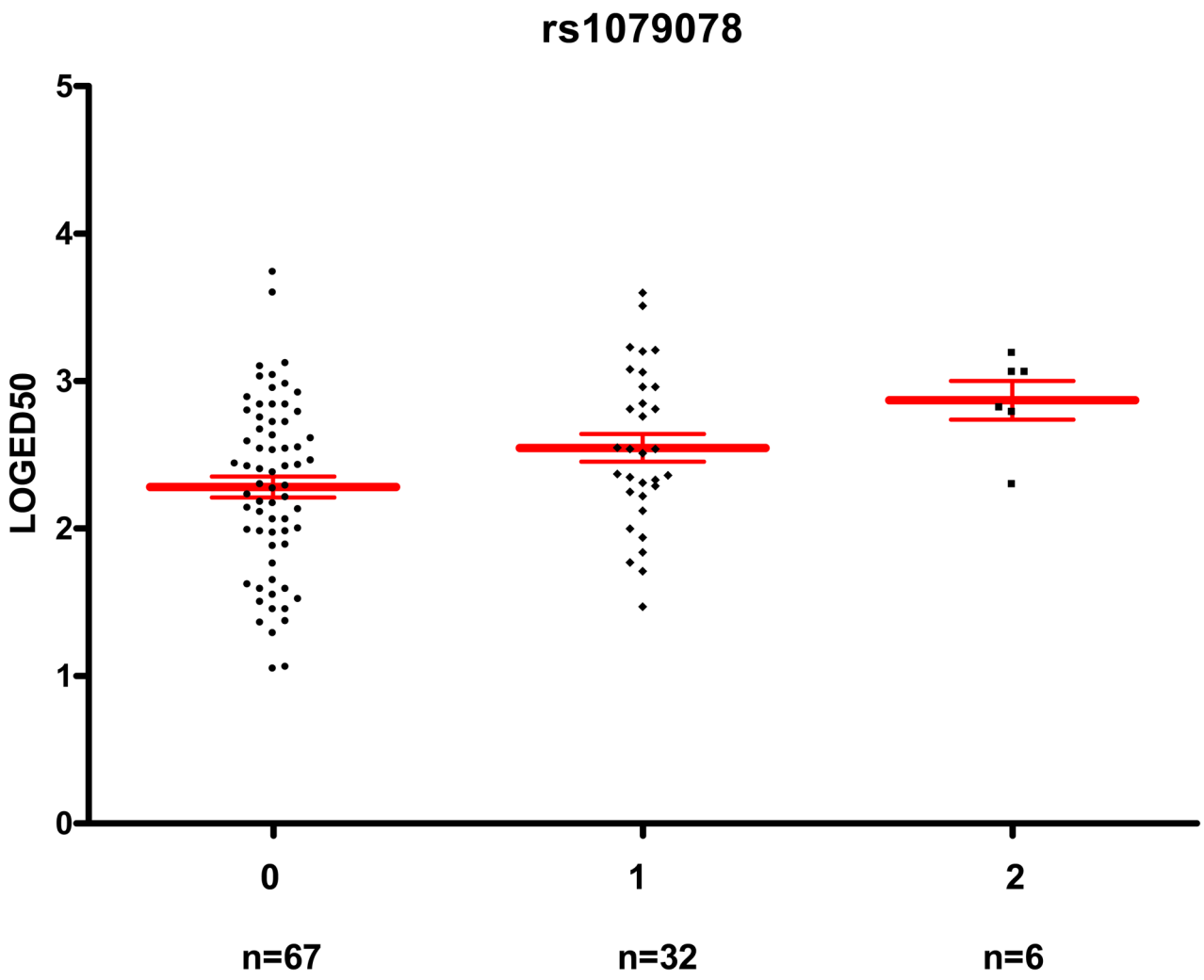

Figure 2. rs1079078 genotype and its association with sensitivity to phenylephrine (ED_{50})

The horizontal lines represent the mean, and the whiskers represent the standard error of the mean. Subjects with variant alleles have significantly higher $LogED_{50}$ (unadjusted P=0.003, adjusted P=0.011).

Table 1

Demographic characteristic and outcome measures

Covariates	Caucasians n = 64	African Americans n = 42	All n = 106
Age, years	26.9 ± 6.8	28.1 ± 8.0	27.3± 7.3
Female sex, n (%)	31 (48)	18 (43)	49 (46)
Family history of Hypertension, n (%)	25 (39)	20 (48)	45 (43)
Resting SBP, mmHg	110.3 ± 11	111.1 ± 9.5	110.6 ± 10.4
Resting DBP, mmHg	60.7 ± 6.8	63.4 ± 8.0	61.8 ± 7.4
Resting HR, bpm*	59.0 ± 8.2	63.7 ± 8.0	60.9 ± 8.4
Baseline Plasma norepinephrine, pg/ml	162.3 ± 58.0	179.5 ± 70.8	168.9 ± 63.3
Baseline Plasma epinephrine, pg/ml	18.5 ± 12.5	21.0 ± 15.6	19.4 ± 13.7
BMI, kg/m^2*	24.3 ± 3.8	26.8 ± 5.4	25.3 ± 4.6
$ED_{50,}$ ng/min, Mean (95%CI)*	310 (222 – 434)	172 (115 – 256)	245 (190 – 318)
E_{max}, %, Median (IQR)	85 (75 – 95)	89 (82 – 98)	87 (76 – 96)

Data presented as mean ± SD except otherwise stated. SBP = Systolic blood pressure; DBP = Diastolic blood pressure; HR = Heart rate

*P value < 0.05 comparing Caucasians and African Americans.

Table 2

Genetic variants of *ADRA1A* associated with sensitivity to phenylephrine (ED_{50})

SNP	Caucasians			P-value* unadjust. (adjusted)	African Americans			P-value* unadjust. (adjusted)	All P-value* unadjust. (adjusted)
	ED_{50}, ng/min, geometric mean (95% CI)				ED_{50}, ng/min, geometric mean (95% CI)				
	0*	1	2		0*	1	2		
rs574647	395 (248–627) N = 28	322 (201–514) N = 32	43 (5–360) N = 4	0.022 (0.064)	214 (131–350) N = 29	98 (46–208) N = 11	468 N = 1	0.283 (0.039)	0.064 (0.008)
rs1079078	244 (156–381) N = 38	365 (199–671) N = 21	963 (593– 1565) N = 5	0.030 (0.055)	139 (85–230) N = 29	329 (162–667) N = 11	201 N = 1	0.094 (0.082)	0.003 (0.011)
rs13270252	269 (186–398) N = 53	646 (316–1318) N = 10	324 N = 1	0.108 (0.039)	186 (112–302) N = 21	204 (87–490) N=14	35 (4–331) N = 3	0.207 (0.027)	0.586 (0.686)
rs580739	394 (245–632) N = 29	241 (140–414) N = 29	336 (77–1468) N = 6	0.360 (0.623)	267 (172–416) N = 29	55 (32–93) N = 11	468 N = 1	0.007 (0.005)	0.131 (0.103)

*0, 1, 2 represent the number of variant alleles for each SNP.

N = number of subjects in each genotype group.

*P values are before (unadjust.) and after adjustment for sex, BMI, baseline norepinephrine values and ethnicity (for analyses of the combined cohort).

Table 3

Genetic variant of *ADRA1A* associated with efficacy to phenylephrine (E_{max}).

SNP	Caucasians				African Americans				All
	$E_{max,}$ %, median (IQR)				$E_{max,}$ %, median (IQR)				
	0	1	2	P value	0	1	2	P value	P value
rs17333700	85 (75–99)	85 (76–92)	65	0.078	89 (83–98)	92 (83–97)	-	0.749	0.040
	N = 35	N = 26	N = 3		N = 36	N = 5			

0, 1, 2 is the number of variant alleles for each SNP.

N = number of subjects with each variant allele.