Heredity of Endothelin Secretion

Human Twin Studies Reveal the Influence of Polymorphism at the Chromogranin A Locus, a Novel Determinant of Endothelial Function

Elizabeth O. Lillie, PhD; Manula Mahata, PhD; Srikrishna Khandrika, PhD; Fangwen Rao, MD; Richard A. Bundey, PhD; Gen Wen, PhD; Yuqing Chen, MD, PhD; Laurent Taupenot, PhD; Douglas W. Smith, PhD; Sushil K. Mahata, PhD; Michael G. Ziegler, MD; Myles Cockburn, PhD; Nicholas J. Schork, PhD; Daniel T. O’Connor, MD

Background—Endothelial dysfunction predisposes to vascular injury in association with hypertension. Endothelin (ET-1) is a potent vasoactive peptide that is synthesized and released by the vascular endothelium and is a marker of endothelial function. Chromogranin A (CHGA) regulates the storage and release of catecholamines and may have direct actions on the microvasculature. CHGA, a candidate gene for intermediate phenotypes that contribute to hypertension, shows a pattern of single nucleotide polymorphism variations that alter the expression and function of this gene both in vivo and in vitro.

Methods and Results—In a study of twins (n=238 pairs), plasma ET-1 was 58±5% (P<0.0001) heritable. Plasma ET-1 was both correlated and associated with chromogranin fragment levels, and the 2 were influenced by shared genetic determination (pleiotropy [ρs]; for the CHGA precursor, ρs=0.318±0.105; P=0.0032). We therefore hypothesized that variation in the CHGA gene may influence ET-1 secretion. Carriers of the CHGA promoter −988G, −462A, and −89A minor alleles showed significantly higher mean plasma ET-1 than their major allele homozygote counterparts (P=0.02, P=0.006, P=0.03, respectively). Analysis of a linkage disequilibrium block that spans these 3 single nucleotide polymorphisms showed a significant association between the GATAAC haplotype and plasma ET-1 (P=0.0075). In cultured human umbilical vein endothelial cells, CHGA caused dose-dependent secretion of ET-1 over a brief (<1 hour) time course at relatively low concentrations of CHGA (10 to 100 nmol/L) with a threshold concentration (10 nmol/L) in the range found circulating in humans in vivo.

Conclusions—These results suggest that common, heritable variation in expression of the human CHGA gene influences endothelial ET-1 secretion in vivo, explained by a CHGA stimulus/ET-1 secretion coupling in endothelial cells in vitro. The findings document a previously unsuspected interaction between the sympathochromaffin system and the endothelium and suggest novel genetic and cell biological approaches to the prediction, diagnosis, and mechanism of endothelial dysfunction in human disease. (Circulation. 2007;115:2282-2291.)

Key Words: endothelin • endothelium • genetics • hypertension • nervous system, sympathetic • vasculature

The endothelium plays a central role in the vascular biology of both health and disease. Early dysfunction in the endothelium may predispose to later adverse clinical events such as hypertension or atherosclerosis.1 The endothelium releases soluble mediators, including endohelins, a family of potent vasoactive peptides,2 onto the adjacent vascular smooth myocytes. The best-characterized endothelin, endothelin-1 (ET-1), is encoded by a discrete genetic locus on human chromosome 6p24-p23 (EDN1). Once released, the actions of ET-1 are diverse, including vasoactivity,3 promotion of mitogenesis/hypertrophy of vascular myocytes,4,5 profibrotic activity,6 and regulation of angiogenesis.7 The vasoactivity provoked by ET-1 may be complex, with either vasoconstriction or vasodilation in particular vascular beds, perhaps governed by the balance of endothelin type A or type B receptors on target cells.8–10

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Targeted ablation of the Edn1 locus in the mouse results in a widespread, pleiotropic spectrum of traits, including respiratory failure, pharyngeal arch (craniofacial and cardiovascu-
lar) malformations, hypertension, and early mortality.\textsuperscript{11} Despite extensive study in genetically altered mice,\textsuperscript{11} mechanisms controlling ET-1 storage and release in humans are incompletely understood.\textsuperscript{12} In particular, hereditary or genetic influences on ET-1 have not been systematically explored. Common genetic variation in the 5\textsuperscript{-}UTR of human \textit{EDN1} may influence ET-1 release and circulating ET-1 concentration,\textsuperscript{13} but genetic variation at \textit{EDN1} has not yet been linked to blood pressure changes in humans.\textsuperscript{14}

Twin pairs represent a unique window into the role of heredity in the determination of any human trait.\textsuperscript{15,16} We therefore undertook a human twin study of ET-1 in plasma, integrating information about ET-1 secretion with comprehensive biochemical and physiological profiling of vascular control systems in the same individuals to identify candidate genes that may influence ET-1 variability in vivo.

Here, we harness the power of the twin approach to demonstrate the heritability (h\textsuperscript{2}) of ET-1 secretion and to discover evidence of genetic codetermination of ET-1 with a multifunctional sympathochromaffin protein, chromogranin A (CHGA).\textsuperscript{17} CHGA is coreleased with other sympathetic transmitters during exocytosis and is then processed to biologically active fragments such as vasostatin, a potent smooth muscle relaxant\textsuperscript{17,18}; catestatin, an inhibitor of catecholamine release; and the dysglycemic fragment pancreastatin. The vasoreactivity of CHGA prompts our investigation of its potential interaction with the endothelium.

Our results implicate hereditary control of endothelial function by the sympathochromaffin system and suggest strategies to probe novel mechanisms in the pathogenesis of endothelial dysfunction in disease states.

Methods

Study Population
The 238 twin pairs are described in the Methods section of the online Data Supplement.

Clinical Phenotyping
Blood pressure is described in the Methods section of the online Data Supplement.

Biochemical Phenotyping
Endothelin, chromogranin/secretogranin, neuropeptide Y (NPY), glucose, lipid, and apolipoprotein biochemical assays are described in the Methods section of the online Data Supplement.

Single Nucleotide Polymorphism Assays
Isolation of genomic DNA and single nucleotide polymorphism (SNP) assay procedures are described in the Methods section of the online Data Supplement.

Human Endothelial Cell Secretion Assays
Procedures for culture of endothelial cells and stimulation of endothelin release by CHGA are described in the Methods section of the online Data Supplement.

Statistical Analyses
Descriptive statistics, twin heritability, pleiotropy, associations (marker on trait), haplotype inference, linkage disequilibrium (LD), multiple comparisons, and cluster analysis are described in the Methods section of the online Data Supplement.

Results

Study Population
The study sample partitioned by sex, relationship, and ethnicity is summarized in Table 1. Genetic analyses were restricted to subjects of European ancestry. A total of 458 white individuals (103 siblings, 121 monozygotic twin pairs, 51 dizygotic twin pairs, 1 monozygotic/dizygotic triplet, and 8 incomplete pairs) had plasma available for ET-1 assays. Of the total study population, 24 white subjects (10 male, 14 female) from 17 families did not have plasma available for ET-1 assay.

Heritability
Mean and heritability (h\textsuperscript{2}) estimates of baseline traits are presented in Table 2. Stratification by sex showed that women were statistically significantly older (P=0.02), had slightly lower body mass index (P=0.02), had lower systolic blood pressure (P=0.002), and had lower plasma ET-1 (P=0.02). No differences in age- and sex-adjusted mean plasma ET-1 were observed when stratified by blood pressure status (P=0.39) or family history of hypertension (P=0.97) (data not shown). Using the complete twin pairs, we estimated that 58\(\pm\)5% (P<0.0001) of the variance in plasma ET-1 can be attributed to additive genetic effects after adjustment for age and sex.

Correlations of Plasma ET-1 With Clinical and Biochemical Traits: Genetic and Environmental Codetermination
Pearson correlations between natural log-transformed plasma ET-1 and both clinical and biochemical phenotypes in 1 individual per family were estimated with adjustment for age and sex. Phenotypes examined were selected on the basis of

TABLE 1. Study Population

<table>
<thead>
<tr>
<th>Group</th>
<th>All,\textsuperscript{a} n</th>
<th>Sample,\textsuperscript{b} n</th>
<th>White Sample,\textsuperscript{c} n</th>
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<tbody>
<tr>
<td>Men</td>
<td>208</td>
<td>183</td>
<td>113</td>
</tr>
<tr>
<td>Women</td>
<td>532</td>
<td>503</td>
<td>345</td>
</tr>
<tr>
<td>Relationship</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siblings</td>
<td>259</td>
<td>216</td>
<td>103</td>
</tr>
<tr>
<td>MZ twins</td>
<td>338</td>
<td>331</td>
<td>246</td>
</tr>
<tr>
<td>DZ twins</td>
<td>140</td>
<td>136</td>
<td>106</td>
</tr>
<tr>
<td>Triplets</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>482</td>
<td>458</td>
<td>458</td>
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<td>Black</td>
<td>77</td>
<td>61</td>
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<tr>
<td>Hispanic</td>
<td>70</td>
<td>65</td>
<td>...</td>
</tr>
<tr>
<td>Other</td>
<td>111</td>
<td>102</td>
<td>...</td>
</tr>
</tbody>
</table>

M2 indicates monozygotic; D2, dizygotic.
\*Some siblings are siblings of twins; the D2 twins include 1 D2 triplet.
\textsuperscript{†}Subjects with plasma available for ET-1 assay.
their relevance to blood pressure, the metabolic syndrome, or endothelial function. Among the clinical phenotypes, plasma ET-1 was positively correlated with age (r=0.26; 95% confidence interval [CI], 0.12 to 0.38; P=0.0002) and negatively correlated with a change in heart rate as a response to cold stress (r=-0.15; 95% CI, -0.28 to -0.01; P=0.03). Among the biochemical phenotypes, plasma ET-1 was positively correlated with plasma glucose (r=0.14; 95% CI, 0.00 to 0.28; P=0.05), plasma CHGA116–327 (catestatin) (r=0.20; 95% CI, 0.03 to 0.35; P=0.02), and the CHGB312–331 fragment (r=0.31; 95% CI, 0.15 to 0.45; P=0.0002). Plasma ET-1 was negatively correlated with apolipoprotein A-1 (r=-0.19; 95% CI, -0.35 to -0.02; P=0.03), plasma NPY (r=-0.26; 95% CI, -0.41 to -0.10; P=0.002), and the CHGA116–130 fragment (r=-0.22; 95% CI, -0.37 to -0.05; P=0.01). Although correlations were observed between plasma ET-1 and CHGA fragments, no correlations were observed with either blood pressure or catecholamines in plasma or urine.

Pleiotropy, the phenomenon in which a single gene influences >1 trait, was assessed by the genetic covariance (\(\rho_g\)) parameter estimated from twin variance components by sequential oligogenic linkage analysis routines. The \(\rho_g\) and environmental covariances were estimated for ET-1 and its biochemical correlates. All of the \(\rho_g\) estimates were statistically significant. These results suggest that the correlation between plasma ET-1 and other biochemical traits (especially CHGA and CHGB) is driven predominantly by genetic covariation or pleiotropy (CHGA116–439: \(\rho_g=0.32\); CHGA361–372, \(\rho_g=0.29\)); the \(\rho_e\) estimates were not statistically significant.

**Association of Plasma ET-1 With Plasma Chromogranins**

To further characterize the statistically significant correlations between plasma ET-1 and plasma chromogranins in all white study subjects, associations were examined by generalized estimating equations (GEEs) to compare mean values of chromogranins across quartiles of plasma ET-1. Results are illustrated in Figure 1. The CHGA116–130 fragment was observed to have lower values with increased level of plasma ET-1 (P for trend=0.01). Conversely, the precursor CHGA116–439 fragment was observed to increase with increasing plasma ET-1 (P for trend=0.002). Significant trends across the quartiles of plasma ET-1 were not observed for the remainder of the CHGA, CHGB, or SCG2 fragments. However, when plasma ET-1 was dichotomized about the median, mean values of CHGA361–372 (catestatin) were statistically significantly higher in subjects with high ET-1 (>1.07 pg/mL) than in subjects with low ET-1 (1.31±0.06 versus 1.16±0.05 nmol/L; P=0.02).

In an examination of the association between ET-1 and other correlated traits, statistically significant associations between elevated plasma ET-1 (>1.07 pg/mL) and decreased plasma NPY (P=0.01) were observed.

**Chromogranin Phenotypic Clustering**

Because CHGA and ET-1 share genetic determination (CHGA116–439: \(\rho_g=0.32\); CHGA361–372, \(\rho_g=0.29\)), we sought to understand how ET-1 might associate with more global profiles of sympathochromaffin activity. Thus, we extended the univariate correlations and associations of plasma ET-1 to several chromogranin fragments, probing how ET-1 might associate in multivariate fashion with several such peptides being considered as a combined phenotypic profile (or cluster). With the analysis restricted to 1 individual per family and to individuals with complete data on all pertinent phenotypes (n=77), cluster analysis was used to create 2 groups of individuals based on how their CHGA, CHGB, and SCG2 fragments clustered (Table I in the online Data Supplement). Individuals who were part of cluster 2 were characterized by significantly higher natural log precursor CHGA116–439 (P=0.0005), lower CHGA361–372 (catestatin) fragment (P<0.0001), higher CHGB312–331 (P=0.049), lower CHGB39–451 (P<0.0001), and lower CHGB39–451 (P<0.0001) compared with cluster 1. No statistically significant differences existed in body mass index, urinary or plasma catecholamines, blood pressure, or heart rate between the 2 clusters; however, cluster 2 had significantly higher natural log plasma ET-1 (0.21±0.08 versus 0.00±0.09 pg/mL; P=0.037).

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**TABLE 2. Mean Values of Baseline Descriptive Traits Stratified by Sex in White Twin and Sib Subjects Assayed for ET-1**

<table>
<thead>
<tr>
<th>Trait</th>
<th>All</th>
<th>Men</th>
<th>Women</th>
<th>Twins</th>
<th>P*</th>
<th>n†</th>
<th>Heritability (SEM)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>458</td>
<td>113</td>
<td>345</td>
<td>0.157</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>457</td>
<td>113</td>
<td>344</td>
<td>0.242</td>
<td>347</td>
<td>0.85 (0.02)</td>
<td></td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>452</td>
<td>112</td>
<td>340</td>
<td>0.0015</td>
<td>344</td>
<td>0.42 (0.07)</td>
<td></td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>452</td>
<td>112</td>
<td>340</td>
<td>0.0564</td>
<td>344</td>
<td>0.31 (0.09)</td>
<td></td>
</tr>
<tr>
<td>HR, bpm</td>
<td>452</td>
<td>112</td>
<td>340</td>
<td>0.3704</td>
<td>344</td>
<td>0.51 (0.07)</td>
<td></td>
</tr>
<tr>
<td>ET-1, pg/ml</td>
<td>458</td>
<td>113</td>
<td>345</td>
<td>0.0208</td>
<td>347</td>
<td>0.58 (0.05)</td>
<td></td>
</tr>
</tbody>
</table>

BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; and HR, heart rate.

*Means estimated and compared by sex with GEE analysis.
†n=172 twin pairs (121 monozygotic, 51 dizygotic), 1 monozygotic/dizygotic triplet.
‡Adjusted for age and sex.

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†n=172 twin pairs (121 monozygotic, 51 dizygotic), 1 monozygotic/dizygotic triplet.
‡Adjusted for age and sex.
The cluster analysis results were confirmed by a phenotypic similarity analysis conducted in the same study sample with the program DISTLM forward 1.3. A distance matrix summarizing the pairwise comparisons of each of the chromogranin traits was created, and explanatory variables were forwardly selected using permutations in a linear regression model. The set of explanatory traits assessed included plasma ET-1, age, sex, CHGA SNP genotypes, blood pressure, heart rate, plasma, and urinary catecholamines. Plasma ET-1 was observed to be the foremost predictor of chromogranin similarity ($P=0.001$).

**Association of Genetic Variants in CHGA With Plasma ET-1**

Based on the statistically significant heritability estimates of plasma ET-1, an evaluation of the association between genetic variation in the CHGA locus and variation in plasma ET-1 was conducted by GEEs. Of the 19 SNPs genotyped, 2 were out of Hardy-Weinberg equilibrium ($P<0.0001$), and 3 had a minor allele frequency of $<5\%$. One SNP was borderline out of Hardy-Weinberg equilibrium at $P=0.01$ and a low minor allele frequency of 6% in our sample, so it was excluded from analyses. Three types of associations of mean plasma ET-1 level were conducted for each SNP: a comparison of carriers of the minor allele to major allele homozygotes, a comparison of minor allele homozygotes to major allele homozygotes, and a test for trend. A T/C SNP in intron 6 (T9179C) showed significant associations for all 3 tests. Three SNPs in the promoter (T-988G, G-462A, C-89A) showed statistically significant probability values for the comparison of heterozygotes to major allele homozygotes; however, no association comparing minor allele homozygotes to major allele homozygotes was observed. The G-462A polymorphism exhibits a threshold effect in which 1 copy of the minor allele is sufficient to raise ET-1. The T-988G and the C-89A SNP associations show a more extreme trait mean in heterozygotes than in either homozygote class (Table 3), a phenomenon commonly referred to as heterosis\(^{21}\); in such cases, however, the minor allele homozygotes were few, and the trait SEM was correspondingly large. A test combining heterozygotes with homozygotes for the minor allele was conducted at 3 loci with relatively small numbers of minor allele homozygotes: T-988G, G-462A, and C-89A (Figure 2). Carriers of the $\sim$988G allele, $\sim$462A allele, and $\sim$89A allele all showed significantly higher mean plasma ET-1 than their major allele homozygote counterparts ($P=0.02, P=0.006, P=0.03$, respectively) (Figure 2). Because catecholamines can trigger exocytosis from endothelial cell storage granules,\(^{22}\) we also evaluated whether catecholamines (as covariates) might account for the prediction of ET-1 by CHGA genotypes; the prediction was not affected.

An analysis of gene-by-sex interaction was conducted for each SNP locus. Some of the statistically significant associations previously observed in the sex-adjusted analyses were no longer statistically significant within the sex strata; however, the differences in mean levels by genotype were still apparent (data not shown). Among the nonsignificant SNPs in the sex-adjusted analyses, the C-57T SNP showed a statistically significant association in the female stratum. Carriers of the T allele (C/T or T/T diploid genotype) had higher mean plasma ET-1 ($0.17\pm0.06$ pg/mL) compared with non–T carriers (C/C homozygotes, $0.03\pm0.04$ pg/mL; $P=0.02$). No differences by genotype at this locus were observed in the male stratum. A test of interaction was suggestive but not statistically significant ($P=0.07$).
On the basis of the observed correlations between plasma ET-1 and other biochemical traits (CHGB and NPY; Table 3), associations between genetic variation in the candidate CHGB and NPY loci were conducted. No statistically significant associations between the SNPs assayed were observed (data not shown). These SNPs

<table>
<thead>
<tr>
<th>SNP Domain</th>
<th>Position (to Cap Site)</th>
<th>RefSNP</th>
<th>Genotype</th>
<th>n (%)</th>
<th>Mean (SEM)†</th>
<th>P‡</th>
<th>P for Trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter −1106 G/A</td>
<td>G-1106A</td>
<td>rs9658628</td>
<td>G/G</td>
<td>203 (52.5)</td>
<td>0.12 (0.06)</td>
<td>0.8256</td>
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<td></td>
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<td></td>
<td>G/A</td>
<td>155 (40.0)</td>
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<td>0.5536</td>
<td>0.6253</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>A/A</td>
<td>29 (7.5)</td>
<td>0.04 (0.14)</td>
<td>0.6376</td>
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</tr>
<tr>
<td>Promoter −1018 A/T</td>
<td>A-1018T</td>
<td>rs9658629</td>
<td>A/A</td>
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<td>0.14 (0.04)</td>
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<tr>
<td></td>
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<td>AT</td>
<td>95 (24.1)</td>
<td>0.09 (0.10)</td>
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<td>T/T</td>
<td>2 (0.5)</td>
<td>0.27 (0.10)</td>
<td>0.9800</td>
<td>0.1920</td>
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<tr>
<td>Promoter −988 T/G</td>
<td>T-988G</td>
<td>rs9658631</td>
<td>T/T</td>
<td>232 (59.3)</td>
<td>0.09 (0.05)</td>
<td>0.1060</td>
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<td></td>
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<td>T/G</td>
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<td>G-462A</td>
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<td>G/G</td>
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<td>G/A</td>
<td>111 (29.9)</td>
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<td>Promoter −89 C/A</td>
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<td>0.09 (0.12)</td>
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<tr>
<td>Intron 2 G/A</td>
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<td>rs941585</td>
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<td></td>
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<td>A/A</td>
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<td>0.40 (0.20)</td>
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<tr>
<td>Intron 4 T/A</td>
<td>A5088T</td>
<td>rs735726</td>
<td>T/T</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>A/A</td>
<td>27 (7.0)</td>
<td>0.13 (0.16)</td>
<td>0.0160</td>
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<tr>
<td>Intron 6 T/C</td>
<td>T9179C</td>
<td>rs2236132</td>
<td>T/T</td>
<td>329 (86.6)</td>
<td>0.15 (0.04)</td>
<td>0.0160</td>
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<tr>
<td></td>
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<td></td>
<td>T/C</td>
<td>48 (12.6)</td>
<td>0.00 (0.07)</td>
<td>0.0160</td>
<td></td>
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<tr>
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<td></td>
<td></td>
<td>C/C</td>
<td>3 (0.8)</td>
<td>0.40 (0.20)</td>
<td>0.0160</td>
<td></td>
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<tr>
<td>Exon 7 T/C (399 Arg/Trp)</td>
<td>C9610T</td>
<td>rs729940</td>
<td>C/C</td>
<td>265 (68.3)</td>
<td>0.14 (0.05)</td>
<td>0.0160</td>
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<tr>
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<td></td>
<td>C/T</td>
<td>105 (27.1)</td>
<td>0.11 (0.05)</td>
<td>0.0160</td>
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<td>T/T</td>
<td>18 (4.6)</td>
<td>0.06 (0.22)</td>
<td>0.0160</td>
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<tr>
<td>3' G/C</td>
<td>C12602G</td>
<td>rs875395</td>
<td>G/G</td>
<td>107 (28.7)</td>
<td>0.14 (0.06)</td>
<td>0.0160</td>
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<tr>
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<td></td>
<td>G/C</td>
<td>195 (52.3)</td>
<td>0.14 (0.05)</td>
<td>0.0160</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>C/C</td>
<td>71 (19.0)</td>
<td>0.13 (0.12)</td>
<td>0.0160</td>
<td></td>
</tr>
<tr>
<td>3' G/A (ITPK1)</td>
<td>C17757T</td>
<td>rs11446</td>
<td>G/G</td>
<td>170 (44.4)</td>
<td>0.13 (0.05)</td>
<td>0.0160</td>
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<tr>
<td></td>
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<td></td>
<td>G/A</td>
<td>155 (40.5)</td>
<td>0.11 (0.06)</td>
<td>0.0160</td>
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<td></td>
<td></td>
<td>A/A</td>
<td>58 (15.1)</td>
<td>0.17 (0.07)</td>
<td>0.0160</td>
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*Adjusted for age and sex.
†Natural log plasma ET-1.
‡χ², 1 df.
included 11 SNPs spanning the CHGB locus and 1 SNP in NPY (A1201G, Ser50Ser, rs5573).

Association of CHGA Haplotypes With Plasma ET-1

Blocks of LD across the 13 common CHGA SNPs were examined with Haplovew (Figure 3). Two blocks of LD were defined by the confidence interval algorithm. The first block spanned 5 SNPs in the promoter and 1 SNP in intron 2. The second block spanned 1 SNP in exon 7 and 1 SNP in the 3’-UTR. The significant SNP association in intron 6 lies between these 2 LD blocks. The other 3 significant SNPs all lie within the first block (Figure 3).

A haplotype analysis of the first LD block was conducted to gain a more comprehensive understanding of how CHGA variation influences plasma ET-1. Twenty-two haplotypes were observed, of which only 5 were relatively “common” (frequency >1%). Association analyses including all observed haplotype probability estimates in the GEE model and restricting the GEE model to the 5 common haplotypes were conducted. None of the score test probability values were statistically significant for either model. However, the individual haplotype parameter estimates were statistically significant for 4 of the 5 common haplotypes in the model, including all haplotypes. When the model was restricted to only parameters for the common haplotype probability estimates, the GATACA haplotype was statistically significantly associated with plasma ET-1 (P=0.0075). When the association between GATACA genotype (0, 1, or 2 copies) was modeled, subjects with 1 copy of the GATACA haplotype had higher mean ET-1 levels; however, the difference was marginally significant. Subjects with 1 copy (n=33) had a mean natural log ET-1 of 0.26±0.08 pg/mL compared with those with wild type (0 copies; n=363), whose mean was estimated at 0.11±0.04 pg/mL (P=0.06). No subjects were observed to carry 2 copies of the GATACA haplotype. A

Figure 2. CHGA promoter SNP genotypes vs plasma ET-1 in twins and siblings. At each SNP locus, the models grouped carriers of the minor allele vs major allele homozygotes. Results were computed from sex- and age-adjusted data in white (of European ancestry) twins and siblings using GEEs.

Figure 3. LD across the 13 CHGA SNP loci, spanning ~19 kbp on human chromosome 14q32. Top, Exon/intron structure of the gene. Bottom right, Haplovew plot of LD blocks. Bottom left, Legend for top. SNP loci in bold were significantly associated with plasma ET-1 concentration. The most 3’ SNP (No. 13) is in the 5’-UTR of the next gene on chromosome 14q32, ITPK1.
Multiple Genetic Comparisons

We analyzed 6 SNPs within CHGA haplotype block 1 (Figure 3) for an association with endothelin. Because SNPs within a haplotype block are correlated, a standard correction for independent multiple tests (eg, Bonferroni’s correction) would be inappropriately conservative. An emerging approach is to consider all of the SNPs within a block of LD simultaneously (ie, to take a haplotype approach). As noted, we found that haplotype GATACA was associated with plasma endothelin (\(P=0.0075\)). A more appropriate correction for multiple SNP loci than Bonferroni’s correction, taking into account correlation among SNPs within a block of LD, is the SNP spectral decomposition method of Nyholt24 (implemented online at http://genepi.qimr.edu.au/general/daleN/SNPSpD/) to yield an “effective” number of markers within a block of LD. Using this method in block 1 (eg, GATACA), we found that the effective number of independent marker loci was 4.5; thus, the experiment-wide significance threshold to maintain the type I error rate at \(\leq5\%\) was \(P=0.011\). This threshold was exceeded by peak promoter SNP G-462A (at \(P=0.0086\)) and by the GATACA haplotype effect (at \(P=0.0075\)).

**CHGA Triggers Endothelin Release From Human Endothelial Cells**

In human endothelial vein endothelial cells, exposure to 10- to 100-nmol/L full-length recombinant/purified human CHGA for 1 hour triggered substantial (up to \(\approx4\)-fold) release of ET-1, with a threshold concentration of 10 nmol/L and the suggestion of saturability beyond 100 nmol/L (Figure 4).

**Discussion**

Endothelin is a potent vasoconstrictor and marker of endothelial function.\(^1\) The objective of the present study was to characterize how ET-1 corresponds to biochemical and clinical phenotypes contributing to blood pressure, the metabolic syndrome, and endothelial function in this family-based study of nondiseased twins and siblings. Associations between both genetic and biochemical variations in the CHGA and CHGA fragments with plasma ET-1 were observed consistently with multiple statistical methodologies. Finally, guided by the initial genetic observations, we were able to demonstrate a direct effect of CHGA on ET-1 release from cultured endothelial cells. Thus, the phenotypic and genetic associations now have a mechanistic grounding in the cell biology of secretion.

**Heterosis**

Two of the CHGA promoter variants (−988G/T and −89C/A) associated with the ET-1 trait displayed an effect for heterozygotes that was more extreme than that for either homozygote group (Table 3), thus fulfilling the fundamental criterion for heterosis.\(^2\) This may be explained by one of several underlying mechanisms, including a U-shaped dose-response relationship for gene on trait, greater “fitness” in heterozygotes, or hidden stratification in 1 homozygote class. One such stratification might be the effect of allelic variation at other (non-CHGA) loci on ET-1 expression such as that previously reported for EDN1 itself (ie, cis-acting quantitative trait loci).\(^3\) Regardless of the mechanism, the phenomenon of heterosis points out the value of trait associations with diploid genotypes rather than simply with alleles, which could not capture such effects.

**Twin Method: Random Sample of the Population**

Because the twinship was the ascertainment criterion for the present study, this cohort may in some sense constitute a random (unbiased) sample of the population.\(^15,16,25\) Indeed, our cohort spans both sexes and a spectrum of ages (from 18 to 81 years). One advantage of this approach is that the results should be generalizable and applicable to the entire population rather than to only 1 disease state.

Compared with the California Twin Program, a population-based registry of twins in California, our twins were similar in mean age, mean body mass index, proportion of whites, and proportion with a family history of hypertension. A higher proportion of our twins were hypertensive (9.5% versus 5.3%; \(P<0.0001\)) or female (76.1% versus 52.6%; \(P<0.0001\)), and a lower proportion reported ever smoking (29.3% versus 42.4%; \(P<0.0001\)). Although no selection bias appears to exist for subjects with a family history of hypertension, our sample appears to be biased toward hypertensive
individuals. The lower smoking prevalence in our twins cannot be explained by the increased proportion of female subjects in our study population but may reflect the current southern California lifestyle because we observe no statistically significant difference in the proportion of those who have ever smoked among our twins compared with the twins within the California Twin Program who reside in Southern California.

The twin method relies on variance component comparisons in monozygotic versus dizygotic twins. One limitation in this design is the potential for misassignment of zygosity. This is unlikely in our study because twin zygosity was based on 3 indexes: self-report, allele sharing at the \( TH \) (TCAT), locus, and analysis of microsatellite whole-genome scan results in twins who were self-identified as dizygotic twins.

One caveat to the twin samples is that the 2 members of each twinship are genetically correlated and hence not independent observations; therefore, statistical methods have been developed to account for and even exploit this dependence such as the heritability estimates of sequential oligogenic linkage analysis routines and the clustered statistics of GEEs. Another caveat to the study of healthy individuals is that the sample does not readily capture any particular disease spectrum such as endothelial dysfunction; thus, whether our CHGA polymorphism is pertinent to the overall function of the endothelium remains to be tested.

**Plasma ET-1**

Endothelin acts in an autocrine and paracrine manner in tissue cells and circulates in the blood in small quantities. It remains to be elucidated whether the variability in measurements of plasma ET-1 correlates with the levels of ET-1 that is active in tissue cells. Our observations of a statistically significant association of circulating plasma ET-1 concentration with not only the CHGA protein (Figure 1) but also the CHGA genetic polymorphism (Figure 3) suggest that CHGA regulates either expression or release of ET-1 in some capacity. Although the circulating CHGA precursor concentration correlated directly with plasma ET-1 (Figure 1b), the relationship of the CHGA fragment CHGA\(_{116-439}\) to ET-1 was inverse (Figure 1a); an inverse relationship between the CHGA precursor and its fragments typically is observed in human plasma and is usually ascribed to interindividual differences in proteolytic cleavage of the prohormone to its peptide fragments.

**CHGA and Endothelial Cell Biology**

Our observations in vitro, using human endothelial vein endothelial cells over a brief (<1 hour) exposure to low concentrations (10 to 100 nmol/L) of CHGA, support a model for a process by which CHGA triggers endothelin release (Figure 4). We have not yet characterized the mechanism whereby the endothelial cell secretes ET-1 in response to CHGA (Figures 4), but successful triggering of ET-1 release by CHGA (Figure 4), coupled with its very low effective concentrations (as low as 10 nmol/L) and short time course (<1 hour), would suggest a specific and perhaps receptor-mediated response; apparent saturability in the 10- to 100-nmol/L dose range also is consistent with such a process. Indeed, precedent exists for potent, biologically active peptides derived from other regions of CHGA. Of particular note is the threshold concentration of CHGA to trigger ET-1 release (Figure 4); 10 nmol/L approximates the plasma CHGA concentration circulating in healthy human subjects, raising the likelihood that CHGA exerts not only local/paracrine effects on the endothelium but also an endocrine/systemic influence. Indeed, in our twin population, the plasma concentrations of CHGA\(_{116-439}\) span the range of 0.04 to 59.8 nmol/L, documenting that healthy humans possess circulating CHGA in the range capable of releasing ET-1. Therefore, these experimental results have novel implications for the biology of human vasoconstriction and genetic risk of cardiovascular events for which endothelial dysfunction is a precursor.

**Compatibility of Results With Previous Reports**

Other studies have examined associations between candidate loci and ET-1 variability. A functional study reports that a 5′-UTR adenine insertion variant in the ET-1 gene (\( EDN1 \)) is associated with increased ET-1 protein expression. A coding SNP in \( EDN1 \), Lys198Asn, has been evaluated for association with cardiovascular risk factors and outcomes. In a study of both normal and preeclamptic pregnant women, the T/T genotype was reported to be associated with increased plasma ET-1. The Prevention of Renal and Vascular End Stage Disease (PREVEND) study, a large study of nondiabetic subjects, did not identify any significant associations between \( EDN1 \) polymorphism and plasma ET-1.

Our group has focused on understanding genetic variation in the \( CHGA \) locus. Contributions of rare and common polymorphisms in \( CHGA \) have been observed to be active in regulating catecholamine physiology. Observations of human \( CHGA \) promoter activity as measured by firefly luciferase reporter show statistically significantly decreased promoter activity in the haplotype containing the GATAAC allele (5′ promoter portion) of the GATACA haplotype compared with all other promoter haplotypes; the key base position conferring differential promoter activity seemed to be G-462A, corresponding to the second SNP in GATACA. The functional compatibility of the promoter results with the observations presented here suggests that further research into understanding the links between CHGA and ET-1 will add to our understanding of the function of the endothelium in health and disease. Furthermore, a more comprehensive analysis of genetic variation in the endothelin system should aid in the understanding of how heredity determines ET-1 and how other genes might interact with the \( CHGA \) associations observed.

**Complex Trait Genetics: Multiple Alleles, Multiple Traits**

Genetic analyses of a complex trait necessitate the consideration of multiple phenotypes and genotypes, raising the
possibility of false-positive (type I) statistical errors. We approached this issue in several ways: establishing a functional relationship between CHGA and endothelin, identifying individual SNP associations, performing pleiotropic/bivariate analyses (1 gene, >1 trait), haplotyping, and using SNP spectral decomposition (determining the effective number of SNPs within a block of LD and thereby adjusting required threshold for significance). Nonetheless, the statistical confidence of our observations was modest; replication in an independent sample is the ultimate guardian against false-positive conclusions.

Conclusions

Our results first established a relationship between plasma chromogranins and plasma ET-1 and a shared genetic determination (ρc, pleiotropy) and then documented an effect of CHGA allelic variation on ET-1. Inspired by the genetic findings, we undertook studies of CHGA in endothelial cell biology and demonstrated a direct effect of CHGA on triggering ET-1 release from cultured endothelial cells (Figure 4). The results document a previously unsuspected interaction between the sympathochromaffin system and the endothelium and suggest novel genetic and unsuspected interaction between the sympathochromaffin system and the endothelium and suggest novel genetic and cell biological approaches to the prediction, diagnosis, and mechanism of endothelial dysfunction in human disease.

Acknowledgments

We appreciate the assistance of the UCSD General Clinical Research Center and its core laboratory (RR00827) in clinical and biochemical phenotyping of the twin pairs.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

Endothelial dysfunction predisposes to vascular injury in association with hypertension. Endothelin is a potent vasoactive peptide synthesized and released by the vascular endothelium. Chromogranin A regulates the storage and release of catecholamines and may have direct actions on the microvasculature. In a study of twins, plasma endothelin-1 was both correlated and associated with chromogranin fragment levels. Carriers of particular chromogranin A promoter alleles showed higher plasma endothelin than their major allele homozygote counterparts. Analysis of genetic variation spanning these 3 variants showed a significant association between the promoter haplotype and plasma endothelin. In cultured endothelial cells, chromogranin A caused dose-dependent secretion of endothelin, with a threshold concentration in the range found circulating in humans in vivo. These results suggest that common, heritable variation in expression of the human chromogranin A gene influences endothelial secretion in vivo, explained by a chromogranin A stimulus/endothelin secretion coupling in vitro. The findings document a previously unsuspected interaction between the sympathochromaffin system and the endothelium and suggest novel genetic and cellular approaches to the prediction, diagnosis, and mechanism of endothelial dysfunction in human disease.
Heredity of Endothelin Secretion: Human Twin Studies Reveal the Influence of Polymorphism at the Chromogranin A Locus, a Novel Determinant of Endothelial Function


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