Human phenylethanolamine N-methyltransferase genetic polymorphisms and exercise-induced epinephrine release

Yuan Ji,1 Eric M. Snyder,2 Brooke L. Fridley,3 Oreste E. Salavaggione,1 Irene Moon,1 Anthony Batzler,3 Vivien C. Yee,5 Daniel J. Schaid,3 Michael J. Joyner,4 Bruce D. Johnson,2 and Richard M. Weinshilboum1

1Division of Clinical Pharmacology, Department of Molecular Pharmacology and Experimental Therapeutics, and Departments of 2Internal Medicine, 3Health Sciences Research, and 4Anesthesiology, Mayo Clinic College of Medicine, Mayo Foundation, Rochester, Minnesota; and 5Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio

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Ji Y, Snyder EM, Fridley BL, Salavaggione OE, Moon I, Batzler A, Yee VC, Schaid DJ, Joyner MJ, Johnson BD, Weinshilboum RM. Human phenylethanolamine N-methyltransferase genetic polymorphisms and exercise-induced epinephrine release. Physiol Genomics 33: 323–332, 2008. First published March 18, 2008; doi:10.1152/physiolgenomics.00248.2007.—Phenylethanolamine N-methyltransferase (PNMT) catalyzes the synthesis of epinephrine from norepinephrine. We previously identified and functionally characterized common sequence variation in the PNMT gene. In the present study, we set out to determine whether common PNMT genetic polymorphisms might be associated with individual variation in circulating epinephrine levels during exercise in 74 Caucasian American subjects. Circulating epinephrine levels were measured in each subject at baseline and during two different levels of exercise, ~40% and ~75% of peak workload. The PNMT gene was resequenced from each subject, and eight novel PNMT polymorphisms were identified, including a C319T (Arg107Cys) nonsynonymous single nucleotide polymorphism (SNP) and I1G(280)A, a SNP located in the first intron of the gene. The I1G(280)A SNP was significantly associated with decreased exercise-induced circulating epinephrine levels and with a decreased epinephrine-to-norepinephrine ratio. The Cys107 recombinant allozyme displayed significantly lower levels of both PNMT activity and immunoreactive protein than the wild-type allozyme after transfection into COS-1 cells, but it did not appear to be associated with level of epinephrine in these subjects. Electrophoretic mobility shift and reporter gene assays performed with the I1G(280)A SNP indicated that this polymorphism could bind nuclear proteins and might modulate gene transcription. Our studies suggest that functionally significant variant sequence in the human PNMT gene might contribute to individual variation in levels of circulating epinephrine during exercise.

PNMT, EC2.1.1.28 catalyzes the synthesis of epinephrine from norepinephrine (13, 14). PNMT is expressed in the adrenal medulla, a small number of nuclei in the central nervous system, and the retina (1, 8). In the adrenal, PNMT is induced by corticosteroids secreted in the adrenal cortex (36). During environmental or physiological stress such as exercise, pituitary corticotrophin (ACTH) release results in the secretion of glucocorticoids that induce PNMT (35). Studies performed with rodents demonstrated that hypophysectomy reduced adrenal medullary PNMT, but it could be restored by the administration of ACTH or the potent synthetic glucocorticoid dexamethasone (35). Glucocorticoid treatment can also induce adrenal PNMT mRNA levels (3, 29). PNMT is encoded by a relatively simple three-exon gene (11). Studies performed with the rat gene showed that binding sites for Egr-1 and the glucocorticoid receptor (GR) in the 5’-flanking region (5’-FR) of the gene were essential for the induction of PNMT during both acute and chronic stress (33). The presence of GR response elements (GREs) and Sp1 binding sites in the human PNMT promoter suggests that similar transcription regulation mechanisms might also apply to the human gene (11).

Virtually all circulating epinephrine present during the “stress response” originates from the adrenal medulla (1). In response to a stress such as exercise, increased levels of epinephrine are associated with increased energy mobilization and the redistribution of blood to organs involved in exercise (16). As a result, functionally important variation in the sequence of the PNMT gene could, in theory, result in alterations in PNMT function or stress-dependent induction, leading to variation in the rate of epinephrine synthesis. Therefore, we chose to use exercise-induced epinephrine release into the peripheral circulation as a “stress test” for possible functional alterations in epinephrine synthesis as a result of common polymorphisms in PNMT. We had previously (9) identified common sequence variation in the human PNMT gene by resequencing ~3.5 kb of DNA that included all PNMT exons and introns, as well as ~1 kb of the 5’-FR. Functional genomic studies were also performed with the four nonsynonymous single nucleotide polymorphisms (SNPs) identified during that study as well as with common 5’-FR haplotypes. The results showed that variations in PNMT sequence might be able to alter enzyme function at a cellular level as a result of either variation in levels of PNMT allozyme activity and/or immunoreactive protein or by alteration in the activity of the PNMT promoter (9). However, to determine whether variations in PNMT sequence might also be functionally important in vivo, a genotype-phenotype association study would be required that would correlate phenotypic variation with PNMT sequence variation. That type of study often involves the direct measurement of enzyme activity in biopsies of tissue in which the enzyme is expressed. However, that approach is not practical for studies of PNMT since the peripheral expression of this enzyme is largely restricted to the adrenal medulla.
Therefore, in the present study, we set out to determine whether variation in PNMT sequence might alter a “physiological phenotype,” exercise-induced changes in circulating epinephrine concentrations in healthy subjects. The measurement of exercise-induced catecholamine changes would have the potential advantage of potentially “unmasking” effects of PNMT-dependent synthesis of epinephrine that would not be evident in the basal state. Specifically, 74 healthy young subjects were subjected to two levels of exercise (40% and 75% of peak workload). Circulating epinephrine was measured at rest and at both levels of exercise by placing a catheter in the radial artery. This phenotype was correlated with the PNMT genotype obtained by resequencing the entire gene. Functional genomics studies were then performed with novel gene sequence variation identified in this group of subjects. We obtained preliminary evidence that functionally significant PNMT sequence variation might influence epinephrine levels during exercise. These studies represent a step toward understanding the possible relationship between polymorphisms in PNMT and response to stress.

METHODS

Exercise Studies

Subjects. One hundred and four Caucasian American (CA) subjects recruited in Rochester, MN expressed a willingness to participate in the exercise physiology studies. However, as described subsequently, only 74 subjects eventually chose to participate in the full exercise study. Exclusion criteria included a history of cardiopulmonary disease, pregnancy, smoking, taking medications, and inability to exercise. Written informed consent was obtained from all participants. This study was reviewed and approved by the Mayo Clinic Institutional Review Board.

Exercise studies. All subjects underwent baseline pulmonary function testing, incremental cycle ergometry testing to exhaustion, a complete blood count to rule out anemia, and, in women, a pregnancy test. This baseline exercise study served as an initial “familiarization” session and was used to determine workload intensities for subsequent sessions. It also acted as a screening test to rule out the possibility of myocardial ischemia and/or cardiac arrhythmias. After these initial tests, subjects met with the Mayo Clinic Clinical Research Unit (CRU) nutritionist and were placed on a controlled-sodium diet (3,450 mg/day) for 3 days, with a 24-h urine collection to confirm sodium intake. The subjects subsequently returned to the CRU on two occasions for exercise testing. The second session consisted of a cycle ergometry test similar to that conducted during the first visit, but with the addition of a measurement of cardiac output by a previously validated open-circuit acetylene uptake method (10). That session served as further familiarization for the final study day. It also allowed for the confirmation of workloads for the final visit. On the final visit, before the study, a 5-cm 20-gauge catheter (Arrow International, Reading, PA) was placed in the radial artery after local anesthesia with 2% lidocaine to obtain samples for catecholamine assays and to measure arterial blood pressure directly. Subjects then exercised for 9 min at ~40% and for 9 min at ~75% of the peak workload that they had achieved during the initial exercise studies. Nine minutes of exercise was performed because pilot data had suggested that this was an adequate time to obtain three sets of measures, and it brought the subjects close to exhaustion during the highest workload.

Catecholamine Assays

Plasma epinephrine and norepinephrine concentrations were assayed in the Mayo Clinic CRU immunochemical core laboratory by high-performance liquid chromatography (HPLC) with electrochemical detection as described by Kissinger et al. (15) and by Sealey (26). This type of assay has become the standard approach for the determination of plasma catecholamine concentrations. The lower limit of detection for both norepinephrine and epinephrine in our laboratory is <10 pg/ml—well below values measured in any of our samples. The intra-assay coefficients of variation (CVs) were 12.2% and 3.6% at 13.8 and 242 pg/ml, respectively, for epinephrine and 4.5% and 3.3% at 224 and 429 pg/ml, respectively, for norepinephrine. Interassay CVs were 6.4% and 8.2% at 61 and 917 pg/ml, respectively, for epinephrine and 4.6% and 8.6% at 235 and 1,096 pg/ml, respectively, for norepinephrine. Twenty-four-hour urinary epinephrine and norepinephrine concentrations were measured in the Mayo Department of Laboratory Medicine and Pathology Endocrinology Laboratory, also by HPLC with electrochemical detection.

PNMT Resequencing

PNMT was resequenced with DNA samples obtained from all 104 initial subjects, 74 of whom completed the exercise study while the remainder of the subjects chose to participate in a different study. Specifically, the entire PNMT gene, including all exons and introns as well as ~1 kb of the 5′-FR, was amplified by overlapping PCR reactions. The amplicons were then sequenced on both strands with dye terminator DNA-sequencing chemistry. Details of the resequencing of PNMT have been described elsewhere (9).

Statistical Analyses

Preliminary genetic analysis and data transformation. Minor allele frequencies, descriptive statistics, linkage disequilibrium (R2 and D′), and tests for Hardy-Weinberg equilibrium were calculated before performing genetic association analysis. Measurements of epinephrine and norepinephrine concentrations were obtained at different exercise intensity levels for all 74 subjects and were used as phenotypes in the genotype-phenotype correlation analysis. The ratio of epinephrine to norepinephrine was also included as a phenotype that would indirectly reflect epinephrine synthesis. The data were logged transformed because of the skewness of the raw values and were adjusted for baseline value (catecholamine levels at rest). All phenotypes used in the analysis were log(value at time t) − log(baseline value).

Single-SNP association test. A linear model was used to test the association of single SNPs with the mean of the three measurements at a low level of exercise (40% of peak workload, Low) or the mean of the three measurements at a high level of exercise (75% of peak workload, High). The single-SNP association test was completed by standard F-test with degrees of freedom of g − 1, where g was the number of genotypes observed for the SNP. Age and sex were included in the statistical model as covariates.

The analysis of data treated as repeated measurements at each workload provided the opportunity to detect genetic effects in the model on dynamic parameters (e.g., interaction of SNP effect with time), as opposed to aggregating measurements taken at different time points into a single number, for example, the sample mean. That is, the repeated-measurements analysis allows one to determine whether the impact of genetic variants differ with workloads of Low or High, represented by time measured in minutes (i.e., does the workload level/time modify the impact of the effect of SNP genotype on epinephrine level?). We fit a model that considered all six measurements of circulating epinephrine, including three at Low and three at High, adjusted for baseline value in a repeated-measurements statistical framework using a mixed model (17, 22), with age and sex as covariates. In a separate repeated-measurements analysis, we also included the baseline value in the model.

Haplotype association test. Haplotype analysis was conducted with an approach similar to that used for the single-SNP analysis, using the mean of the three measurements at Low adjusted for baseline value and the mean of the three measurements at High adjusted for baseline.
level, with age and sex included in the model. The haplotype for each DNA sample was either observed or inferred with the EM algorithm described by Schaid et al. (25), as implemented in the Splus library Haplo.stat (http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm). With this approach, whole gene haplotypes, as well as four-locus haplotypes containing the four common PNMT 5'-FR SNPs, C(−968)G, G(−591)T, A(−390)G, and A(−184)G (see Table 1), were determined for the 74 subjects who participated in the exercise test. A global test was used to determine the association of these haplotypes with phenotypes (epinephrine,norepinephrine, and their ratio).

The most common haplotype was set as the reference haplotype for the repeated-measurements analysis. The repeated-measurements haplotype models were modifications of the single-SNP repeated-measurements models, in which haplotype posterior probabilities, computed under an additive haplotype model, replaced the SNP genotypes and the fixed “genetic” effects. Age and sex were also included in this model as covariates. Tests for the global significance of haplotypes were completed with a likelihood ratio test.

Functional Genomic Studies

PNMT allozyme functional genomic studies. The functional consequence of a novel PNMT nonsynonymous SNP discovered during the resequencing of DNA samples obtained from these subjects was studied with recombinant enzyme. Specifically, wild-type (WT) and variant PNMT expression constructs were cotransfected into COS-1 cells with a β-galactosidase construct. β-Galactosidase activity was measured as a control for transfection efficiency. Supernatant preparations from cells transfected with these constructs were used to assay PNMT activity and to measure immunoreactive protein levels with quantitative Western blots, as described previously (9). Structural analysis using the WT PNMT structure (PDB ID: 1HNN) (20) was also performed to analyze the possible structural effects of each amino acid substitution within the context of the local chemical and steric environment (7). The novel nonsynonymous SNP observed in this study (Cys107), as well as those observed during our previous PNMT resequencing study (Ser9, Ala98, Cys112, and Thr175), were included in the structural analysis (9).

Functional genomics of I1G(280)A. The novel I1G(280)A SNP observed during our resequencing study was located within the first PNMT intron. That intron is ~1 kb in length. Included among possible functional consequences of an intron polymorphism are alternative mRNA splicing or an effect on transcription (5, 6). Therefore, functional genomic studies were performed with I1G(280)A and minigene constructs, electrophoretic mobility shift (EMS) assays, and reporter gene experiments.

A minigene construct was created by cloning the entire WT PNMT gene sequence, including all exons, introns, and ~1 kb of the 5'-FR, into the mammalian expression vector pcDNA4/HisMax (Invitrogen, Carlsbad, CA). This construct was then used as template to perform site-directed mutagenesis to create a variant minigene construct with a G to A change in sequence at nucleotide I1(280). These constructs were then transiently expressed in COS-1 cells, followed by the isolation of total RNA that was used to perform RT-PCR to compare mRNA splicing patterns between WT and variant sequences. The primers used to perform these studies are listed in Supplemental Table S1.1

EMS assays were performed with rat pheochromocytoma PC-12 cell nuclear extract and oligonucleotides that contained WT, I1(280)G, or variant, I1(280)A, sequences (see Supplemental Table S1). Details of the annealing and labeling steps have been described elsewhere (31). 32P-labeled annealed oligonucleotides were then incubated with 5 μg of nuclear extract and 5× binding buffer (Promega, Madison, WI) for the binding reaction. Nuclear extract was not present in the experimental control reaction; 250×, 150×, and 50× excess of unlabeled specific oligonucleotides was added to the competition reaction mixture. All reactions were incubated at room temperature for 20 min and then subjected to electrophoresis with a 5% nondenaturing acrylamide gel.

For the reporter gene studies, the entire WT PNMT intron 1 (~1 kb) was amplified from genomic DNA with the PCR and was cloned into the reporter gene vectors pGL3-Basic, pGL3-Control, and pGL3-Enhancer (Promega). Acc65I and Xhol restriction sites had been added to the 5'-ends of the forward and reverse primers, respectively, to facilitate subcloning these ampiclons upstream of the firefly luciferase open reading frame (ORF). The inserts were sequenced in both directions to ensure that the correct sequence was present and were then used as template for site-directed mutagenesis to create variant pGL3 reporter gene constructs. The primers used to perform these experiments are also listed in Supplemental Table S1.

RESULTS

Exercise Studies

Epinephrine in the peripheral circulation is released from chromaffin granules in the adrenal medulla. These chromaffin granule stores might serve as a “reservoir” of epinephrine—making it more difficult to detect the functional effects of subtle alterations in epinephrine synthesis as a result of PNMT polymorphisms. Therefore, we used exercise as a “stress” in an attempt to unmask individual variation in PNMT-catalyzed epinephrine synthesis. A total of 104 CA subjects who satisfied study enrollment criteria volunteered for the exercise protocol, but only 74 eventually completed the study. Most of the remaining subjects participated in other ongoing studies. However, DNA from all 104 initial subjects was used to resequence PNMT to define the range of common sequence variation present in this gene. Subjects using tobacco or medications other than oral contraceptives were excluded from the exercise study. Blood samples were obtained, and the entire PNMT gene was resequenced with DNA isolated from those blood samples. As stated above, 74 subjects (52.7% women) completed the exercise study and had physiological parameters measured, including circulating epinephrine levels determined at all stages of exercise (rest, Low, and High) (Fig. 1).

PNMT Resequencing

The PNMT gene was resequenced with DNA from each of the 104 subjects who originally volunteered for the exercise...
study. During resequencing of the gene, we identified a total of 14 SNPs. The locations and minor allele frequencies for those SNPs are listed in Table 2 and are shown schematically in Fig. 2. Figure 2 also shows the SNPs identified during our previous PNMT resequencing study (9) from Coriell Institute CA subjects. Several novel SNPs were identified, including a nonsynonymous SNP, C319T, that resulted in an Arg107Cys amino acid change, and an intron 1 SNP, I1G(280)A, both of which were observed in two heterozygous CA subjects. Several novel SNPs were identified, including a nonsynonymous SNP, C319T, that resulted in an Arg107Cys amino acid change, and an intron 1 SNP, I1G(280)A, both of which were observed in two heterozygous CA subjects. Several novel SNPs were identified, including a nonsynonymous SNP, C319T, that resulted in an Arg107Cys amino acid change, and an intron 1 SNP, I1G(280)A, both of which were observed in two heterozygous CA subjects.

These polymorphisms include those identified in the present study with DNA samples from 104 subjects at Mayo Clinic as well as those identified during our previous PNMT resequencing study (9) from Coriell Institute CA subjects as well as those identified previously with 60 CA subjects (9) are shown. Arrows indicate the locations of polymorphisms, with different colors indicating minor allele frequencies. Black rectangles represent portions of exons encoding the open reading frame, and open rectangles represent portions of exons encoding 5′- and 3′-untranslated regions. The green oval represents the location of a possible Erg-1-responsive sequence and the red oval a possible glucocorticoid receptor response element (GRE) sequence, both located in intron 1 (12). Coriell represents the Coriell Institute, from which the DNA samples used in the previous resequencing study were obtained. *Non-synonymous coding single nucleotide polymorphisms (cSNPs).

Table 2. Human PNMT polymorphisms in CA subjects

<table>
<thead>
<tr>
<th>Region</th>
<th>Contig Position</th>
<th>rs No.</th>
<th>Nucleotide</th>
<th>Sequence Change</th>
<th>Amino Acid Change</th>
<th>MAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′-FR</td>
<td>1548057</td>
<td></td>
<td>−968</td>
<td>C→G</td>
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<td>0.005</td>
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<tr>
<td>5′-FR</td>
<td>1548187</td>
<td></td>
<td>−838</td>
<td>G→T</td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>5′-FR</td>
<td>1548382</td>
<td></td>
<td>−643</td>
<td>G→T</td>
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<td>0.005</td>
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<tr>
<td>5′-FR</td>
<td>1548434</td>
<td>rs2934966</td>
<td>−591</td>
<td>G→T</td>
<td></td>
<td>0.036</td>
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<tr>
<td>5′-FR</td>
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<td></td>
<td>−445</td>
<td>G→A</td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>5′-FR</td>
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<td></td>
<td>−392</td>
<td>G→C</td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>5′-FR</td>
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<td>rs3764351</td>
<td>−390</td>
<td>A→G</td>
<td></td>
<td>0.633</td>
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<td>5′-FR</td>
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<td>rs876493</td>
<td>−184</td>
<td>A→G</td>
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<td>0.525</td>
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<tr>
<td>Exon1</td>
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<td>26</td>
<td>A→G</td>
<td>Asn9Ser</td>
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<td>IVS1</td>
<td>1549227</td>
<td></td>
<td>I1(1)</td>
<td>G→T</td>
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<td>0.008</td>
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<td>IVS1</td>
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<td>rs200173</td>
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<td>I1(280)</td>
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<td>I1(360)</td>
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<td>I1(484)</td>
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<td>Exon2</td>
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<tr>
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<td>3′-UTR</td>
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<td></td>
<td>941</td>
<td>G→A</td>
<td></td>
<td>0.008</td>
</tr>
</tbody>
</table>

These polymorphisms include those identified in the present study with DNA samples from 104 subjects at Mayo Clinic as well as those identified during our previous resequencing study (9) with 60 Caucasian American (CA) DNA samples obtained from the Coriell Institute. Alternations in nucleotide and encoded amino acid sequences, as well as their minor allele frequencies (MAFs), are listed. The numbering scheme for nucleotides was described previously (9).

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Genotype-Phenotype Correlations

Association test for single SNPs or haplotypes using mean values of epinephrine, norepinephrine, and epinephrine-to-norepinephrine ratio. When a single-SNP association test was performed using the mean value for the three measurements at Low exercise or the three measurements obtained during High exercise (see Fig. 1), the I1G(280)A SNP in the first intron of PNMT was significantly associated with decreased levels of epinephrine and the ratio at both Low ($P = 0.0004$ and $0.0068$, respectively) and High ($P = 0.0234$ and $0.0168$, respectively) exercise levels compared with the WT PNMT genotype. This SNP did not appear to be associated with the level of circulating norepinephrine. Figure 3 shows percent change of phenotype [level of epinephrine (Fig. 3A), level of norepinephrine (Fig. 3B), epinephrine-to-norepinephrine ratio (Fig. 3C)] from baseline to Low and High for the two individuals heterozygous for I1G(280)A compared with the other 72 subjects. Figure 4 shows a comparison of values for epinephrine change from baseline levels in these two subjects, once again compared with the remaining 72 participants in the exercise study, over the entire time course of the study. No significant haplotype associations were observed during analyses for either the haplotype of the entire gene or the four-locus 5′-FR haplotypes listed in Table 1. We also measured basal 24-h urinary catecholamine excretion values in 61 of these subjects for whom 24-h urine samples were available—including the 2 subjects heterozygous for I1G(280)A. These two subjects with variant I1A(280) alleles had a lower average urinary epinephrine 24-h excretion than the remaining 59 subjects for whom basal 24-h urine samples were available (Table 3).

Repeated-measurements SNP and haplotype tests. The repeated-measures model used in the single-SNP test considered all six measurements (3 at Low, 3 at High; see Fig. 1) together. I1G(280)A was not found to be associated with epinephrine level during that analysis. However, I1G(280)A was associated with baseline epinephrine ($P = 0.0002$) as well as the epinephrine-to-norepinephrine ratio ($P = 0.0002$). When the baseline measurement was included in the model together with the three values at Low and the three values at High, there was a significant association with I1G(280)A ($P < 0.0001$) as well as a dynamic effect of I1G(280)A (interaction of genotype with time; $P = 0.034$). The four-locus haplotype (see Table 1) association test with the log(epinephrine) − log(baseline epinephrine) phenotype for all six measurements of epinephrine, three Low and three High, showed a significant global haplotype association ($P = 0.032$), with haplotype H1 (GCAG) having a significant association ($P = 0.004$) compared with the most common haplotype (CGAA). Because of the lack of stability of the model with rare haplotypes, haplotypes H2 and H4 were pooled into a “rare” haplotype for this analysis.

Since the nonsynonymous C319T (Arg107Cys) SNP and the I1G(280)A SNP were both discovered by resequencing DNA samples from these study subjects and since the I1G(280)A SNP was associated with both baseline and exercise-induced epinephrine levels as well as epinephrine-to-norepinephrine ratios, we performed functional genomic experiments to test the possible effects of these two novel SNPs on PNMT function.

Functional Genomic Studies

PNMT variant allozyme studies. PNMT had been resequenced in DNA samples from all 104 CA subjects who initially volunteered for the exercise study. Included among the 14 SNPs observed within the 3.5 kb of DNA resequenced was one novel nonsynonymous SNP, C319T, that resulted in an Arg107Cys alternation in the encoded amino acid. Functional genomic studies were performed with the Cys107 allozyme, using recombinant protein obtained by transient expression in COS-1 cells. Those experiments showed that this variant allozyme displayed significantly reduced levels of both PNMT activity and immunoreactive protein compared with the WT allozyme (11.7% and 47.1% of levels for WT, respectively). Substrate kinetic studies showed that the Cys107 allozyme had an apparent $K_m$ value for octopamine (21.7 ± 3.4 μM; $n = 3$) that was similar to that of the WT allozyme (23.5 ± 3.1 μM);
n = 3), but a twofold higher $K_m$ value for the methyl donor cosubstrate, $S$-adenosyl-$l$-methionine (AdoMet) (12.4 ± 2.2 μM for Cys107 vs. 6.2 ± 0.4 μM for WT; $n = 3$, $P < 0.05$).

**PNMT structural analysis.** Structural analysis was performed for the protein encoded by the allele containing the C319T, Arg107Cys nonsynonymous SNP, as well as nonsynonymous SNPs that had been identified during our previous PNMT gene resequencing study (Asn9Ser, Thr98Ala, Arg112Cys, and Ala175Thr) (9). Levels of both PNMT allozyme activity and immunoreactive protein for those variant PNMT allozymes are displayed graphically in Fig. 5, which shows, as we have found for many other enzymes (21, 24), a significant correlation between levels of protein and enzyme activity, implying that a major mechanism by which nonsynonymous SNPs influence function for PNMT is an alteration in protein quantity.

The human PNMT X-ray crystal structure is shown in Fig. 6 as a gray ribbon structure, with the positions of amino acids altered as a result of genetic polymorphisms highlighted (codon 9 was not included in the crystal structure) (20). The light pink spheres represent atoms of the methyl donor cosubstrate AdoMet, and the yellow spheres are atoms of the PNMT inhibitor SK&F29661 (20). None of the four genetically variant residues shown in the crystal structure was located within the active site of the enzyme that binds AdoMet, with the variant residue at codon 107 that was observed during the present study lying nearest to the active site. Among the four variant residues pictured in Fig. 6, Thr98 is most deeply “buried” and forms hydrogen bonds to Arg153 and to a water molecule. The water molecule, in turn, H-bonds to the side chain of Asp96 and to the carbonyl group of Leu75. The buried water molecule and its electrostatic interactions might be important for protein structure/folding/stability. This Thr to Ala alteration at residue 98 would be easily accommodated in terms of space since Ala is smaller than Thr. However, the H-bonding interaction would be lost, leaving a cavity as a result of the smaller Ala side chain, both of which could be unfavorable, which may explain the striking effect of this polymorphism on function (a reduction of >80% in level of immunoreactive protein; Fig. 5). Arg107 is partially buried and partially solvent accessible. It forms H-bonds with the carbonyl group of Asp101 in the preceding turn, and its side chain packs against residues 154–156. The smaller Cys107 substituted as a result of the genetic polymorphism at codon 107 would remove

### Table 3. Twenty-four-hour urinary catecholamine levels for the 61 subjects for whom 24-h urine samples were available

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Epinephrine, µg/24 h</th>
<th>Norepinephrine, µg/24 h</th>
<th>Epinephrine-to-Norepinephrine Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>G/G (n = 59)</td>
<td>5.0±0.24</td>
<td>34.3±0.93</td>
<td>0.15±0.005</td>
</tr>
<tr>
<td>G/A (n = 2)</td>
<td>2.6</td>
<td>28.5</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Values are means [subjects heterozygous for I1G(280)A] and means ± SE (remaining subjects) for epinephrine, norepinephrine, and epinephrine-to-norepinephrine ratio.
of32P-labeled oligonucleotides were added (Fig. 7A). These “shifts” were inhibited when unlabeled specific oligonucleotides were added to the reaction mixtures. The shifted bands resulting from variant oligonucleotides that contained either the WT (G) or the variant (A) nucleotide at the I1(280) locus, using rat pheochromocytoma PC-12 cell nuclear extract. Both WT and variant oligonucleotides bound nuclear proteins, resulting in similar “gel shift” patterns (Fig. 7A). These “shifts” were inhibited when unlabeled specific oligonucleotides were added to the reaction mixtures. The shifted bands resulting from variant oligonucleotide binding appeared to be of higher density than those with the WT oligonucleotides, even when equal amounts of 32P-labeled oligonucleotides were added (Fig. 7A). These results were compatible with the location of this SNP in an area of high cross-species sequence homology in primates and its location within an area predicted by rVISTA to bind to multiple transcription factors. We followed these EMS experiments with reporter gene studies.

pGL3 reporter gene constructs were created by cloning the first intron of PNMT into a series of pGL3 reporter gene vectors (Promega), including pGL3-Basic, pGL3-Promoter, and pGL3-Enhancer. The pGL3-Enhancer vector contains an SV40 enhancer located downstream of the firefly luciferase ORF, which aids in identifying a functional promoter in the presence of an enhancer. The pGL3-Promoter vector was designed to aid in the identification of enhancer or repressor elements cloned into the upstream region of an SV40 promoter. In contrast, the widely used pGL3-Basic vector lacks both eukaryotic promoter and enhancer sequences, thus allowing the regulatory activity of DNA sequences cloned upstream of the luciferase ORF to be tested. Variant reporter gene constructs with “A” at position I1(280) were then created by site-directed mutagenesis. The results of dual-luciferase assays performed after the transfection of PC-12 cells are shown in Fig. 7B. Neither WT nor variant constructs showed activity in either pGL3-Basic or pGL3-Enhancer constructs, indicating that the cloned sequence might not contain sequences that could function as a promoter in those constructs. However, when cloned into a pGL3-Promoter vector, both WT and variant PNMT intron 1 sequences appeared to have enhancer effects that upregulated promoter activity by ~40–50% (P < 0.001). In addition, luciferase activity for the variant was significantly higher than that for the WT (110.6 ± 2.7%, mean ± SE; P = 0.0006). However, this effect was not in a direction that could explain the observed decrease in circulating epinephrine and ratio of epinephrine to norepinephrine during exercise that we observed to be associated with the variant nucleotide.

**DISCUSSION**

PNMT catalyzes the reaction required to convert the neurotransmitter norepinephrine to the neurohormone and neurotransmitter epinephrine (35, 36). However, very little is known with regard to the possible contribution of common DNA sequence variation within the gene encoding PNMT to varia-
tion in epinephrine regulation and function. We set out to test the hypothesis that common polymorphisms in PNMT might influence the function of this enzyme in humans by combining gene sequencing with assays of circulating epinephrine in response to vigorous exercise as a dynamic physiological phenotype. Exercise stress can upregulate PNMT gene expression, largely as a result of the action of glucocorticoids (1, 13, 35, 36). Several previous studies have suggested that two common PNMT promoter SNPs, A(-390)G and A(-184)G (both included in our analyses), might be associated with risk for diseases such as essential hypertension, early-onset Alzheimer disease, or multiple sclerosis (4, 18, 19, 23, 34). However, there have not been direct genotype-phenotype correlation data in support of a role for inheritance in PNMT function in humans. In the present study, we used a strenuous exercise protocol to alter circulating epinephrine levels in healthy subjects—an approach that not only avoided the need for tissue (i.e., adrenal) biopsy but also provided a dynamic, sequential, stepwise phenotype. Our study also made it possible to monitor possible effects throughout the entire course of the exercise test. Since adrenal epinephrine is stored in chromaffin granules, we speculated that there might be a variation in the effect of PNMT-dependent epinephrine synthesis on catecholamine stores available for release during exercise. Therefore, we attempted to determine whether this dynamic phenotype might be associated with variation in the sequence of the PNMT gene—a gene that we completely sequenced in all subjects who participated in the study.

PNMT is a relatively small gene that contains three exons and spans only 3 kb, including ~1 kb of 5'-FR (Fig. 2). That structure and short length made it possible for us to resequence the entire “core” PNMT gene to obtain gene sequence information rather than merely genotyping previously identified polymorphisms. A total of 14 SNPs were identified during this gene resequencing effort, including 6 that were observed during our original resequencing of PNMT in 60 DNA samples (120 alleles) from CA subjects (Ref. 9; Table 2 and Fig. 2). Together, these two studies resulted in the identification of a total of 19 polymorphisms in the 328 alleles from CA subjects that were studied.

We also performed functional genomic studies with the novel Cys107 variant allozyme as well as the novel I1G(280)A intron SNP—both first observed during the present study. Those experiments showed that the change from Arg to Cys at codon 107 results in reductions in levels of both enzyme activity and immunoreactive protein (Fig. 5), as well as a twofold increase in the apparent $K_m$ value for AdoMet. Structural analysis indicated that the Arg107 residue is closest of the four polymorphisms modeled to the AdoMet binding site in the human PNMT crystal structure (Fig. 6) and this residue is partially “buried” in the structure of the protein. Therefore, the alterations in function of the Cys107 allozyme that we observed could be explained, at least in part, by structural changes induced by the variant residue. Our previous study (9) showed that the Thr98Ala polymorphism that we observed in one heterozygous DNA sample from a African American subject resulted in a dramatic reduction in both PNMT activity and immunoreactive protein levels—which were associated with accelerated protein degradation. Our structural modeling in the present study indicated that Thr98 was deeply buried in the PNMT molecule (Fig. 6), so that polymorphism might significantly disrupt the structure of PNMT. A similar phenomenon has been observed for the clinically important thiopurine S-methyltransferase (TPMT)*3A variant allozyme in which two changes in amino acid sequence as a result of genetic polymorphisms lead to misfolding, accelerated degradation, and a striking reduction in the level of TPMT protein (30, 32).

Our genotype-phenotype association analyses showed a significant association of the I1G(280)A SNP with decreased epinephrine levels during exercise. This SNP was located in the initial PNMT intron. By transiently expressing a minigene construct that contained the entire PNMT gene, including all introns and exons, we attempted to evaluate the possible effect of I1G(280)A on mRNA splicing by the use of RT-PCR, but no changes were observed (data not shown). However, EMS and reporter gene assays both suggested that this intron SNP might influence PNMT transcription. The sequence containing the variant “A” nucleotide resulted in more intense shifted bands during EMS than the WT “G” sequence (Fig. 7A). In addition, when the entire first intron of PNMT was cloned into the pGL3-Promoter vector, we observed an enhancer effect. The variant sequence (Promoter-V in Fig. 7B) showed slightly but significantly higher enhancer activity than the WT sequence (Promoter-WT; P = 0.0012). I1G(280)A is not located within a putative GRE that has been described in the middle of PNMT intron 1 (2) but does lie within a “cluster” of transcription factor binding sites based on prediction by rVISTA. Although these functional genomic data did not explain the decrease in circulating epinephrine levels that we observed in association with this polymorphism, they do suggest a possible role for the I1(280) polymorphism in the regulation of PNMT transcription, especially since intron 1 of PNMT displayed enhancer effect activity during luciferase reporter gene assays. Our data are also compatible with the in silico analysis reported by Kepp et al. (12) with regard to the possible role of PNMT intron 1 in transcription regulation. Obviously, studies involving a larger number of subjects will be needed both to confirm our observations with regard to I1G(280)A and to increase our ability to detect the possible functional effects of other PNMT polymorphisms such as that resulting in the Cys107 allozyme. In addition, since circulating levels of epinephrine are regulated by many enzymes other than PNMT, e.g., monoamine oxidase and catechol O-methyltransferase, a pathway-based approach that includes the assay of epinephrine metabolites and the genotyping of genes encoding catecholamine metabolic enzymes and uptake proteins might also be informative if applied during future studies of genetic factors that could influence stress-induced epinephrine release.

Exercise requires numerous physiological adjustments in order to deliver oxygen and compounds involved in energy generation to working muscles. Those adjustments include increased cardiac output and muscle blood flow, bronchodilation, glycogenolysis, and lipolysis (16). In addition, vasoconstriction occurs in visceral organs and nonactive muscles. However, there is marked heterogeneity in the cardiopulmonary and metabolic responses to exercise, even among healthy adults of the same sex, age, and fitness level. Factors that account for this variation remain unclear, but may be due, in part, to variation in genes that encode proteins important in regulating sympathetic outflow and related postjunctional effects. During heavy exercise, there is a significant increase in circulating catecholamines that plays an important role in
cardiopulmonary, vascular, and metabolic responses to exercise. Previous studies have highlighted the importance of sequence variation in genes that encode adrenergic receptors in heterogeneous response to exercise (27, 28). The present study focused on variation in a gene that encodes a protein critical in the synthesis of, and, as a result, the quantity of circulating epinephrine that may be available to adrenergic receptors.

In summary, we set out to study the possible role of PNMT genetic polymorphisms in exercise-induced alternation in circulating epinephrine levels. Our study utilized an exercise protocol to generate a dynamic physiological phenotype, changes in circulating epinephrine during vigorous exercise—thus making it possible to perform a PNMT genotype-phenotype association study. We also performed functional genomic studies of two novel PNMT genetic polymorphisms that were observed in the course of the study. As a result, this study represents a step toward a more complete understanding of the possible contribution of genetic variation in PNMT to human physiology and/or disease risk. The results not only expand our understanding of PNMT functional variants, but they also provide a basis for the future application of a similar strategy to study the contribution of inheritance to other important physiological phenotypes.

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REFERENCES


24. Pelleymounter for performing the cross-species sequence homology analysis.


